The Role of the Selenoprotein Glutathione Peroxidase-1 in T Cell Activation and Differentiation

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Abstract: Glutathione peroxidase-1 (GPx-1) is an antioxidant enzyme that plays an important role in reducing cellular oxidative stress. The expression of *GPX1* has previously been shown to be upregulated upon T cell activation *in vitro* and $CD4^+$ T cells that lack GPx-1 have also been shown to preferentially differentiate into the T_H1 effector subtype. These observations suggest that the activity of GPx-1 may play a role in T cell activation and differentiation. In order to determine the relationship between GPx-1 expression and T cell activation, we used a combination of *in vitro* and *in vivo* experiments to correlate GPx-1 expression with the activation marker CD44 and to address whether inhibition of GPx-1 impacts the differentiation of induced regulatory T cells. Gene expression analysis done on naïve $CD4^+$ T cells stimulated *in vitro* reveals that the number of *GPX1* transcripts is reduced in cells cultured in the presence of recombinant PD-L1 when compared to cells cultured in the presence of control human Ig-Fc. In contrast to these data, flow cytometry data does not reveal any significant differences between the overall abundance of intracellular GPx-1 over the course of 72 hours when comparing $CD4^+$ T cells cultured in the presence and absence of recombinant PD-L1. Using *in vivo* immunization and tumor models, we demonstrate that the overall expression of GPx-1 is higher both in $CD44^+$ T cells derived from the draining lymph node and tumor infiltrating lymphocytes when compared to $CD44^-$ T cells from each microenvironment. To assess the role of GPx-1 during activation and differentiation of naïve $CD4^+$ T cells *in vitro*, we used a small molecule inhibitor of GPx-1, mercaptosuccinate (MS). Our data reveal that the addition of MS reduces the overall number of $CD44^+$ T cells and that the presence of MS increases the oxidative state of $CD4^+$ T cells in a dose dependent manner. These

data support the role of GPx-1 as an important cytosolic and mitochondrial antioxidant enzyme. Our data also reveal that the presence of MS in cell culture media induces the expression of the transcription factor Forkhead box P3 (FoxP3) in a dose-dependent manner that is independent of the presence of TGF-β. Overall, these data suggest that GPx-1 is an important antioxidant enzyme that may play a role in regulating T cell activation and differentiation. Additionally, we provide preliminary evidence to support the idea that the redox tone of the cell may be important for fate determination. Further work is needed to assess the functionality and durability of these FoxP3 expressing T cells, and to address whether or not these cells exhibit the typical characteristics of induced regulatory T cells.

Table of Contents

Figures

Figure 1.1: Selenoprotein synthesis. The SECIS element in the 3' untranslated region of the mRNA (stem loop) recruits SBP (green), which in turn recruits Elongation Factor Selenocysteine (EFSec) (blue) and $tRNA^{Sec}$ (yellow and red). The complex interacts with the ribosome to decode the UGA stop codon as selenocysteine. Selenoproteins are represented as light blue chains, where selenocysteine is magenta. Figure adapted from Berry et al., 2005 (Berry, 2005).

Figure 1.2: Naïve $CD4^+$ T cell differentiation. Naïve $CD4^+$ T cells (labeled as T_H0 cell) can differentiate into several T helper (T_H) cell subsets. The legend for the molecular cartoons represented on each cell can be found in the lower left corner. The diagram above shows the cytokines required for T cell polarization *in vitro*. Polarized cells (center) are labeled by helper cell subtype (T_H1, T_H2, T_H17, and T_{reg}) and by the canonical transcription factors (TBET, Gata-3, RORγt, and Foxp3) that drive their differentiation and effector functions. Additionally, the cytokines produced and their immunological functions are listed on the right for each subset. Adapted from Tato and O'Shea, 2006 (Tato and O'Shea, 2006).

Figure 1.3: **Glutathione peroxidase-1 and proposed catalytic mechanism. (A)** Ribbon representation of the monomeric unit of bovine glutathione peroxidase-1 (PDB ID: 1GP1)(Epp et al., 1983). The catalytic triad is conserved in selenocysteine-containing glutathione peroxidases and is composed of a tryptophan, glutamine, and selenocysteine residue. The catalytic triad is represented as sticks and labeled as trp158 (W158), gln80 (Q80), and sec45 (U45). **(B)** Inactivation of peroxides by GPx-1 involves the formation of a several stable intermediary modifications that are made directly to the active-site selenocysteine. Active GPx-SeH reacts with peroxides (ROOH or H_2O_2) to form selenic acid (SeOH) (no. 1 in figure). One molecule of glutathione (GSH) reduces

GPx-SeOH to GPX-Se-SG and releases water (no. 2 in figure). An additional molecule of GSH reacts with GPx-Se-SG to further reduce the enzyme back to GPx-SeH and release oxidized glutathione (GSSG) (no. 3 in figure). The net reaction is shown in the lower part of the figure. Figure adapted from Lubos et al 2011 (Lubos et al., 2011).

Figure 3.1: $CD4^+$ T cells cultured in the presence of PD-L1-Fc express lower levels of genes that encode for the selenoproteins GPx-1, Sep15, and TR3. $CD4^+$ T cells were activated with 4 μg/mL of each αCD3 and αCD28 in the presence of 5 μg/mL of hIg-Fc or 10 μg/mL of PD-L1-Fc. Cells were harvested after 24 and 48 hrs. Total RNA was isolated and gene expression analysis was performed using quantitative PCR. (A) Expression analysis of genes that encode for selenoproteins using Rpl13a as a reference gene. (B) Expression analysis of genes involved in selenoprotein synthesis. Statistical analysis was performed on triplicates for each condition. ($^*p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$).

Figure 3.2: $CD4^+$ T cells upregulate expression of GPx-1 upon stimulation with anti-CD3 and anti-CD28. MACS purified $CD4^+$ T cells were isolated and cultured $CD4^+$ T cells were activated with 4 μg/mL of each αCD3 and αCD28 in the presence of 5 μg/mL of hIg-Fc or 10 μg/mL of PD-L1-Fc (**A**) Shown here is the gating strategy to isolate live, single $CD4^+$ T cells. (**B**) Histograms of GPx-1 expression in live $CD4^+$ T cells after 24 hrs of stimulation. $CD4^+$ T cells cultured in the presence of recombinant hIg-Fc or PD-L1-Fc are shown in green and red, respectively. Untreated cells (black) are reported after 24 hrs only because cell death was so significant in the untreated controls for 48 and 72 hrs. (**C**) Data generated from the same experiment after 48 and 72 hrs of stimulation.

Figures 3.3: Activated T cells derived from the dLN express higher levels of GPx-1 in two in vivo models. (**A**) Gating strategy to isolate $CD4^+$ live-single cells for FACS analysis. (**B**) Representative plots for $CD4^+$ T cells plotted for the expression of GPx-1 vs. CD44 in LN of four control mice (left) and nine mice immunized with NP-Ova (center). Quantitative analysis was performed on the percentages of $CD44^+$ $CD4^+$ T cells from control mice and immunized mice (Right). (**C**) Shown on the left is a representative flow plot of $CD4^+$ T cells from the dLN of immunized mice showing the expression of CD44 and GPx-1. In the center panel, $CD44^+$ T cells from the dLN of mice immunized with NP-Ova (purple) were compared to $CD44^-$ T cells (cyan) from the same dLN. The MFI for GPx-1 expression of $CD4^+$ T cells from the dLN of nine mice are quantified on the right. (**D**) Shown on the left is a representative flow plot for $CD4^+$ T cells harvested from the dLN of mice challenged with MC38 tumor 24 days after s.c. implantation. Histograms of $CD44^+$ (purple) and $CD44^-$ (cyan) from the same tumor are shown in the middle panel. The MFI for GPx-1 expression of $CD4^+$ T cells that were derived from the dLN of four mice are quantified on the right ($^*p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$).

Figure 3.4: GPx-1 correlates with CD44 expression but not with the expression of CD62L or FoxP3. Shown here are representative plots of $CD4^+$ T cells from the dLN of mice implanted with MC38 cells. (**A**) Expression of GPx-1 in activated versus naïve CD4 T cells from the dLN. On the left, $CD4^+$ T cells are plotted for the expression of CD44 vs. CD62L. $CD44^+$ $CD62L^-$ cells are considered activated. A histogram for each quadrant is plotted in the center panel to compare the relative expression of GPx-1 in each population. The isotype control shown is for bulk $CD4^+$ T cells. The results are quantified on the right. (B) Expression of GPx-1 in $FoxP3^+$ versus $FoxP3^-$ $CD4^+$ T cells derived from the dLN of mice implanted with MC38 cells. On the left, $CD4^+$ T cells are plotted for CD44 vs. FoxP3 expression. A histogram for each quadrant is plotted in the center

panel to compare the relative expression of GPx-1 in each population. The results are quantified on the right. The data presented here are representative of two individual experiments (*$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$).

Figure 3.5: Tumor infiltrating $CD4^+$ T cells express higher levels of GPx-1 than $CD4^+$ T cells from the draining lymph node. Representative histograms of $CD4^+$ T cells from the dLN and tumors of mice implanted s.c. with MC38 tumor cells. (**A**) On the left, bulk $CD4^+$ from the dLN (blue) and tumor (red) are compared for GPx-1 expression in a histogram. In the right panel, the geometric mean fluorescence intensity (MFI) for each sample is quantified. (**B**) $CD44^+$ T cells harvested from the dLN and tumor are compared for their expression of GPx-1. On the left, a representative histogram of $CD44^+$ $CD4^+$ T cells from the dLN (blue) and tumor (red) are compared for expression of GPx-1. On the right, the geometric mean fluorescence intensity (MFI) for each sample is quantified. (**C**) $CD44^+$ $FoxP3^+$ T cells harvested from the dLN and tumor are compared for their expression of GPx-1. On the left, a representative histogram of $CD44^+$ $FoxP3^+$ $CD4^+$ T cells from the dLN (blue) and tumor (red) are compared for expression of GPx-1. On the right, the geometric mean fluorescence intensity (MFI) for each sample is quantified. The MFI for GPx-1 expression for each isotype control is reported for every population (*$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$).

Figure 3.6: Naïve $CD4^+$ T cells cultured in the presence of the GPx-1 inhibitor, mercaptosuccinate, induces FoxP3 in naïve $CD4^+$ T cells in a dose dependent manner that is independent of TGF-β. Naïve $CD44^-$ $CD62L^+$ $FoxP3^-$ $CD4^+$ T cells were sorted from FoxP3.GFP reporter mice and stimulated with αCD3 and αCD28 in the presence or absence of hIG-Fc or PD-L1-Fc and plus or minus 2 ng/mL of TGF-β. T cell activation was measured in the presence and absence of the GPx-1 inhibitor, mercaptosuccinate (MS) at concentrations of 0, 1, 5, and 10 mM

at time zero (**A**) $CD4^+$ T cells activation was assessed by quantifying the number of Live $CD4^+$ T cells/sample. (**B**) $CD4^+$ T cell activation was measured by the percentage of $CD44^+$ $CD4^+$ live T cells. (**C**) Oxidative stress was measured using cellROX deep red reagent and quantified by reporting the mean geometric fluorescence intensity for each condition and plotted in a bar graph (**D**) Representative plots of live $CD4^+$ T cells cultured in the presence of PD-L1-Fc or hIg-Fc plus or minus the addition of TGF-β. FoxP3 expression is represented on the y-axis and CD4 expression is represented on the x-axis. In the bar graphs on the right, the percentage of $FoxP3^+$ T cells are reported as percentage of live-$CD4^+$ T cells (top panel). The MFI of FoxP3 of live-$CD4^+$ $FoxP3^+$ T cells is quantified (bottom panel). (**E**) The percentage of live-$CD4^+$ $CD25^+$ T cells is quantified (left) and the MFI for CD25 is quantified (right) for each condition.

Tables

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Chapter 1: Background

1.1. Introduction:

The intersection between nutrition and human health has been studied for decades. Technological advances in bioinformatics, metabolomics, and epidemiology have helped elucidate the role that nutrient availability and processing plays in a plethora of human disease-states and conditions (Astarita and Langridge, 2013; Boeing, 2013; Mayne et al., 2016). Unlike most tissues that assume a more rigid metabolic status, the immune system is enriched for cells that not only exhibit diverse metabolic profiles but also extraordinary plasticity in their ability to redirect nutrient metabolism and adapt to their local environments (Green, 2012; Verbist et al., 2012). The goal of this thesis was to determine how T lymphocytes (T cells) regulate selenium metabolism during their initial activation. Microarray analysis of T cells activated in the presence of ligands for the surface protein programmed cell death-1 (PD-1) could modulate pathways involved in selenium metabolism during T cell activation *in vitro*. These microarray analyses suggested changes in pathways modulating selenium metabolism in T cells when PD-1 was engaged by its ligands under conditions that induced naïve $CD4^+$ T cells to become $FoxP3^+$ regulatory T cells. Thus, we tested the hypothesis that the PD-1 pathway regulates the expression of selenoprotein genes. One selenoprotein gene that appeared to be highly regulated during differentiation *in vitro* was the *GPX1* gene, which encodes for the selenoprotein glutathione peroxidase-1 (GPx-1). Our results led us to investigate the relationship between the selenoprotein glutathione peroxidase-1 and T cell activation. We focused on determining the expression of GPx-1 in different models of T cell activation and correlated its expression with the activation marker CD44. Additionally, we asked what impact GPx-1 inhibition would have on T cells during activation *in vitro*. To provide a context for these studies, the background will be divided into eight sections. First, I will introduce

selenoproteins (Section 1.2) and highlight key aspects of the immune system that pertain to my research (Section 1.3). Additionally, I will provide information on costimulatory and coinhibitory receptors (e.g. PD-1) that regulate T cell activation (Section 1.4) and I will review what is already known about selenoproteins and GPx-1 in the context of T cell biology (Sections 1.5 and 1.6, respectively). Finally, I will close the introduction with a summary of the questions that are addressed in this thesis (Section 1.7).

1.2. Selenoproteins

Selenium is an essential trace element for a number of organisms, including humans. Selenium is important for both proper mammalian development (Kohrle, 2000) and immune function (Huang et al., 2012), and its deficiency has been correlated with a number of pathophysiological conditions such as heart disease (Benstoem et al., 2015), cancer (Fernandes and Gandin, 2015; Wrobel et al., 2016), and inflammation (Huang et al., 2012). Its biological function is mediated by the amino acid selenocysteine, which is incorporated into a discrete set of proteins, known as selenoproteins, that perform a number of functions essential to the cell and the organism. Selenoproteins are present in all three evolutionary domains of life: Eukarya, archea, and eubacteria. The pathway for the biosynthesis of selenocysteine was resolved only a decade ago thanks to technological advances in genomics and comparative biology (Barrett et al., 2013; Yuan et al., 2006). Selenocysteine is the only amino acid whose synthesis occurs on its own tRNA, Sec $tRNA^{[Ser]Sec}$ (Xu et al., 2007). Sec $tRNA^{[Ser]Sec}$ is encoded by the *trsp* gene. As its shorthand notation implies, Sec $tRNA^{[Ser]Sec}$ is initially aminoacylated with serine and is subsequently converted into selenocysteyl-tRNA by the enzyme selenocysteine synthase, which replaces the hydroxyl group of serine with selenophosphate to form selenocysteyl-tRNA (sec-tRNA). In addition to its unique biosynthesis, selenocysteine is alternatively encoded by an in-frame UGA

stop codon in a manner that is dependent on the presence of a *cis*-acting element known as the Selenocysteine Insertion Sequence (SECIS) located in the 3' untranslated region of the selenoprotein mRNA (Berry et al., 1991; Grundner-Culemann et al., 1999). In addition to the SECIS element, successful incorporation of selenocysteine into the growing peptide strand is dependent on the expression of SECIS-binding proteins (SBP1/2), Sec-specific elongation factor (EFsec), and for a number of additional elongation factors, accessory proteins, and regulatory mechanisms that are unique to each selenoprotein (**Figure 1.1**) (Labunskyy et al., 2014). The synthesis of several selenoproteins is regulated by selenium availability, thereby resulting in the hierarchal expression of different selenoproteins in the context of selenium deficiency, where housekeeping selenoproteins are preferentially translated over stress-related selenoproteins (i.e. a selenoproteins whose function is not absolutely necessary for viability or survival but help reduce cellular stress) (Carlson et al., 2007).

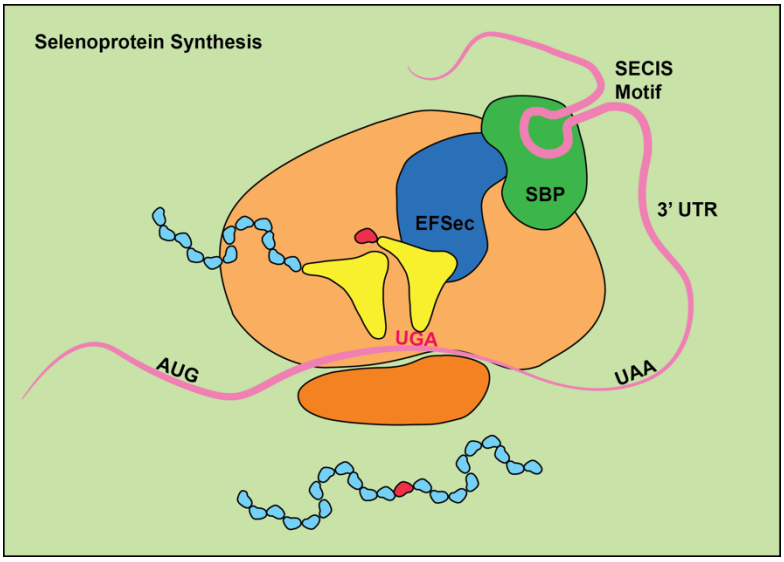


Figure 1.1: Selenoprotein synthesis. The SECIS element in the 3' untranslated region of the mRNA (stem loop) recruits SBP (green), which in turn recruits Elongation Factor Selenocysteine (EFSec) (blue) and $tRNA^{Sec}$ (yellow and red). The complex interacts with the ribosome to decode the UGA stop codon as selenocysteine. Selenoproteins are represented as light blue chains, where selenocysteine is magenta. Figure adapted from Berry et al., 2005 (Berry, 2005).

There are more than 50 known families of selenoproteins and their distribution varies greatly between species. Although selenoproteins can be found in all three domains of life, a number of organisms have lost selenoproteins from their genomes altogether (Lobanov et al.,

2008). The selenoproteome of humans is composed of 25 proteins (24 in mice). The majority of these selenoproteins serve to catalyze redox reactions (Labunskyy et al., 2014). Despite this singular functionality, selenoproteins serve a variety of important biological roles (**Table 1.1**). The selenoproteome of different tissue types varies by patterns of gene expression and how their expression is regulated (Labunskyy et al., 2014; Reeves and Hoffmann, 2009). In mammals, the selenoprotein iodothyronine deodinases are absolutely necessary for the activation and deactivation of the thyroid hormones, thyroxine and 3,5,3'-triiodothyronine (Labunskyy et al., 2014). Furthermore, the selenoproteins glutathione peroxidase and thioredoxin enzymes are responsible for mitigating oxidative stress at the cellular and organismal level(Arnér and Holmgren, 2000; de Haan et al., 1998). Given the diverse roles of known selenoproteins, our goal was to determine the role of selenoproteins in the immune system.

1.3 T Lymphocytes and their Effector Subtypes

The immune system serves to both protect organisms from foreign invaders and transformed cells as well as to promote tolerance to self and commensal organisms. These dual functions are regulated at the systemic and local level via the efforts of both immune and non-immune cells using diverse mechanisms of communication (e.g. cell-to-cell contact and the secretion of soluble factors). The immune system is divided into the innate and adaptive branches, where both work together to eliminate pathogens, transformed cells, or dying tissue. Innate immune cells are primarily derived from the myeloid progenitor cells and consist of macrophages, dendritic cells, and granulocytes (e.g. basophils and neutrophils). In contrast, adaptive immune cells are derived from lymphoid progenitors and are composed only of T lymphocytes (T cells) and B lymphocytes (B cells). Notable exceptions include the lymphoid natural killer (NK) cells, which function as innate immune cells that help clear infected and transformed cells (Zúñiga-

Pflücker, 2004). If a pathogen cannot be cleared by innate defenses, innate immune cells will elicit an inflammatory response that activates dendritic cells that are equipped to present antigen to cells of the adaptive immune system and are thus named antigen-presenting cells (APCs). Dendritic cells convey important information to the adaptive immune system (such as the location of the infection and the type of pathogen) and are important regulators of the adaptive immune response. B cells, macrophages, and some stromal cells also act as APCs in important biological contexts (e.g. T cell-dependent B cell activation and thymic selection of T cells). When activated by antigen,

Selenoprotein	Abbreviation(s)	Function
Glutathione peroxidase 1**	GPx1, GPX1	Cytosolic and mitochondiral glutathione peroxidase
Glutathione peroxidase 2	GPx2, GPX2	Gastrointestinal glutathione peroxidase
Glutathione peroxidase 3	GPx3, GPX3	Plasma glutathione peroxidase
Glutathione peroxidase 4**	GPx4, GPX4	Phospholipid hydroperoxide glutathione peroxidase
Glutathione peroxidase 6	GPx6, GPX6	Olfactory glutathione peroxidase
Iodothyronine deodinase 1	DI1, D1, DIO1	Thyroid hormone-activating iodothyronine deodinase
Iodothyronine deodinase 2	DI2, D2, DIO2	Tissue-specific thyroid hormone-activating iodothyronine deodinase
Iodothyronine deodinase 3	DI3, D3, DIO3	Tissue-specific thyroid hormone-deactivating iodothyronine deodinase
Thioredoxin reductase 1**	TR1, TrxR1, TXNRD1	Reduction of cytosolic thioredoxin
Thioredoxin reductase 2	TGR, TR2, TrxR3, TXNRD3	Testis-specific thioredoxin reductase
Thioredoxin reductase 3**	TR3, TrxR2, TXNRD2	Reduction of mitochondrial thioredoxin and glutaredoxin
Methionine-R-sulfoxide reductase	MsrB1, SelR, SelX, MSRB1	Reduction of oxidized methionine residues
Selephosphate synthetase 2*	SPS2, SEPHS2	Involved in the synthesis of selenoproteins
Selenoprotein W	SelW, SEPw1	Unknown
Selenoprotein T*	SelT	Unknown
Selenoprotein H*	SelH	Unknown
Selenoprotein V	SelV	Unknown
Selenoprotein I	SelI, SEPI, EPT1	Unknown
15 kDa Selenoprotein**	Sep15	Putative role in quality control of protein folding in the ER
Selenoprotein M	SelM, SEPM	Unknown
Selenoprotein K*	SelK	Putative role in ER-associated degredation
Selenoprotein S*	SelS, SEPS1, VIMP	Putative role in ER-associated degredation
Selenoprotein O	SelO	Unknown
Selenoprotein N	SelN, SepN, SEPN1	Putative role during muscle development
Selenoprotein P	SelP, SEPP1	Se transport

Table 1.1: List of known human selenoproteins. *Selenoproteins documented to be expressed in T cells at the level of mRNA. **Selenoproteins documented to be expressed at the protein level. Table and information adapted from Labunsky et al., 2014 and Carlson et al., 2010 (Carlson et al., 2010; Labunskyy et al., 2014)

T and B cells give rise to antigen-specific clones, which differentiate into both effector cells and memory cells. The induction of memory lymphocytes distinguishes the adaptive immune system from the innate immune system, in that memory lymphocytes are capable of responding more quickly when re-challenged with the same pathogen.

T and B cells evolved mechanisms to produce variable-antigen receptors, known as the T cell receptor (TCR) and B cell receptor (BCR), respectively. T cells are integral players of the adaptive immune system. They directly mediate cellular immunity and enhance humoral immunity by optimizing B cell responses. Unlike B cells, which mature in the bone marrow, precursor T cells migrate from the bone marrow to the thymus where they complete development. T cell development is a complex process that culminates with the successful rearrangement of a set of genes that encode for the TCR and commitment to either the $CD4^+$ or $CD8^+$ lineage. This process is orchestrated by a number of receptor-ligand interactions that occur between T cells and the thymic stroma, where the latter present self-antigen in the form of peptide fragments via Major Histocompatibility Complex (MHC) proteins I and II. Successful signaling through the rearranged TCR by ligation with MHC-I or -II expressed on thymic stromal cells is necessary for survival (Zúñiga-Pflücker, 2004). T cell selection occurs via both positive and negative selection, where positive selection occurs when the TCR recognizes MHC and peptide (pMHC). Commitment to either the $CD8^+$ or $CD4^+$ lineage occurs during positive selection and depends on the ability of the TCR to recognize MHC-I or MHC-II, respectively. Negative selection occurs when the TCR interacts too strongly with pMHC, thereby inducing cell death. This is an important process by which the thymus eliminates T cells that react against self-antigens. T cells that survive both positive and negative selection and receive the proper survival factors enter the periphery as naïve T cells, where they circulate between lymphoid organs surveying for their cognate pMHC.

T cell activation occurs when a T cell interacts with an APC (e.g. dendritic cells) in the context of inflammation. T cell activation requires at least two signals. The first signal occurs when the T cell engages with pMHC on the surface of an APC via its TCR. During an infection, APCs will take up foreign antigen and present it on their MHC-I and -II as peptide fragments. TCR ligation with its cognate pMHC induces T cell intrinsic signal cascades, which promote clonal expansion and survival (Langhoff and Steinman, 1989). Additional signals through the co-stimulatory receptor CD28 are also necessary for proper T cell activation, and link innate and adaptive immunity by providing the essential second signal. Activation of innate immune cells during inflammation results in the up-regulation of ligands for CD28, such as CD80/B7-1 and CD86/B7-2. Signaling through CD28 provides important survival signals to T cells and promotes their function. Additionally, this second signal or "danger signal" is a pivotal checkpoint that helps reduce the chance that auto-reactive T cells will get activated in the periphery. in part by inducing T cell anergy (Matzinger, 2002; Medzhitov and Janeway, 2000). T cells that receive only signal one are desensitized to antigen and considered anergic (Schwartz, 2003). Some activated T cells will differentiate into effector T ($T_{\mathbf{eff}}$) cells, which directly assist with pathogen clearance, whereas others will differentiation into memory cells, which are major drivers of immunological memory and a characteristic feature of the adaptive immune response.

Seeing as $CD8^+$ and $CD4^+$ T cells respond to different pMHCs, and that they exhibit unique transcriptomes, activation induces distinct responses and effector functions in each lineage. Activated $CD8^+$ T cells are called cytotoxic T cells (CTLs) and their primary function is to mediate cellular immunity by targeting infected or transformed cells for cell death. In contrast, activated $CD4^+$ T cells or T helper ($T_{\mathbf{H}}$) cells help orchestrate the immune response so that it can most optimally clear and resolve infection. $T_{\mathbf{H}}$ cells promote cytotoxic cellular immunity, enhance

antibody production, and produce cytokines that serve to polarize the immune response so that the least amount of collateral damage is done at the site of infection and to the organism at large. Cytokines are a broad category of secreted proteins that are produced by immune and non-immune cells to maintain cellular homeostasis and to help coordinate immune responses. Binding of cytokines with their cognate membrane bound receptors provides signals to cells that alter their behavior (e.g. modulate gene expression, alter cell morphology and mobility, and can promote survival). T cells both produce and respond to cytokines during their lifetime. During an infection, many cytokines work in a positive feedback loop by which they promote the differentiation of T_H cells into particular T_H cell subsets (e.g. T_H1, T_H2, T_H17, etc). Each T_H cell subset perpetuates this signal by producing the same cytokine and/or similarly functioning cytokines that polarize the immune response against specific types of pathogens (**Figure 1.2**). For example, T_H1 cells promote the clearance of intracellular pathogens by producing cytokines that activate macrophages and CTLs. In contrast, T_H2 and T_H17 cells promote the clearance of extracellular pathogens by secreting cytokines that enhance barrier functions and promote tissue inflammation, respectively (Hirahara and Nakayama, 2016). Each T_H cell subset is characterized by a canonical transcription factor as shown in **Figure 1.2**. However, T_H cells exhibit a certain level of transcriptional plasticity that allows them to adapt to an infection over the course of time (Bluestone et al., 2009). T_H cells are necessary for the proper clearance of a number of infections and their depletion results in severe immunodeficiency (Okoye and Picker, 2013). Importantly, when the activity of T_H cells is left unchecked, severe immunopathology and even autoimmunity can ensue, which can lead to irreversible tissue damage and compromise organ function (Hirahara and Nakayama, 2016).

Regulatory T (T_{regs}) cells are a distinct subset of $CD4^+$ T cells that directly counter these pro-inflammatory immune responses. There are several types of T_{regs} cells, but most are $CD4^+$ cells

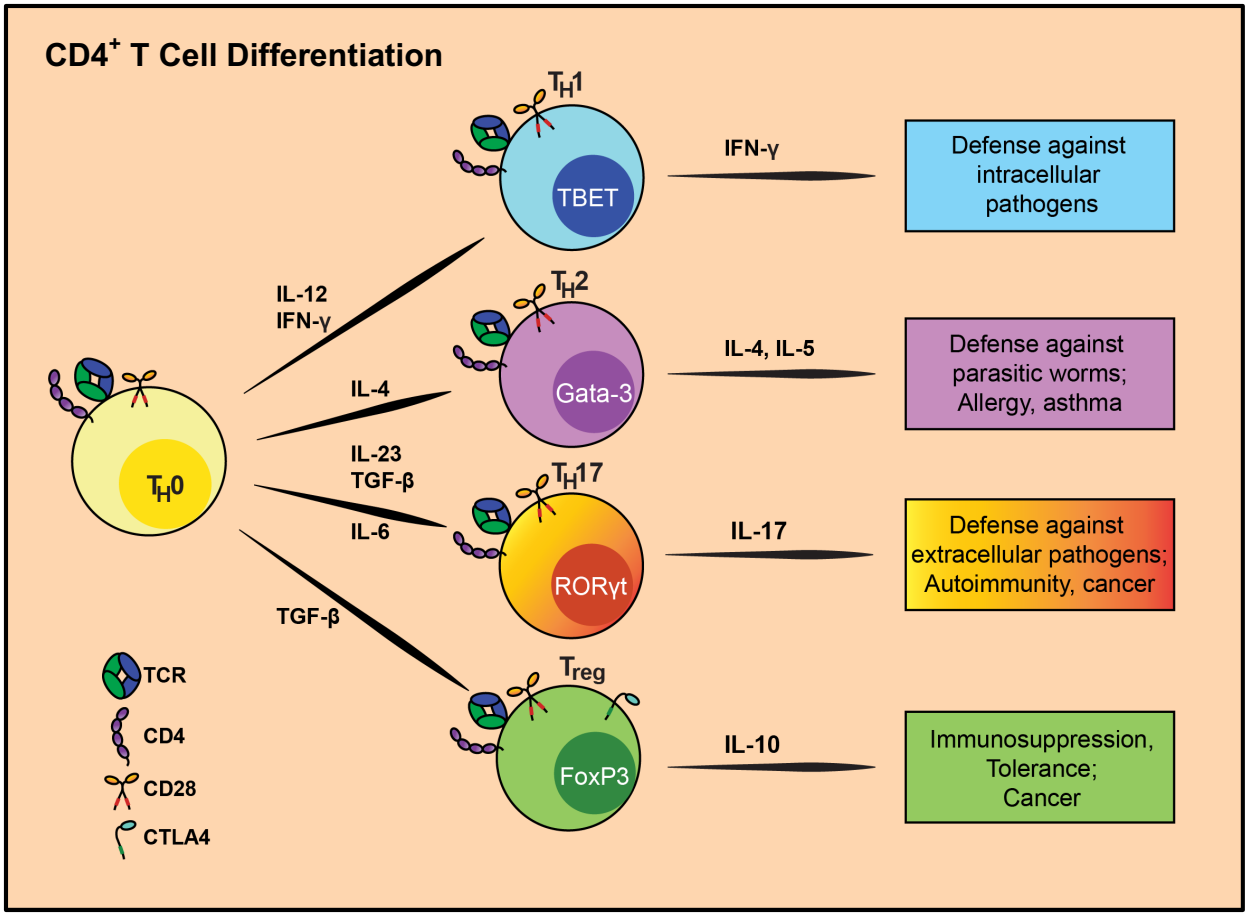


Figure 1.2: Naïve CD4$^+$ T cell differentiation. Naïve CD4$^+$ T cells (labeled as T_H0 cell) can differentiate into several T helper (T_H) cell subsets. The legend for the molecular cartoons represented on each cell can be found in the lower left corner. The diagram above shows the cytokines required for T cell polarization *in vitro*. Polarized cells (center) are labeled by helper cell subtype (T_H1, T_H2, T_H17, and T_{reg}) and by the canonical transcription factors (TBET, Gata-3, RORγt, and Foxp3) that drive their differentiation and effector functions. Additionally, the cytokines produced and their immunological functions are listed on the right for each subset. Adapted from Tato and O'Shea, 2006 (Tato and O'Shea, 2006).

that produce the anti-inflammatory cytokine, interleukin-10 (IL-10) (Fontenot et al., 2003; Fontenot et al., 2005; Rubtsov et al., 2008). They inhibit both CTL and T_H cell responses directly by suppressing their activity and indirectly by suppressing the activity of APCs (Fontenot et al., 2005; Hou et al., 2015). T_{reg} cells are necessary not only for contracting immune responses after an infection is cleared but also for promoting tolerance to self and commensal organisms. The transcription factor forkhead box P3 (FoxP3) drives the immunoregulatory phenotype of T_{reg} cells, and its deficiency causes the severe immune pathology observed in immunodysregulation

polyendocrinopathy enteropathy X-linked (IPEX) syndrome, a fatal autoimmune syndrome that normally presents during infancy and is caused by mutant FoxP3 or aberrantly expressed FoxP3 (Bennett et al., 2000; Brunkow et al., 2001). It is characterized by lymphocytic infiltrate in the small intestine and inflammation of the kidneys, liver, or pancreas (Wildin et al., 2002). This phenotype highlights the functional role that T_{reg} cells play in modulating immune responses. FoxP3 expressing T_{reg} cells can originate directly from the thymus (cT_{reg}) or they can be derived from FoxP3$^-$ naïve CD4$^+$ T cells in the periphery (pT_{reg}). While all of the mechanisms responsible for the induction of T_{reg} cells *in vivo* are not resolved, the *in vitro* differentiation of naïve CD4$^+$ T cells into FoxP3 expressing T_{reg} cells requires the growth factor transforming growth factor-β (TGF-β). Signaling through the TGF-β receptor activates both the transcription factors nuclear factor of activated T cells (NFAT) and SMAD-3, which work together to stabilize and promote histone acetylation in the the *FoxP3* enhancer region (Tone et al., 2008).

1.4. Costimulatory and Coinhibitory Receptors in T lymphocytes

One key mechanism by which immune homeostasis is achieved is through the activity of T cell costimulatory and coinhibitory pathways. A number of costimulatory and coinhibitory receptors have been characterized, many of which exhibit synergistic functions that either promote T cell activation, survival, and inflammation or attenuate T cell function by suppressing activation, effector function, and by promoting cell death (Gergely et al., 1999; Unkeless and Jin, 1997). In addition to the costimulatory receptor CD28, inducible T cell costimulator (ICOS) is another example of a costimulatory receptor that is important for maintaining both the T_H1 and T_H2 cell phenotype (Khayyamian et al., 2002). Additionally, there are a number of coinhibitory receptors that help dampen the immune response in the context of systemic or chronic infection (Greenwald et al., 2002; Leibson, 2004; Wang and Chen, 2004). Cytotoxic T-cell-associated protein 4

(CTLA4), programmed cell death 1 (PD-1), and lymphocyte activation gene-3 (LAG3) are all examples of coinhibitory receptors that function as important immunoregulatory checkpoints that serve to limit T cell activation, proliferation, and inflammation. In addition to coinhibitory pathways being essential to the resolution of immune responses, they also mediate T cell tolerance towards self-antigen and restore immune homeostasis. Importantly, these immunoregulatory checkpoints can be exploited by microbes and tumors to evade immune eradication (Frazier et al., 2010; Spranger, 2016; Ye et al., 2015). Temporal and differential patterns of expression of both costimulatory and coinhibitory receptors on the surfaces of both T_{eff} cells and T_{reg} cells is essential for modulating the immune response so that the organism as a whole endures the least amount of tissue damage during the course of an infection (Liu et al., 2015; Zhou et al., 2008).

The coinhibitory molecule Programmed Cell Death-1 (PD-1) plays an important immunoregulatory role by reducing T cell activation and effector function (Bennett et al., 2000; Bennett et al., 2003; Freeman et al., 2000). Inhibitory signals through the PD-1 pathway control induction and maintenance of tolerance to self-antigens (Nishimura et al., 2001; Sharpe et al., 2007). The inhibitory effect of PD-1 is mediated by engagement with its ligands PD-L1 and PD-L2 (Latchman et al., 2001; Yamazaki et al., 2002). which can be expressed by immune and non-immune cells. The pro-inflammatory cytokine, interferon-γ is a powerful driver of PD-L1 expression (Freeman et al., 2000). Importantly, the PD-1 pathway has been shown to induce T_{reg} cells from naïve $CD4^+$ T cells and T_H1 cells *in vitro* and *in vivo* (Francisco et al., 2009). The PD-1 pathway exerts important immunoregulatory effects during infection, cancer, and autoimmunity and this knowledge has been translated to therapy to treat some forms of cancer. While clinical outcomes have been promising, the basic science behind how PD-1 regulates T cell activation, differentiation, and effector function is only beginning to be elucidated. Recent evidence suggests

that PD-1 may regulate T cell responses in part by altering various aspects of cellular metabolism (Parry et al., 2005; Patsoukis et al., 2015; Tkachev et al., 2015).

1.5. Selenoproteins and T Lymphocytes

As mentioned earlier, activation of T cells in the periphery requires both ligation of the TCR with its cognate pMHC and co-stimulatory signals. Successful signaling through the TCR requires the production of reactive oxygen species (ROS) (e.g. H_2O_2 and NO), where both under and over-production of ROS can limit T cell activation, function, and viability. (Hildeman, 2004; Kwon et al., 2003; Williams and Kwon, 2004). Seeing as a number of selenoproteins are important antioxidant enzymes, it is possible that these enzymes may function to not only protect T cells from oxidative stress but also modulate T cell responses. A number of cell-specific and germline deficient mouse models have been used to determine the role that selenoproteins and ROS play in T cell responses, as summarized below (Carlson et al., 2010; Shrimali et al., 2008).

Studies of T cell specific ablation of the *trsp* gene (the gene that encodes for Sec $tRNA^{[Ser]Sec}$) demonstrate that the selenoproteome is important for T cell development and activation *in vivo* (11). *Trsp* deficient mice exhibit decreased pools of mature T cells, a smaller fraction of circulating $CD8^+$ T cells, and defective T cell-dependent antibody responses (Shrimali et al., 2008). *Trsp* deficient T cells have abrogated proliferation potential, produce higher levels of ROS, and exhibit higher levels of oxidative stress (i.e., ROS-induced damage to proteins, membrane lipids, etc.) at basal states (11). These data illustrate the role of selenoproteins as antioxidant enzymes and support the notion that ROS levels may impact both T cell viability and function (Labunskyy et al., 2014; Wrobel et al., 2016). Importantly, the addition of the antioxidant N-acetylcysteine to cell media rescued the ability for *trsp* deficient T cells to proliferate in a dose-

dependent manner, thereby demonstrating the importance of the antioxidant function of selenoproteins in regulating T cell responses.

Murine T cells express twenty of the 24 selenoprotein genes encoded by the mouse genome; however, only a handful can be detected at the protein level (Carlson et al., 2010; Gladyshev et al., 1999). Among the selenoproteins detected at both mRNA and protein levels are glutathione peroxidase-1 (GPx-1), 15 kDa selenoprotein (Sep15), glutathione peroxidase-4, selenoprotein P, selenoprotein K, selenoprotein T, and selenoprotein H (Carlson et al., 2010). GPx-1 is both transcribed and translated at high levels of expression in T cells (Gladyshev et al., 1999). Given its expression pattern in T cells, we focused on investigating the function of GPx-1 in T cell activation.

1.6. Glutathione Peroxidase-1

GPx-1 is a member of the glutathione peroxidase family of enzymes, which are responsible for catalyzing the reduction of H_2O_2 or organic peroxides to water or alcohol. Mammalian GPx-1 exists as a homotetramer with a molecular mass between 83 and 95 kDa, where each monomer hovers around 200 amino acids depending on the species and allelic variant (Lubos et al., 2011). GPx-1 is a crucial antioxidant enzyme that localizes both to the cytosol and mitochondria (Esworthy et al., 1997; Singh et al., 1994). In selenocysteine-containing GPx enzymes, the redox cycle involves interconversion between selenol (GPx-SeH), selenic acid (GPx-SeOH), and selenenyl sulfide (GPx-SeSG) intermediates, where GPx-SeH is the active form of the enzyme (**Figure 1.3**). It is important to note that GPx-1 activity depends on the availability of glutathione (GSH) in the cell, whereby GSH acts as the reducing agent to restore the oxidized GPx-SeSG intermediate back into the active GPx-SeH form (Amir Aslani and Ghobadi, 2016).

GPx-1 expression and activity is regulated at the transcriptional, post-transcriptional, translational, and post-translational levels (Lubos et al., 2011). For example, oxygen tension and ROS can induce the transcription of *GPx-1* via pathways dependent on the presence of oxygen response elements (ORE) and the transcription factor nuclear factor κB (NFκB), respectively (Cowan et al., 1993; Zhou et al., 2001). Furthermore, inhibition of the nutrient-sensing signaling protein, mammalian target of rapamycin (mTOR) can increase GPx-1 protein levels in lymphocytes (Reinke et al., 2014).

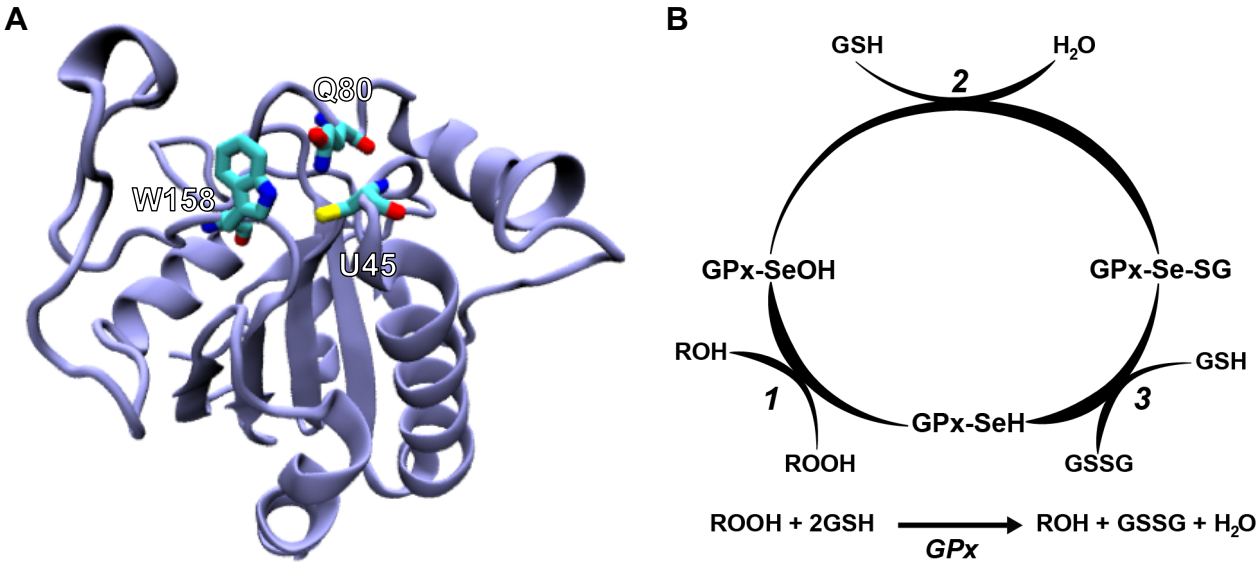


Figure 1.3: **Glutathione peroxidase-1 and proposed catalytic mechanism. (A)** Ribbon representation of the monomeric unit of bovine glutathione peroxidase-1 (PDB ID: 1GP1)(Epp et al., 1983). The catalytic triad is conserved in selenocysteine-containing glutathione peroxidases and is composed of a tryptophan, glutamine, and selenocysteine residue. The catalytic triad is represented as sticks and labeled as trp158 (W158), gln80 (Q80), and sec45 (U45). **(B)** Inactivation of peroxides by GPx-1 involves the formation of a several stable intermediary modifications that are made directly to the active-site selenocysteine. Active GPx-SeH reacts with peroxides (ROOH or H_2O_2) to form selenic acid (SeOH) (no. 1 in figure). One molecule of glutathione (GSH) reduces GPx-SeOH to GPX-Se-SG and releases water (no. 2 in figure). An additional molecule of GSH reacts with GPx-Se-SG to further reduce the enzyme back to GPx-SeH and release oxidized glutathione (GSSG) (no. 3 in figure). The net reaction is shown in the lower part of the figure. Figure adapted from Lubos et al 2011 (Lubos et al., 2011).

1.7. Glutathione Peroxidase-1 and T Lymphocyes

There are few publications that address the impact of germline GPx-1 deficiency on T cells. Homozygous null GPx-1 deficient ($^{-/-}$) mice are viable, exhibit no gross phenotypic differences when compared to their wild-type counterparts, but they are sensitized to oxidative stress-inducing

agents such as paraquat or hydrogen peroxide (de Haan et al., 1998). Importantly, GPx-1$^{-/-}$ mice exhibit altered immune responses (Kim et al., 2014a; Kim et al., 2014b; Won et al., 2010). For example, GPx-1 deficient mice are protected from ovalbumin-induced allergic asthma. Eosinophil infiltration, goblet cell hyperplasia, collagen deposition, and airway hyperresponsiveness are all attenuated in the lungs of the GPx1 deficient mice when challenged with intranasal injections of ovalbumin when compared to wild-type mice (Won et al., 2010). After *in vitro* stimulation with anti-CD3 and anti-CD28, GPx-1 deficient CD4$^+$ T cells exhibit a hyperoxidative state, produce higher levels of the cytokine interleukin-2 (IL-2), and proliferate more quickly than wild-type cells. Additionally, GPx-1 deficient CD4$^+$ T cells cultured in T_H17 or T_H2 skewing conditions produce less subtype specific cytokines (e.g. interleukin-17 or interleukin-4) and exhibit a T_H1-like phenotype. Taken together, these data suggest that GPx-1-dependent regulation of intracellular ROS is important not only for regulation of CD4$^+$ T cell viability and proliferation but also for modulating differentiation into T cell effector subtypes (Won et al., 2010).

In addition, GPx-1 deficient mice exhibit attenuated disease in several autoimmune models (Kim et al., 2014a; Kim et al., 2014b). Double knockout mice lacking both GPx-1 and the glutathione-independent antioxidant enzyme Catalase (Cat) show an attenuated response to dextran sodium sulfate (DSS)-induced colitis and exhibit hyperfunctional T_{reg} cells. Interestingly, administration of the antioxidant, N-acetylcysteine (NAC) aggravated DSS-induced colitis and decreased T_{reg} cell function in double knockout mice, thereby supporting a role for ROS as an important mediator of T cell effector function (Kim et al., 2014b). Similarly, in the imiquimod-induced psoriatic dermatitis model GPx-1$^{-/-}$ mice also exhibit hyperfunctional T_{reg} cell responses. Consistent with the DSS-induced colitis model, T_{reg} cell function was enhanced in GPx-1$^{-/-}$ mice as measured by their ability to suppress CD8$^+$ T cell responses (Kim et al., 2014a).

Section 1.8: Questions Addressed in this Thesis

In this thesis, we investigated the role that the PD-1 pathway plays in regulating the expression and function of selenoproteins. Microarray data generated in our lab from *in vitro* induced into T_{reg} cells suggested that engagement of PD-1 on naïve $CD4^+$ T cells modulates the expression of pathways involved in selenoprotein synthesis. Thus, we sought to determine more specifically whether PD-1 engagement on naïve T cells impacts the expression of both selenoprotein genes and genes involved in selenium metabolism. We focused on GPx-1 because it is one of the top selenoprotein genes expressed upon T cell activation. We first compared the expression pattern of GPx-1 at the protein level during T cell activation and differentiation *in vitro* in both the presence and absence of the PD-1 ligand PD-L1. Additionally, we sought to determine GPx-1 expression *in vivo* using two models of T cell mediated immunity. Our results led us to assess the role that GPx-1 plays in T cell activation and differentiation, thus we employed a GPx-1 inhibitor to determine the effect that GPx-1 inhibition has on T cell activation and differentiation *in vitro*.

Chapter 2: Materials & Methods

Section 2.1: Mice. Six to 12-week-old wild-type C57BL/6 mice or Foxp3.GFP reporter C57BL/6 mice were used for *in vitro* experiments (Bettelli et al., 2006). Wild-type C57BL/6 mice were used for MC38 tumor experiments and C57BL/6-Tg OT-II (TcraTcrb) mice were used for the immunization studies. The genotypes of the mice were verified by PCR and flow cytometry. Harvard Medical School is accredited by the American Association of Accreditation of Laboratory Animal Care. Mice were maintained in a pathogen-free facility and used according to the Harvard Medical School Standing Committee on Animals and National Institutes of Health Animal Care Guidelines. Animal protocols were approved by the Harvard Medical School Standing Committee on Animals.

Section 2.2: Cell Purification. $CD4^+$ T cells were isolated from the spleens of C57BL/6 male or female mice by magnetic-activated cell sorting (MACS). Single cell suspensions were made by mechanical dissociation in FACS Buffer (1% FCS, PBS, 2 mM EDTA; Invitrogen). Cells were washed and isolated by incubation with 25 µl of CD4 microbeads per spleen and positively selected through LS columns (Miltenyi Biotec) according to the manufacturer's instructions. When indicated, naïve $CD4^+CD62L^+CD44^-$ Foxp3.GFP^- T cells were purified from the spleens of male or female C57BL/6 Foxp3.GFP reporter mice by sorting on the FACSaria Cell Sorter after staining with anti- CD4+ Brilliant Violet 510 (clone RM4-5; BioLegend), anti-CD62L PE (clone MEL-14; BioLegend), and anti-CD44 APC (clone IM7; BioLegend). Naïve $CD4^+CD62L^+CD44^-$ Foxp3.GFP- T cells were always >99% pure. When staining, approximately 1 µg of antibody was used per 10^6 cells.

Section 2.3: *In Vitro* Stimulation. Twenty-four well or 96-well flat-bottom tissue culture plates (Falcon) were coated with 4 μg/ml of each anti-CD3 (clone 2C11) and anti-CD28 (clone 37.51) and molar equivalents of recombinant Human IgG_1-Fc (R&D Systems) and PD-L1-Fc (R&D Systems) (5 μg/ml and 10 μg/ml, respectively) in phosphate buffered saline (PBS; Invitrogen) overnight at 4°Celsius. Before plating the purified $CD4^+$ T cells, the coated tissue culture plates were washed once with sterile PBS. The purified $CD4^+$ T cells were plated at 10^6/ml in the coated 24-well or 96-well flat-bottom tissue culture plates in complete media consisting of RPMI-1640 with L-glutamine (Invitrogen) supplemented with 10% FCS (Sigma-Aldrich), penicillin-streptomycin (100 U penicillin and 100 μg streptomycin (Invitrogen), 12 mM HEPES (Invitrogen), and 50 μM β-mercaptoethanol (Sigma-Aldrich) for 1, 2, 3, or 4 days at 37°C with 5% CO_2. Triplicates for each condition were plated unless otherwise indicated. When indicated, recombinant TGF-β (R&D Systems) was supplemented into the media at a final concentration of 2 ng/mL.

Section 2.4: Quantitative PCR. Total RNA was isolated using the RNEasy Mini Plus kit (QIAGEN) according to the manufacturer's instructions and quantified using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). cDNA was synthesized by reverse transcription with random hexamer primers using the High Capacity cDNA Synthesis kit (Applied Biosystems). Real-time qPCR was performed using SYBR Green chemistry (Roche) on a LightCycler 480 instrument. All samples were run as triplicates. Please see **appendix II** for primer sequences.

Section 2.5: Flow Cytometry. Cell surface staining was performed in the dark at 4°C in FACS Buffer (1% FCS, PBS, 2 mM EDTA; Invitrogen). The following anti–mouse antibodies were used for cell surface staining: anti-CD16/CD32 (Fc Block), anti-CD4 Brilliant Violet 510 (clone RM4-5; BioLegend), anti-CD62L PE (clone MEL-14; BioLegend), anti-CD25 PE (clone PC61;

BioLegend), anti-CD44 APC (clone IM7; BioLegend), and anti-PD-1 PE-Cy7 (clone RMPI-30; BioLegend). For intracellular detection of reactive oxygen species (ROS), cells were stained in 2.5 μM of CellROX deep red or CellROX green (Thermo Fisher) in complete media for 30 minutes at 37°C with 5% CO_2 before staining for surface markers. If the cells were subsequently going to be fixed and permeabilized for intracellular staining, cellROX green was used. Intracellular staining for FoxP3 and/or GPx-1 was performed using the following antibodies: anti-FoxP3 APC or anti-FoxP3 FITC (clone FJK-16s; BioLegend), anti-GPx-1 (polyclonal; PA5-26323; Thermo Fischer), and donkey anti-rabbit Brilliant Violet 421 (polyclonal; Poly4064; BioLegend). Cells were fixed and permeabilized using the FoxP3 fix/perm kit (eBioscience) according to the manufacturer's instructions. Flow cytometry was performed on an LSR II instrument (BD Biosciences) and data were analyzed using FlowJo v10.1 (FlowJo, LLC).

Section 2.6: Mouse Immunization. Six to twelve-week-old C57BL/6-Tg OTII (TcraTcrb) mice were immunized with 100 μg NP_{16}-OVA (Biosearch Technologies) in a 1:1 H37RA CFA (DIFCO) emulsion. The emulsion was prepared manually at room temperature and stored at 4°C. The emulsion was injected subcutaneously into the left and right flank of each experimental mouse. The draining lymph nodes from each mouse were harvested seven days later. The tissue was then disaggregated in complete media, passed through a cell strainer (70 μm), and assessed for the expression of GPx-1 using flow cytometry. All samples were processed on ice and were kept protected from light while staining for extracellular and intracellular molecules. The data presented are representative of two independent experiments.

Section 2.7: MC38 Tumor Experiments. On day 0, WT mice were injected subcutaneously on the flank with 100,000 MC38 adenocarcinoma cells. After seven days, mice were monitored every three days for tumor growth. On the day of harvest (~20 days post-injection), both the tumor and

the corresponding draining lymph node were dissected. Tumor samples were subjected to collagenase type I (400 U/ml; Worthington Biochemical) digestion for 30 to 45 min at 37°C, after which they were passed through a cell strainer (70 μm). The draining lymph nodes were disaggregated in complete media and passed through a cell trainer (70 μm). Mononuclear cells from each tumor were isolated by centrifugation through a Percoll gradient (30 and 70%). The interface was removed, washed and re-suspended in culture medium for analysis. Cells were subsequently moved into FACS buffer and stained for both ROS and GPx-1 as described above (**Section 2.5**).

Section 2.8: GPx-1 Inhibition Assay. We used used mercaptosuccinate to inhibit GPx-1 *in vitro*. 96-well tissue culture plates were coated with 4 μg/mL of anti-CD3 and anti-CD28 and either 5 μg/mL of recombinant Human IgG_1-Fc or 10 μg/mL of recombinant PD-L1-Fc. We duplicated these conditions and added TGF-β up to a final concentration of 2 ng/mL to assess the impact that TGF-β has on T cell activation during GPx-1 inhibition. Thus, there were a total of four different culture conditions, anti-CD3, anti-CD28, and Human IgG_1-Fc (+/- TGF-β) and CD3, anti-CD28, and PD-L1-Fc (+/- TGF-β). Naïve $CD4^+$ T cells ($CD4^+CD44^-CD62L^+$) were isolated from the spleens of 8-12 week old Foxp3.GFP reporter C57BL/6 mice using the previously described cell sorting protocol (**Section 2.2**). The isolated naïve cells were diluted in complete media with or without TGF-β at final concentration of 2 ng/mL. Cells were diluted to a concentration of 10^6 cells/mL in complete media and 100 μL was added to each well to obtain 100,000 cells/well. A stock solution of 1mM of mercaptosuccinate (MS) was prepared in complete media and the pH was adjusted to 7.2 using concentrated sodium hydroxide. The MS preparation was then filtered through a 40-micron filter using a 10-mL syringe. The filtered stock solution was then used to make a dilution series two-fold the desired final concentration. To obtain the desired 1X

concentration of MS, 100 µl of the appropriate MS dilution was added to each well for a final volume of 200 µl/well. The final concentrations of MS used to treat cells were 0, 1, 5, and 10 mM. Cells were cultured for three days at 37°C with 5% CO_2 before staining for ROS and surface markers.

Section 2.9: Statistical Analysis. Statistical analysis was performed using Prism 6. Unpaired Student's t test was used for all comparisons, unless otherwise indicated in the figure legends. Data are represented as mean ± SD or SE. p Values <0.05 were considered statistically significant (*$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$).

Chapter 3: Results

Section 3.1: $CD4^+$ T cells cultured in the presence of PD-L1 express lower levels of genes encoding the selenoproteins GPx-1, Sep15, and TR3. In order to assess the impact of PD-1 engagement on selenoprotein expression, we stimulated $CD4^+$ T cells with anti-CD3/CD28 in the presence or absence of recombinant PD-L1-Fc and harvested cells for gene expression analysis at 24 and 48 hours. Bulk splenic $CD4^+$ T cells were isolated using MACs purification. Untreated cells were processed on the day of the harvest and the experimental groups were treated with anti-CD3, anti-CD28, and human Ig-Fc or anti-CD3, anti-CD28, and PD-L1-Fc. We found that genes encoding the T cell specific selenoproteins were upregulated less in $CD4^+$ T cells cultured with PD-L1-Fc compared to those cultured with control human Ig-Fc. This difference was observed primarily within the first 48 hours. The expression level of some genes, such as GPx-1 and sep15, appeared to be more highly influenced by the presence of PD-L1-Fc than other selenoprotein genes such as GPx-4 and TR1 (**Figure 3.1A**). Given the complexity of selenoprotein synthesis, we also assessed the expression of genes involved in seleno-amino acid metabolism and protein elongation. The expression level of CTH2 (an enzyme involved in both cysteine and selenocysteine metabolism) was notably downregulated in the presence of PD-L1 at 48 hours, while other genes involved solely in selenium metabolism, such as SECIS-binding protein-2 (SBP2) and selenophosphate synthetase-2 (SPS2) were less directly affected by the presence of PD-L1-Fc (**Figure 3.1B**).

Section 3.2: $CD4^+$ T cells upregulate expression of GPx-1 upon stimulation with anti-CD3 and anti-CD28. Given our gene expression analysis and previously published data on selenoprotein expression in bulk T cells (Carlson et al., 2010), we next examined GPx-1 protein expression *in vitro* to validate the results that we obtained from our gene expression analysis. We

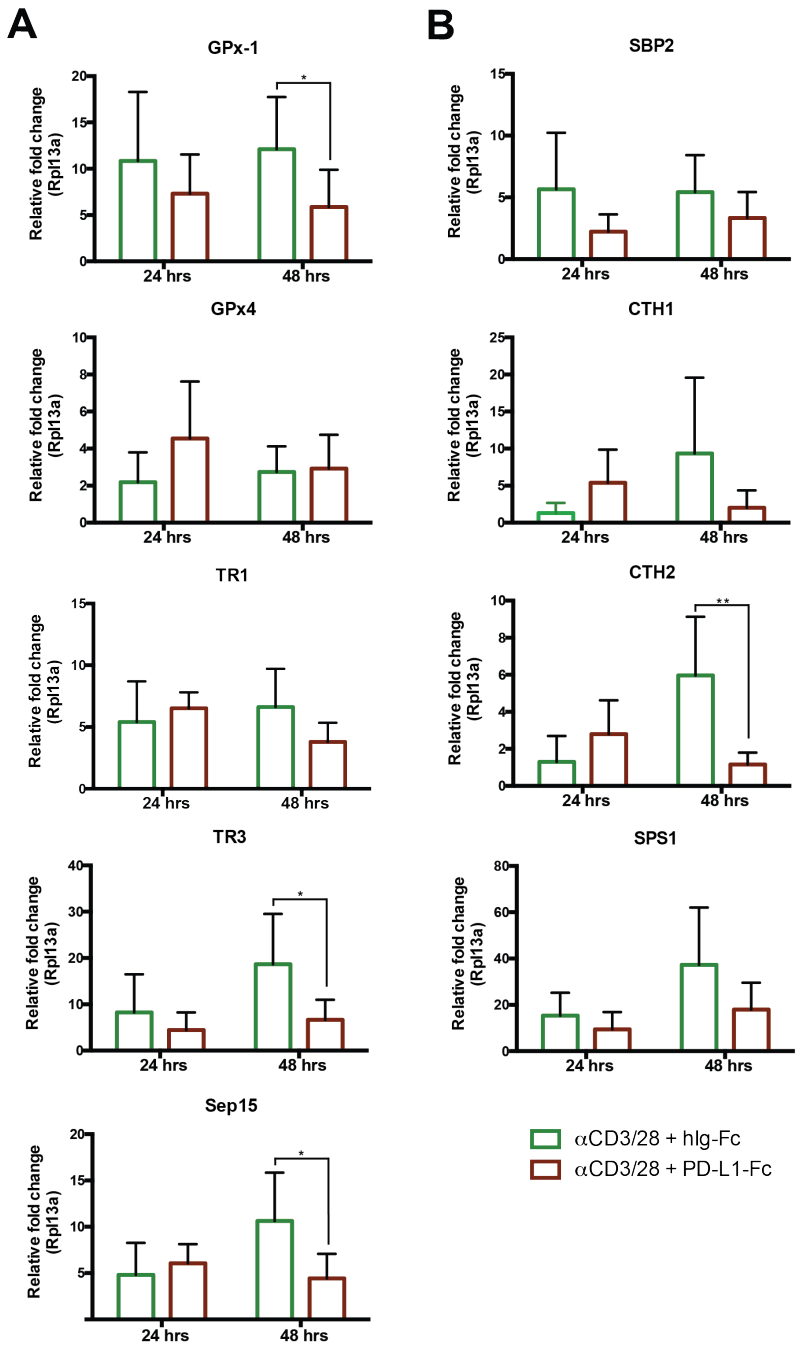


Figure 3.1: CD4$^+$ T cells cultured in the presence of PD-L1-Fc express lower levels of genes that encode for the selenoproteins GPx-1, Sep15, and TR3. CD4$^+$ T cells were activated with 4 μg/mL of each αCD3 and αCD28 in the presence of 5 μg/mL of hIg-Fc or 10 μg/mL of PD-L1-Fc. Cells were harvested after 24 and 48 hrs. Total RNA was isolated and gene expression analysis was performed using quantitative PCR. (A) Expression analysis of genes that encode for selenoproteins using Rpl13a as a reference gene. (B) Expression analysis of genes involved in selenoprotein synthesis. Statistical analysis was performed on triplicates for each condition. (*$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$).

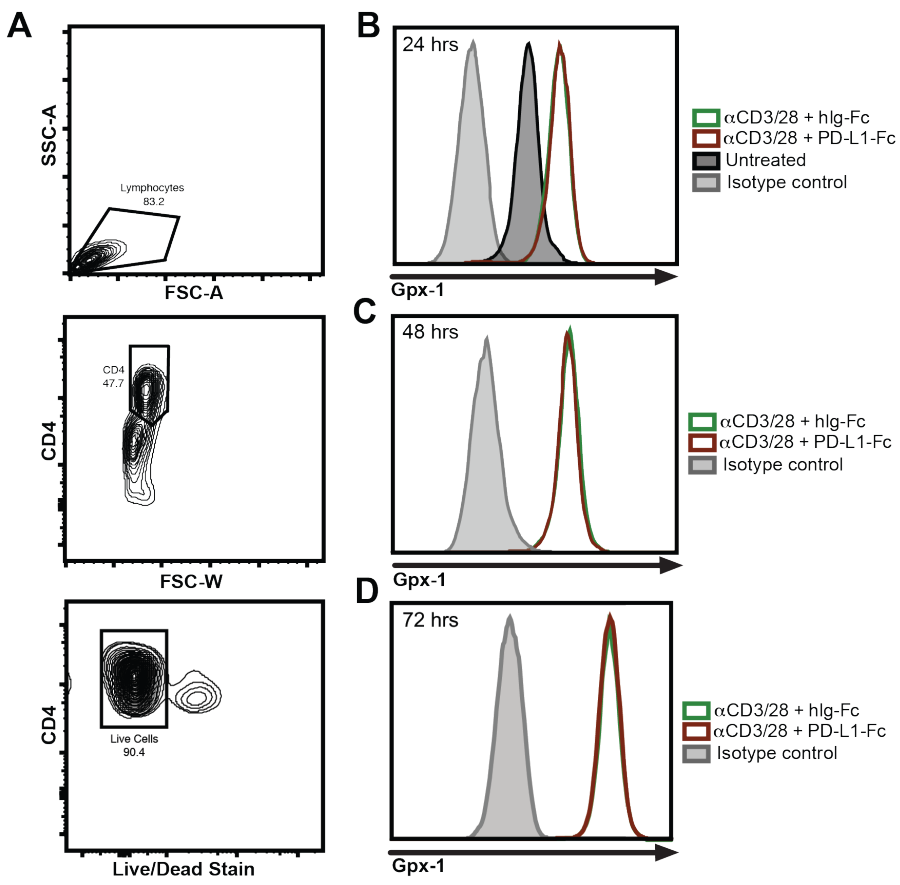
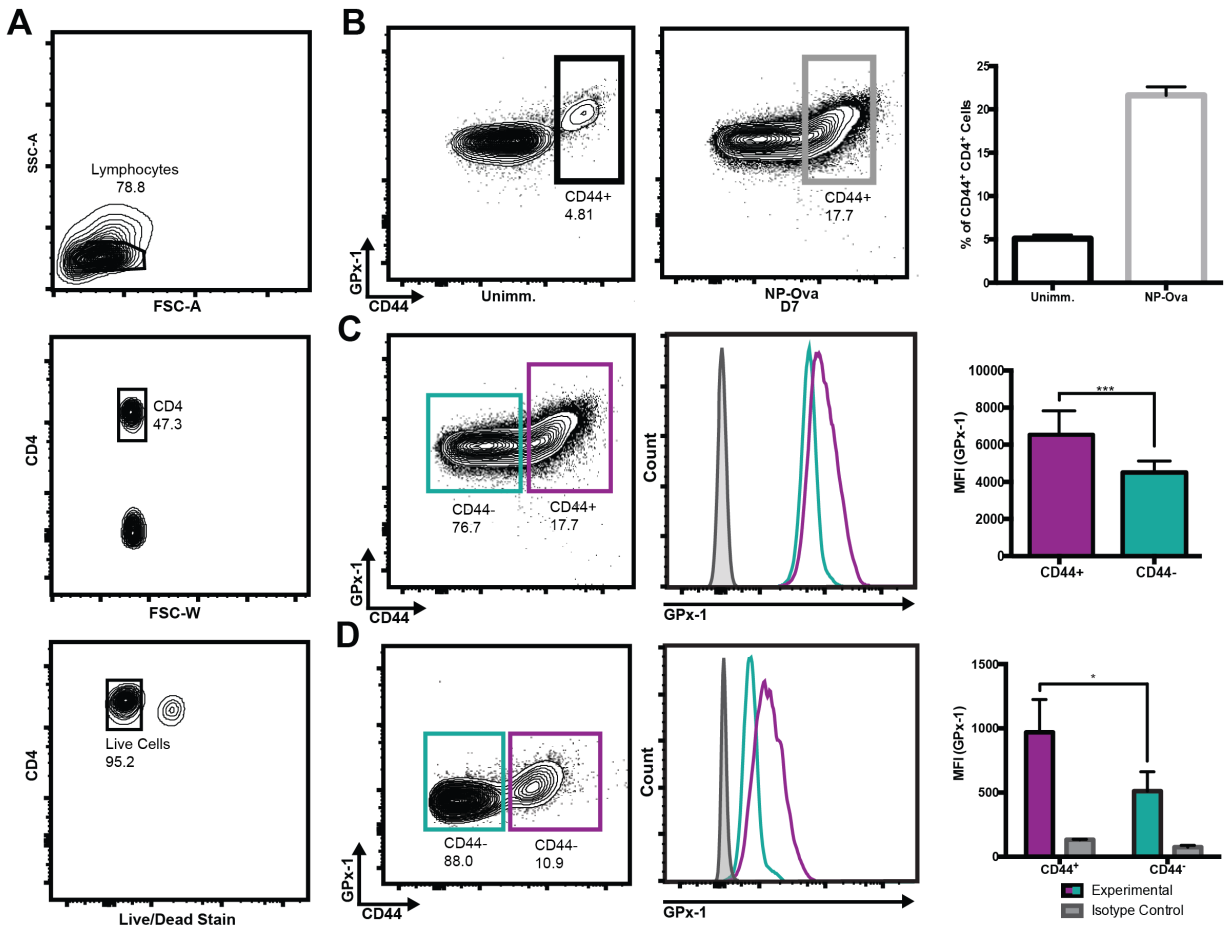


Figure 3.2: CD4$^+$ T cells upregulate expression of GPx-1 upon stimulation with anti-CD3 and anti-CD28. MACS purified CD4$^+$ T cells were isolated and cultured CD4$^+$ T cells were activated with 4 μg/mL of each αCD3 and αCD28 in the presence of 5 μg/mL of hIg-Fc or 10 μg/mL of PD-L1-Fc (**A**) Shown here is the gating strategy to isolate live, single CD4$^+$ T cells. (**B**) Histograms of GPx-1 expression in live CD4$^+$ T cells after 24 hrs of stimulation. CD4$^+$ T cells cultured in the presence of recombinant hIg-Fc or PD-L1-Fc are shown in green and red, respectively. Untreated cells (black) are reported after 24 hrs only because cell death was so significant in the untreated controls for 48 and 72 hrs. (**C**) Data generated from the same experiment after 48 and 72 hrs of stimulation.

analyzed the expression of GPx-1 every 24 hours for three days using flow cytometry. To detect the expression of GPx-1, we used a polyclonal antibody against the C-terminus of GPx-1 that has been reported by the manufacturer to detect GPx-1 in flow cytometry. We found that the level of GPx-1 expression increased upon stimulation with anti-CD3 and anti-CD28 compared to untreated control cells (**Figure 3.2B**). However, we observed little difference in the expression level of GPx-1 in cells cultured with PD-L1-Fc or control human Ig-Fc after 48 hrs (**Figure 3.2C**) and 72 hrs (**Figure 3.2D**).

Section 3.3: Activated T cells express higher levels of GPx-1 in two models of immunity. Given the observation that GPx-1 expression in CD4$^+$ T cells appeared to be higher in cells treated



Figures 3.3: Activated T cells derived from the dLN express higher levels of GPx-1 in two in vivo models. (**A**) Gating strategy to isolate $CD4^+$ live-single cells for FACS analysis. (**B**) Representative plots for $CD4^+$ T cells plotted for the expression of GPx-1 vs. CD44 in LN of four control mice (left) and nine mice immunized with NP-Ova (center). Quantitative analysis was performed on the percentages of $CD44^+$ $CD4^+$ T cells from control mice and immunized mice (Right). (**C**) Shown on the left is a representative flow plot of $CD4^+$ T cells from the dLN of immunized mice showing the expression of CD44 and GPx-1. In the center panel, $CD44^+$ T cells from the dLN of mice immunized with NP-Ova (purple) were compared to $CD44^-$ T cells (cyan) from the same dLN. The MFI for GPx-1 expression of $CD4^+$ T cells from the dLN of nine mice are quantified on the right. (**D**) Shown on the left is a representative flow plot for $CD4^+$ T cells harvested from the dLN of mice challenged with MC38 tumor 24 days after s.c. implantation. Histograms of $CD44^+$ (purple) and $CD44^-$ (cyan) from the same tumor are shown in the middle panel. The MFI for GPx-1 expression of $CD4^+$ T cells that were derived from the dLN of four mice are quantified on the right (*$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$).

with anti-CD3 and anti-CD28 compared to the untreated controls, we next assessed whether GPx-1 expression correlated with an activation phenotype *in vivo*. We examined the co-expression of GPx-1 and CD44 in $CD4^+$ T cells derived from the dLN of OTII transgenic mice immunized with NP-Ova. CD44 is a well-studied marker of T cells activation and its expression is tied to both

effector and memory-like phenotypes (Baaten et al., 2010a; Baaten et al., 2010b; Shimizu et al., 1989). Mice immunized with NP-Ova when compared to control mice exhibited a higher percentage of $CD44^+$ T cells in the dLN (**Figure 3.3A**). In terms of GPx-1 expression, when we compared $CD44^+$ to $CD44^-$ cells we see that $CD44^+$ T cells derived from the lymph nodes of both control and immunized mice express higher levels of GPx-1 when compared to $CD44^-$ T cells (**Figure 3.3B**). We also challenged mice with subcutaneous injections of MC38 tumor. In agreement with our immunization data, activated $CD44^+$ $CD4^+$ T cells derived from the dLN near the tumor injection site expressed higher levels of GPx-1 when compared to $CD44^-$ cells (**Figure 3.3C**).

Section 3.4: GPx-1 expression correlates with CD44 expression and does not correlate with the expression of CD62L or FoxP3. Seeing as there are a number of $CD44^+$ T cells that do not express high levels of GPx-1, we further characterized the GPx-1^{hi} population of cells using additional markers. We examined whether the downregulation of CD62L or the expression of FoxP3 correlated with increased expression of GPx-1. We gated on double negative, single positive, and double positive populations (CD44 vs. CD62L or CD44 vs. FoxP3) and assessed GPx-1 expression. When we examined activated $CD4^+$ T cells in the dLN, we observe that GPx-1 expression correlates with the expression of CD44 and not with the downregulation of CD62L (**Figure 3.4A**). Next, we examined the expression of GPx-1 in T_{reg} cells. Again, we observe that GPx-1 expression correlates only with CD44 expression and does not correspond with the expression of FoxP3 (**Figure 3.4B)**. Taken together, these data suggest that GPx-1 expression correlates most directly with the upregulation of CD44 upon T cell activation. However, the upregulation of CD44 does not necessarily predict high levels of GPx-1 expression.

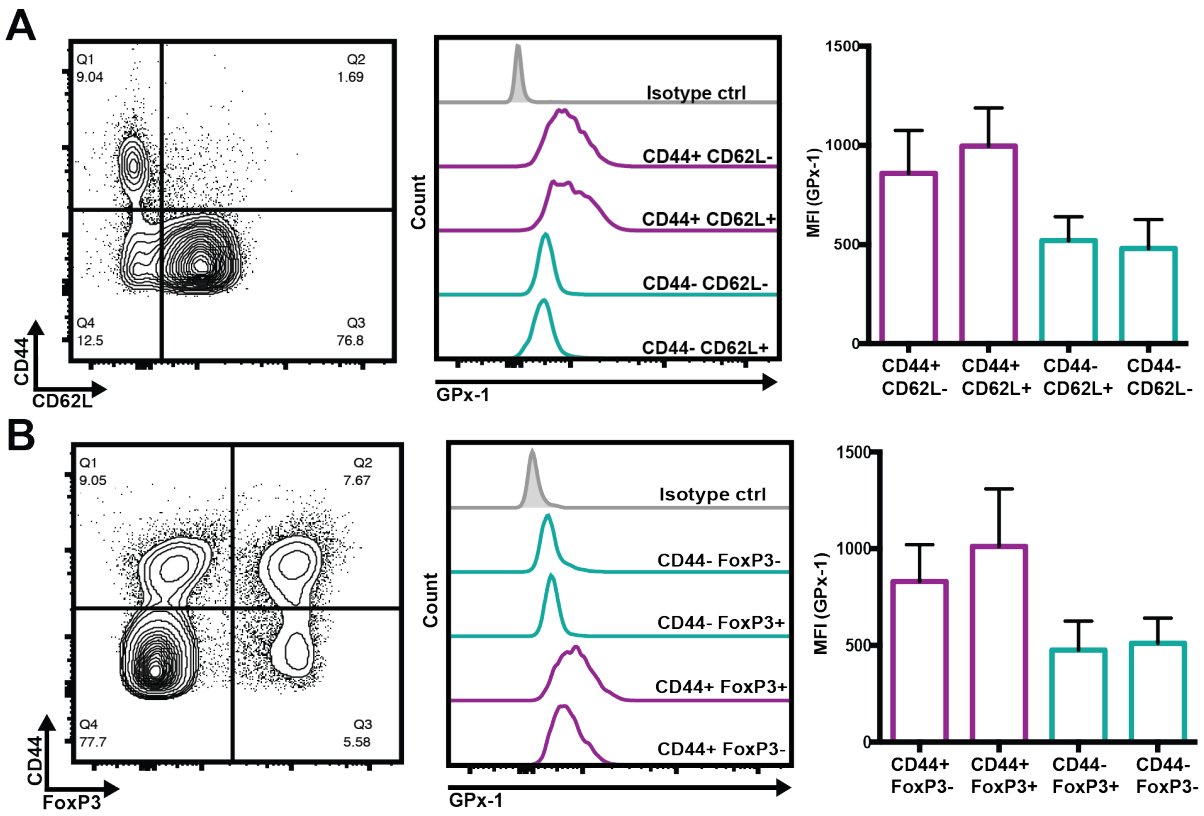


Figure 3.4: GPx-1 correlates with CD44 expression but not with the expression of CD62L or FoxP3. Shown here are representative plots of $CD4^+$ T cells from the dLN of mice implanted with MC38 cells. (**A**) Expression of GPx-1 in activated versus naïve CD4 T cells from the dLN. On the left, $CD4^+$ T cells are plotted for the expression of CD44 vs. CD62L. $CD44^+$ $CD62L^-$ cells are considered activated. A histogram for each quadrant is plotted in the center panel to compare the relative expression of GPx-1 in each population. The isotype control shown is for bulk $CD4^+$ T cells. The results are quantified on the right. (B) Expression of GPx-1 in $FoxP3^+$ versus $FoxP3^-$ $CD4^+$ T cells derived from the dLN of mice implanted with MC38 cells. On the left, $CD4^+$ T cells are plotted for CD44 vs. FoxP3 expression. A histogram for each quadrant is plotted in the center panel to compare the relative expression of GPx-1 in each population. The results are quantified on the right. The data presented here are representative of two individual experiments (*$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$).

Section 3.5: Tumor-infiltrating $CD4^+$ T cells express higher levels of GPx-1. To further investigate the functional role of GPx-1, we compared its expression in $CD4^+$ T cells taken from the dLN and the tumor microenvironment of mice implanted with MC38 tumor cells. GPx-1 expression was higher in tumor infiltrating lymphocytes (TILs) than T cells derived from the dLN (**Figure 3.5A**). Furthermore, when we gated on $CD44^+$ $CD4^+$ T cells, the difference in GPx-1 expression decreased between T cells derived from the tumor microenvironment and the dLN, albeit only slightly (**Figure 3.5B**). We also evaluated $FoxP3^+$ $CD44^+$ T cells for GPx-1 expression.

There was greater expression of GPx-1 in FoxP3$^+$ T cells derived from tumor microenvironment when compared to FoxP3$^+$ T cells from dLN (**Figure 2.5C**). These data suggest that although GPx-1 expression may correlate with the expression of CD44, there are additional factors that regulate GPx-1 expression. These data suggest that the local environment may influence GPx-1 expression.

Section 2.6: Naïve CD4$^+$ T cells cultured in the presence of the GPx-1 inhibitor, mercaptosuccinate, express FoxP3 in a dose dependent manner that is independent of TGF-β. Although the effect GPx-1 deficiency on T cell differentiation into T_H cell subsets has been examined (Won et al., 2010), whether GPx-1 deficiency affects the differentiation of naïve CD4$^+$ T cells into T_{regs} cells is not known. Since we do not have access to germline or cell specific GPx-1 deficient mice, we used a well-studied inhibitor of GPx-1 to examine whether GPx-1 can modulate the differentiation of naïve T cells into T_{reg} cells *in vitro*. Mercaptosuccinic acid (2-sulfanylbutanedioic acid) is a dicarboxylic acid that contains a thiol functional group. At physiological pH, it primarily exists as the conjugate base mercaptosuccinate (MS). MS has been used extensively to inhibit GPx-1 function (Baud et al., 2004; Chaudiere et al., 1984; Zhang et al., 2005) and irreversibly binds to the selenocysteinyl residue harbored in the active site of GPx-1 (Hall et al., 2014). In this *in vitro* stimulation assay, we analyzed cells after 72 hours of stimulation with anti-CD3, anti-CD28, and Human IgG_1-Fc (+/- TGF-β) and anti-CD3, anti-CD28, and PD-L1-Fc (+/- TGF-β). Cells treated with MS exhibited a reduced proliferation potential as measured by cell count per sample (**Figure 3.6A**) and fewer cells were CD44$^+$ when compared to control conditions without the addition of MS (**Figure 3.6B**). Predictably, the cellular oxidative stress increased with the concentration of MS in a dose-dependent manner (**Figure 3.6C**). Interestingly, the addition of MS increased the percentage of FoxP3 expressing cells and the MFI of FoxP3 in a

dose-dependent manner independent of the addition of TGF-β (**Figure 3.6D**). The percentage of $CD25^+$ T cells was higher in T cells cultured in the absence of TGF-β. Additionally, cells cultured in the presence of MS and/or TGF-β exhibited lower MFIs for CD25 when compared to cells cultured in the absence of both MS and TGF-β. (**Figure 3.6E**). These results suggest that GPx-1 may play an important immunoregulatory role in T_{reg} cell induction, possibly by modulating signaling through ROS.

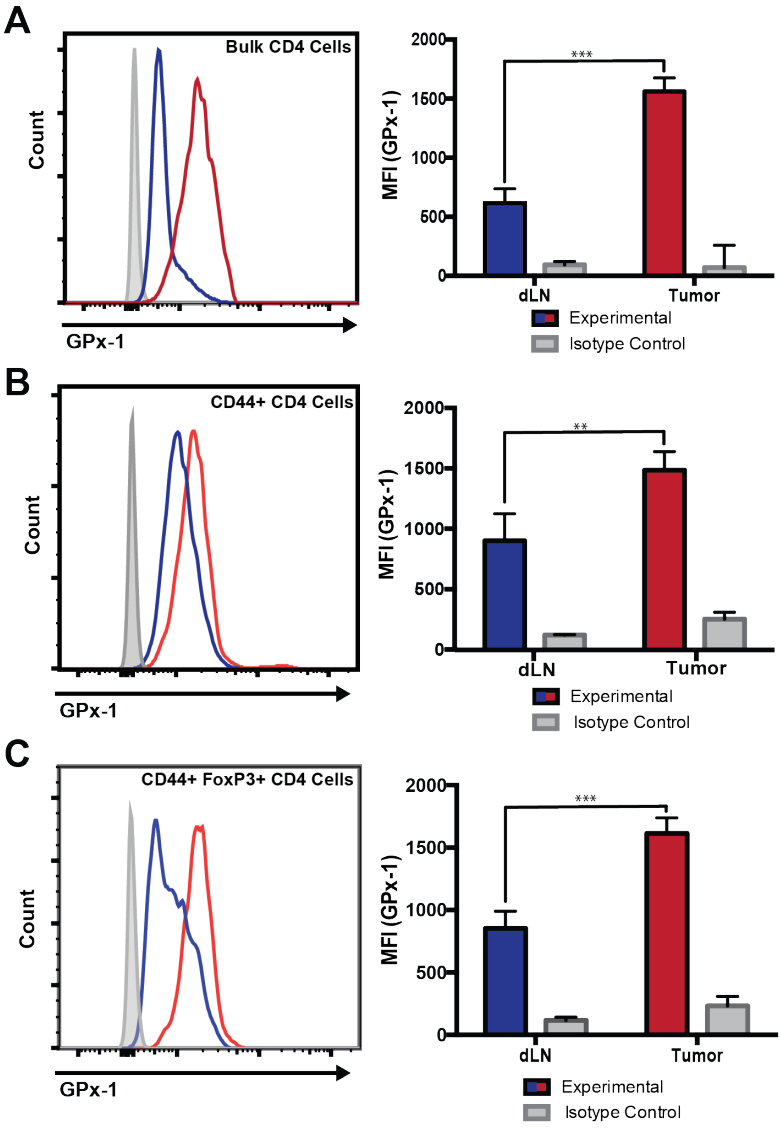


Figure 3.5: Tumor infiltrating $CD4^+$ T cells express higher levels of GPx-1 than $CD4^+$ T cells from the draining lymph node. Representative histograms of $CD4^+$ T cells from the dLN and tumors of mice implanted s.c. with MC38 tumor cells. (**A**) On the left, bulk $CD4^+$ from the dLN (blue) and tumor (red) are compared for GPx-1 expression in a histogram. In the right panel, the geometric mean fluorescence intensity (MFI) for each sample is quantified. (**B**) $CD44^+$ T cells harvested from the dLN and tumor are compared for their expression of GPx-1. On the left, a representative histogram of $CD44^+$ $CD4^+$ T cells from the dLN (blue) and tumor (red) are compared for expression of GPx-1. On the right, the geometric mean fluorescence intensity (MFI) for each sample is quantified. (**C**) $CD44^+$ $FoxP3^+$ T cells harvested from the dLN and tumor are compared for their expression of GPx-1. On the left, a representative histogram of $CD44^+$ $FoxP3^+$ $CD4^+$ T cells from the dLN (blue) and tumor (red) are compared for expression of GPx-1. On the right, the geometric mean fluorescence intensity (MFI) for each sample is quantified. The MFI for GPx-1 expression for each isotype control is reported for every population ($^*p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$).

Chapter 4: Discussion

In this thesis, we investigated the impact that the PD-1 pathway has on the expression of selenoprotein genes and genes involved in selenium metabolism. We found that T cells activated in the presence of recombinant PD-L1 express lower levels of some selenoprotein genes, while the expression of others did not change. Additionally, we determined that few genes involved in selenium metabolism were also downregulated in the presence of PD-L1. Seeing as GPx-1 was one of the selenoprotein genes that was most highly expressed upon T cell activation *in vitro* gene expression analysis, we sought to determine its expression profile at the protein level using flow cytometry. We found that GPx-1 expression increases upon T cell activation *in vitro* and that activated T cells derived from *in vivo* models of immunity also expressed high levels of GPx-1. Lastly, using a GPx-1 inhibitor, we discovered a novel role for GPx-1 in regulating the expression of FoxP3 during T cell activation.

Using an *in vitro* activation assay, we activated T cells using anti-CD3 and anti-CD28 in the presence of recombinant human IgG_1-Fc or PD-L1-Fc. We determined the expression of a number of selenoprotein genes that have been previously shown to be expressed by T cells (Carlson et al., 2010). We performed our gene expression analysis after 24 and 48 hours of activation *in vit*ro after isolating total cellular RNA and performing quantitative PCR. We discovered that T cells cultured in the presence of PD-L1-Fc, express lower levels of the selenoprotein genes GPx-1, sep15, and TR3 when compared to those activated in the presence of the human IgG_1-Fc. Additionally, we examined the expression of genes involved in selenium metabolism during T cell activation in the presence and absence of PD-L1-Fc. The T cells cultured in the presence of PD-L1-Fc did not significantly downregulate genes involved in selenium

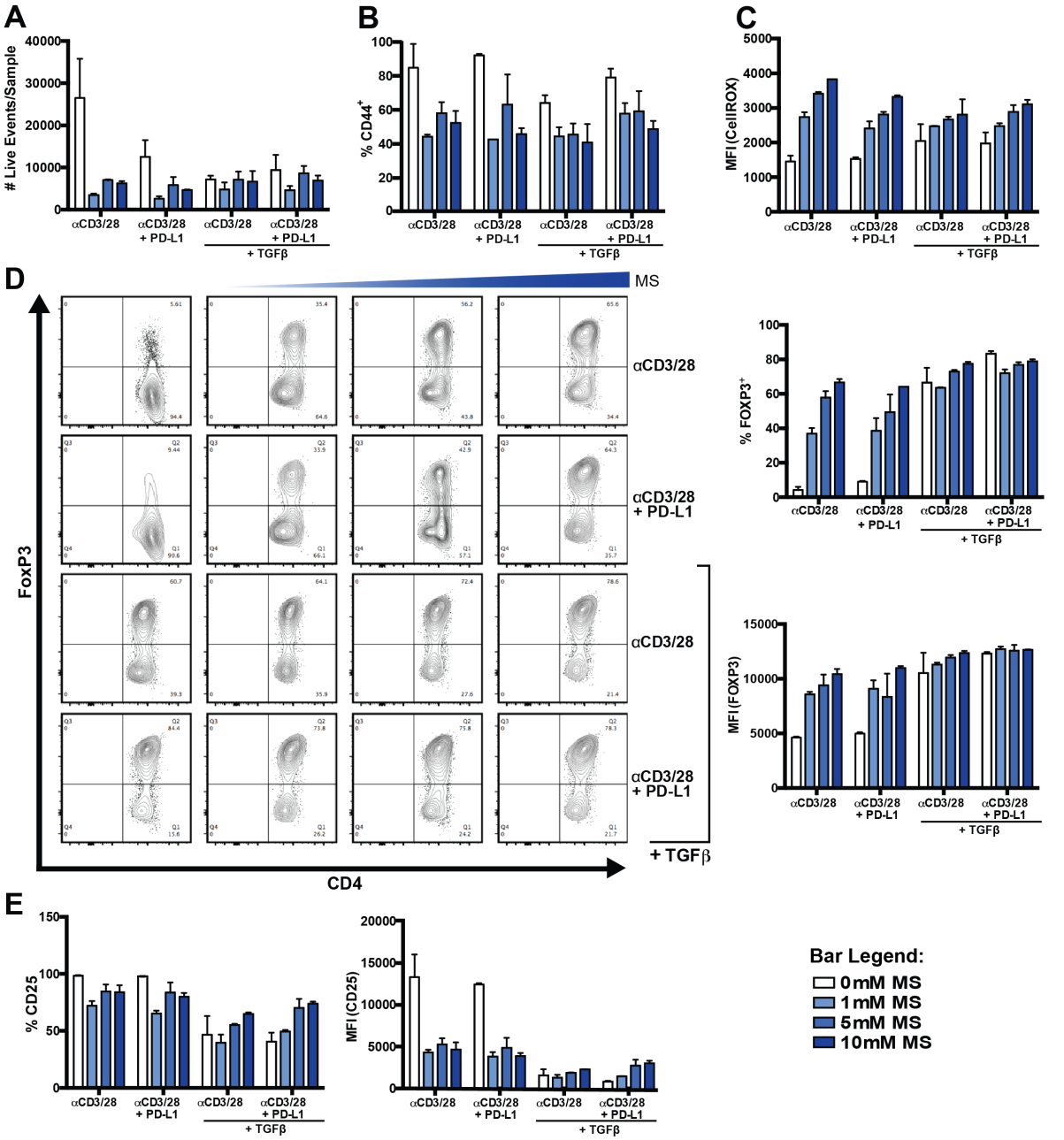


Figure 3.6: Naïve CD4$^+$ T cells cultured in the presence of the GPx-1 inhibitor, mercaptosuccinate, induces FoxP3 in naïve CD4$^+$ T cells in a dose dependent manner that is independent of TGF-β. Naïve CD44$^-$ CD62L$^+$ FoxP3$^-$ CD4$^+$ T cells were sorted from FoxP3.GFP reporter mice and stimulated with αCD3 and αCD28 in the presence or absence of hIG-Fc or PD-L1-Fc and plus or minus 2 ng/mL of TGF-β. T cell activation was measured in the presence and absence of the GPx-1 inhibitor, mercaptosuccinate (MS) at concentrations of 0, 1, 5, and 10 mM at time zero (**A**) CD4$^+$ T cells activation was assessed by quantifying the number of Live CD4$^+$ T cells/sample. (**B**) CD4$^+$ T cell activation was measured by the percentage of CD44$^+$ CD4$^+$ live T cells. (**C**) Oxidative stress was measured using cellROX deep red reagent and quantified by reporting the mean geometric fluorescence intensity for each condition and plotted in a bar graph (**D**) Representative plots of live CD4$^+$ T cells cultured in the presence of PD-L1-Fc or hIg-Fc plus or minus the addition of TGF-β. FoxP3 expression is represented on the y-axis and CD4 expression is represented on the x-axis. In the bar graphs on the right, the percentage of FoxP3$^+$ T cells are reported as percentage of live-CD4$^+$ T cells (top panel). The MFI of FoxP3 of live-CD4$^+$ FoxP3$^+$ T cells is quantified (bottom panel). (**E**) The percentage of live-CD4$^+$ CD25$^+$ T cells is quantified (left) and the MFI for CD25 is quantified (right) for each condition.

synthesis, with the exception of cystathione gamma-lyase-2 or CTH2 (an enzyme that is involved in both the metabolism of organic sulfur and selenium compounds). While our data suggest that the PD-1 pathway may regulate the expression of some selenoprotein genes, it is difficult to determine whether this downregulation is mediated directly by signaling through PD-1 or is secondary to the reduced activation state due to PD-1 immunoinhibitory signals. Further work is needed to better assess whether the PD-1 pathway directly regulates selenoprotein gene expression and protein synthesis. Since GPx-1 is one of the most highly expressed selenoprotein genes in T cells, we focused on its expression in T cells during activation.

We observed reduced transcript levels of *GPX1* in cells stimulated in the presence of PD-L1-Fc, but did not observe differences in GPx-1 protein expression. Since selenoprotein synthesis is highly regulated at the levels of transcription and translation, it is premature to draw conclusions about the effects of PD-1 signaling on selenoprotein synthesis, specifically GPx-1 expression and synthesis. Further work is needed to better assess whether PD-1 signaling directly impacts selenoprotein synthesis and metabolism. One approach that could be used to examine whether signaling through PD-1 impacts selenoprotein gene expression would be to stimulate T cells using different types of activation stimuli (e.g. PMA + Ionomycin or PHA). For example, PMA + Ionomycin activate T cells downstream proximal T cell signaling by activating Protein Kinase C and inducing Ca^{2+} efflux from the ER. Thus, if engagement of PD-1 counters proximal T cell signaling it is plausible that selenoprotein gene expression may not change in T cells cultured with PMA + Ionomycin in the presence of PD-1 ligands. This experiment may help elucidate if T cell signaling pathways are necessary for changes in selenoprotein gene expression and whether signaling through PD-1 interferes with multiple pathways of activation or just one.

When translating the observation that GPx-1 expression increases upon T cell activation from *in vitro* to *in vivo* models, we examined whether its expression correlated with markers of activation. We determined the relationship between GPx-1 and the known marker of activation CD44. The fact that GPx-1 expression correlates with CD44 expression, suggests that it may serve a functional role in activated T cells. Our data show that some $CD44^+$ cells express higher levels of GPx-1 than $CD44^-$ cells. This observation, led us to better define the population of cells that upregulate the expression of GPx-1. Using the downregulation of L-selection (CD62L) as an additional marker of activation and the expression of $FoxP3^+$ to differentiate between T_{eff} cells and T_{reg} cells, we further characterized the cells that increase GPx-1 expression after activation. Our data revealed that the only marker of activation that correlates with GPx-1 expression is CD44. It is possible that the upregulation of GPx-1 in $CD44^+$ T cells is transient, regulated by intrinsic or extrinsic stimuli (e.g. excess peroxide signaling or the presence of pro-oxidants, respectively), or both. Thus, identifying a stable population of T cells (as defined by the expression of canonical transcription factors and/or surface markers) that express hi levels of GPx-1 may be futile.

Support for the argument that the local microenvironment impacts GPx-1 expression comes from our *in vivo* experiments where we observed that $CD4^+$ T cells from the tumor microenvironment (TILs) express higher levels of GPx-1 than $CD4^+$ T cells isolated from the dLN. Additionally, GPx-1 expression was enriched in $CD44^+$ TILs. These observations suggest a role for GPx-1 in T cell effector function. As GPx-1 expression is induced and upregulated in the context of various environmental stimuli (e.g. oxidative stress, nutrient deprivation, etc.) (Cowan et al., 1993; Lubos et al., 2011), it is reasonable to hypothesize that the increase of GPx-1 expression observed in TILs was induced in part by environmental stressors characteristic of the

tumor microenvironment (e.g. in the presence of activated cancer associated fibroblasts or macrophages, hypoxia, etc.) (Fiaschi and Chiarugi, 2012; Policastro et al., 2013).

While there is evidence that the selenoproteome of T cells is necessary for their proper development and function, the exact roles and mechanisms by which these unique proteins contribute to T cell development, activation, and effector function are not thoroughly understood. The T cell selenoproteome functions in part by mitigating oxidative stress (Shrimali et al., 2008). Since GPx-1 is an important antioxidant enzyme, we hypothesized that it functions in T cells to reduce oxidative stress. The findings that $CD4^+$ T cells derived from GPx-1 deficient mice exhibit increased levels of ROS and a reduced proliferation potential *in vitro* support this hypothesis. However, GPx-1 deficient $CD4^+$ T cells also exhibit a skewed T_H cell differentiation potential, suggesting that GPx-1 may impact $CD4^+$ T cell responses and differentiation. GPx-1 has previously been described to modulate ROS signaling by neutralizing H_2O_2 (Veal et al., 2007). Additionally, GPx-1 activity in platelets has been shown to indirectly modulate phospholipase Cγ2 activity by regulating the oxidation state of the src homology 2 domain-containing tyrosine phosphatase 2 (SHP2) in an redox dependent manner (Jang et al., 2014). Thus, it is possible that GPx-1 may serve to modulate ROS signaling downstream TCR signaling in T cells.

Data from our GPx-1 inhibition assay provide additional support for the role that ROS play in modulating T cell activation and differentiation potential. In our GPx-1 inhibition assay, we observed lower cell counts and reduced expression of activation markers in cells that were treated with MS. Additionally, the level of ROS detected in each condition increases in a manner that is dependent on the concentration of MS. These data support the role of GPx-1 as an antioxidant enzyme that acts to reduce oxidative stress. However, we observed also that the addition of MS during T cell activation induced the expression of FoxP3 in a dose-dependent manner that was

independent of the presence of TGF-β. Although preliminary, these data suggest that GPx-1 activity may either directly or indirectly impact T cell activation or differentiation. GPx-1 may act to modulate T cell activation and/or differentiation rather than acting as a switch that when turned on or off determines T cell fate. This idea is supported by the findings that T cells derived from GPx-1 deficient mice are viable and respond to activating stimuli (e.g. treatment with anti-CD3 and anti-CD28). However, they exhibit both a limited ability to differentiate into T_H cell subtypes and preferentially exhibit a T_H1 cell-like effector phenotype *in vivo* models of allergy (Won et al., 2010). Additionally, GPx-1 deficient mice exhibit attenuated autoimmune disease and hyperfunctional T_{reg} cells in models of autoimmunity and inflammatory disease (Kim et al., 2014a; Kim et al., 2014b). Taken together, these data support a role for GPx-1 in modulating T cell responses. Seeing as peroxide signaling is an important signaling molecule downstream TCR activation, it is tempting to speculate that GPx-1 may play a role in modulating this signal cascade. Strong TCR signaling in the periphery is associated with the induction of pT_{reg} cells (Delpoux et al., 2014; Gabrysova and Wraith, 2010). If ROS signaling is an important measure of TCR signal strength, it fits with our data that by inhibiting the activity of GPx-1 in T cells that are stimulated *in vitro*, more ROS would accumulate during activation and thereby recapitulate a strong TCR signal. Interestingly, TGF-β signaling has been shown to induce mitochondrial ROS production and mitochondria-targeted antioxidants have been shown to inhibit TGF-β mediated gene transcription downstream Smad3 (Jain et al., 2013; Liu and Gaston Pravia, 2010). Seeing as Smad3 both stabilizes and promotes histone acetylation in the enhancer region of *Foxp3*, it is tempting to speculate that local ROS production may be an important player in regulating FoxP3 expression downstream TGF-β signaling (Jana et al., 2009; Tone et al., 2008). Further work is needed to

validate the role that GPx-1 and its substrates (e.g. H_2O_2) play in modulating T cell responses and whether this mechanism of action can induce the expression of FoxP3.

Chapter 5: Limitations & Perspectives

Our data suggest that selenoprotein gene expression and metabolism may be downregulated in the presence of PD-L1-Fc, but it is hard to draw definite conclusions about the magnitude of this regulation, and whether it is due to abrogated TCR signaling in the presence of PD-L1 or directly due to signaling through PD-1. Since selenoprotein synthesis is a highly regulated process, protein expression analysis using Western Blot and mass spectrometry could be employed to more carefully assess the expression and regulation of these proteins during T cell activation, and better resolve patterns of expression in the presence and absence of co-stimulation through PD-1.

Likewise, since GPx-1 activity is highly regulated at levels of transcription and translation, further work is needed to assess whether GPx-1 activity is regulated by T cell activation and whether PD-1 impacts GPx-1 activity. Although we measured a significant increase in the MFI for GPx-1 in cells treated with anti-CD3 and anti-CD28 compared to untreated control cells, we did not determine if GPx-1 activity increases upon T cell activation. We need to assess the activity of GPx-1 in a controlled experiment. At present we can only conclude that there is a change in the number of protein copies in the cell upon T cell activation. A number of GPx-1 activity assays have been developed to measure the reductive capacity of cell lysate. Employing one of these assays might provide us with a better understanding of whether GPx-1 activity is regulated at the post-translational level downstream T cell activation as well as whether PD-1 engagement alters GPx-1 activity.

Our results suggest that increases in GPx-1 expression correlate with CD44 expression in $CD4^+$ T cells. These data are congruent across two *in vivo* models of T cell activation. However,

it is important to recognize that the immunological response seven days after immunization with NP-Ova is considerably different than the immune response four-weeks post-transplantation with a subcutaneous tumor cells. When comparing $CD4^+$ T cells from the dLN and tumor microenvironment (TILs) of the same animal, we found differences in the expression level of GPx-1, where TILs expressed higher levels of GPx-1 than those derived from the dLN. Higher expression of GPx-1 in TILs might suggest a functional role for GPx-1 in mitigating oxidative stress. Further work is needed to clarify whether this increase in expression is due to cell intrinsic or environmental factors. Future work using models of infection (e.g. LCMV) may serve to better resolve the function of GPx-1 in newly activated $CD4^+$ T cells and effector cells. Importantly, these models would provide larger numbers of cells that could be isolated for *ex vivo* characterization and GPx-1 activity assays.

There remain a number of unanswered questions regarding how inhibiting GPx-1 induces the expression of FoxP3. As GPx-1 is a well-described antioxidant enzyme and previously published research supports the role for the selenoproteome of T cells to reduce oxidative stress, it is tempting to speculate that inhibiting GPx-1 and thereby increasing ROS is in part responsible for inducing T_{reg} cells *in vitro*. However, it is possible that the inhibition of GPx-1 induces FoxP3 expression through a different mechanism of action. One way to address whether ROS directly induces the expression of FoxP3 is to perform *in vitro* GPx-1 inhibition assays in the presence of increasing concentrations of membrane permeable antioxidants, such as N-acetylcysteine. Data from such experiments would help better resolve whether the induction of FoxP3 is ROS dependent or if GPx-1 inhibition induces FoxP3 via a novel mechanism of action. Additional experiments that employ inhibitors of other antioxidant enzymes could also be employed to both test the hypothesis that ROS play a role in inducing FoxP3 expression and whether the subcellular

location of ROS production is important. Seeing as GPx-1 localizes both to the cytoplasm and the mitochondria, studies that examine antioxidant enzymes that localize to the cytoplasm (e.g. peroxiredoxin I or II) or to the mitochondria (e.g. peroxiredoxins III) may shed light upon the mechanism by which ROS induces FoxP3 expression. Resolving the location of ROS accumulation in the cell may narrow down the number of pathways that could potentially be involved in inducing FoxP3 expression in $CD4^+$ T cells.

Furthermore, it is important to consider how the temporal inhibition of GPx-1 could impact FoxP3 expression. For example, does inhibiting GPx-1 before or after T cell activation change the induction of FoxP3, if at all? Answering these questions may help elucidate the role that GPx-1 plays in T cell activation and what aspects of T cell activation are important for inducing T_{reg} cells *in vivo* (i.e. is the concentration of ROS an important determinant of TCR signal strength?). Resolving the mechanism of action by which inhibiting GPx-1 induces T_{reg} cells may provide insight into how and why $CD4^+$ T cells can be induced to express FoxP3 and effectively differentiate into T_{reg} cells.

While elucidating the mechanism by which GPx-1 inhibition induces FoxP3 may prove valuable and insightful, it is also important for us to assess the functionality of $FoxP3^+$ T cells induced via GPx-1 inhibition. Using naïve $CD4^+$ T cells derived from C57BL/6 Foxp3.GFP reporter mice, *in vitro* differentiated $FoxP3^+$ T cells could be sorted after stimulation with TGF-β or MS and subsequently analyzed for IL-10 production and CTLA4 expression. Additionally, sorted cells could also be used in an *in vitro* suppression assay with T_{eff} cells, such as CTLs. CTL effector function could then be measured by cytokine production or staining for granzyme after incubation with the sorted $FoxP3^+$ T cells for a given period of time. Such an experiment might shed light on the viability and functionality of the $FoxP3^+$ $CD4^+$ T cells derived from GPx-1

inhibition. These data would inform future experiments aimed at assessing the role of GPx-1 in T cell activation and differentiation.

Concluding statements:

In this thesis, we tested the hypothesis that the PD-1 pathway regulates the expression of selenoprotein genes. We found that T cells activated in the presence of ligands for PD-1 express lower levels of several selenoproteins, including GPx-1. Upon further examination of GPx-1 expression *in vitro*, we found little difference between GPx-1 expression at the protein level in naïve T cells activated in the presence or absence of PD-L1 over the course of three days. When we examined GPx-1 expression *in vivo* models of immunity (e.g. NP-Ova immunization and MC38 tumor injection), we found that some, but not all, $CD44^+$ T cells express high levels of GPx-1. We also observed that the expression of GPx-1 varies between cells derived from different tissue environments (i.e. the draining lymph node versus the tumor microenvironment). Interestingly, we discovered that inhibiting GPx-1 *in vitro* induces the expression of FoxP3 in naïve $CD4^+$ T cells, suggesting that GPx-1 may be involved in modulating T cell activation and effector responses. Further work needs to be done to address the mechanism by which GPx-1 inhibition induces FoxP3 expression in naïve T cells and whether these are functional T_{reg} cells. While a number of questions remain, our findings give impetus to more thoroughly investigate the role of selenoproteins in T cell responses.

References:

1. Amir Aslani, B., and S. Ghobadi. 2016. Studies on oxidants and antioxidants with a brief glance at their relevance to the immune system. *Life Sci* 146:163-173.
2. Arnér, E.S.J., and A. Holmgren. 2000. Physiological functions of thioredoxin and thioredoxin reductase. *Eur. J. Biochem.* 267:6102-6109.
3. Astarita, G., and J. Langridge. 2013. An emerging role for metabolomics in nutrition science. *J Nutrigenet Nutrigenomics* 6:181-200.
4. Baaten, B.J., C.R. Li, and L.M. Bradley. 2010a. Multifaceted regulation of T cells by CD44. *Commun Integr Biol* 3:508-512.
5. Baaten, B.J., C.R. Li, M.F. Deiro, M.M. Lin, P.J. Linton, and L.M. Bradley. 2010b. CD44 regulates survival and memory development in Th1 cells. *Immunity* 32:104-115.
6. Barrett, C.W., K. Singh, A.K. Motley, M.K. Lintel, E. Matafonova, A.M. Bradley, W. Ning, S.V. Poindexter, B. Parang, V.K. Reddy, R. Chaturvedi, B.M. Fingleton, M.K. Washington, K.T. Wilson, S.S. Davies, K.E. Hill, R.F. Burk, and C.S. Williams. 2013. Dietary selenium deficiency exacerbates DSS-induced epithelial injury and AOM/DSS-induced tumorigenesis. *PLoS One* 8:e67845.
7. Baud, O., A.E. Greene, J. Li, H. Wang, J.J. Volpe, and P.A. Rosenberg. 2004. Glutathione peroxidase-catalase cooperativity is required for resistance to hydrogen peroxide by mature rat oligodendrocytes. *J Neurosci* 24:1531-1540.
8. Bennett, C.L., R. Yoshioka, H. Kiyosawa, D.F. Barker, P.R. Fain, A.O. Shigeoka, and P.F. Chance. 2000. X-linked syndrome of polyendocrinopathy, immune dysfunction, and diarrhea maps to Xp11.23-Xq13.3. *Am. J. Hum. Genet.* 66:461-468.
9. Bennett, F., D. Luxenberg, V. Ling, I.M. Wang, K. Marquette, D. Lowe, N. Khan, G. Veldman, K.A. Jacobs, V.E. Valge-Archer, M. Collins, and B.M. Carreno. 2003. Program Death-1 Engagement Upon TCR Activation Has Distinct Effects on Costimulation and Cytokine-Driven Proliferation: Attenuation of ICOS, IL-4, and IL-21, But Not CD28, IL-7, and IL-15 Responses. *The Journal of Immunology* 170:711-718.
10. Benstoem, C., A. Goetzenich, S. Kraemer, S. Borosch, W. Manzanares, G. Hardy, and C. Stoppe. 2015. Selenium and its supplementation in cardiovascular disease--what do we know? *Nutrients* 7:3094-3118.
11. Berry, M.J. 2005. Insights into the hierarchy of selenium incorporation. *Nature Genetics* 37:1162-1163.
12. Berry, M.J., L. Banu, Y. Chen, S.J. Mandel, J.D. Keiffer, J.W. Harney, and R. Larsen. 1991. Recognition of UGA as a selenocysteine codon in Type I deiodinase requires sequences in the 3' untranslated region. *Nature* 353:
13. Bettelli, E., Y. Carrier, W. Gao, T. Korn, T.B. Strom, M. Oukka, H.L. Weiner, and V.K. Kuchroo. 2006. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 441:235-238.
14. Bluestone, J.A., C.R. Mackay, J.J. O'Shea, and B. Stockinger. 2009. The functional plasticity of T cell subsets. *Nat Rev Immunol* 9:811-816.
15. Boeing, H. 2013. Nutritional epidemiology: New perspectives for understanding the diet-disease relationship? *Eur J Clin Nutr* 67:424-429.
16. Brunkow, M.E., E.W. Jeffery, K.A. Hjerrild, B. Paeper, L.B. Clark, S.A. Yasayko, J.E. Wilkinson, D. Galas, S.F. Ziegler, and F. Ramsdell. 2001. Disruption of a new

forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nature Genetics* 27:68-73.
17. Carlson, B.A., M.E. Moustafa, A. Sengupta, U. Schweizer, R. Shrimali, M. Rao, N. Zhong, S. Wang, L. Feigenbaum, B.J. Lee, V.N. Gladyshev, and D.L. Hatfield. 2007. Selective restoration of the selenoprotein population in a mouse hepatocyte selenoproteinless background with different mutant selenocysteine tRNAs lacking Um34. *J Biol Chem* 282:32591-32602.
18. Carlson, B.A., M.H. Yoo, R.K. Shrimali, R. Irons, V.N. Gladyshev, D.L. Hatfield, and J.M. Park. 2010. Role of selenium-containing proteins in T-cell and macrophage function. *Proc Nutr Soc* 69:300-310.
19. Chaudiere, J., E.C. Wilhelmsen, and A.L. Tappel. 1984. Mechanism of Selenium-Glutathione Peroxidase and Its Inhibition by Mercaptocarboxylic Acids and other Mercaptans. *J Biol Chem* 259:1043-1050.
20. Cowan, D.B., R.D. Weisel, W.G. Williams, and D.A.G. Mickle. 1993. Identification of Oxygen Responsive Elements in the & Flanking Region of the Human Glutathione Peroxidase Gene. *The Journal of biological chemistry* 268:26904-26910.
21. de Haan, J.B., C. Bladier, P. Griffiths, M. Kelner, R.D. O'Shea, N.S. Cheung, R.T. Bronson, M.J. Silvestro, S. Wild, S.S. Zheng, P.M. Beart, P.J. Hertzog, and I. Kola. 1998. Mice with a homozygous null mutation for the most abundant glutathione peroxidase, GPx1, show increased susceptibility to the oxidative stress-inducing agents paraquat and hydrogen peroxide. *The Journal of biological chemistry* 273:
22. Delpoux, A., P. Yakonowsky, A. Durand, C. Charvet, M. Valente, A. Pommier, N. Bonilla, B. Martin, C. Auffray, and B. Lucas. 2014. TCR signaling events are required for maintaining CD4 regulatory T cell numbers and suppressive capacities in the periphery. *J Immunol* 193:5914-5923.
23. Epp, O., R. Ladenstein, and A. Wendel. 1983. The Refined Structure of the Selenoenzyme Glutathione Peroxidase at 0.2-nm Resolution. *Eur. J. Biochem.* 133:51-69.
24. Esworthy, R.S., Y.S. Ho, and F.F. Chu. 1997. The Gpx1 Gene Encodes Mitochondrial Glutathione Peroxidase in the Mouse Liver. *Archives of Biochemistry and Biophysics* 340:59-63.
25. Fernandes, A.P., and V. Gandin. 2015. Selenium compounds as therapeutic agents in cancer. *Biochim Biophys Acta* 1850:1642-1660.
26. Fiaschi, T., and P. Chiarugi. 2012. Oxidative stress, tumor microenvironment, and metabolic reprogramming: a diabolic liaison. *Int J Cell Biol* 2012:762825.
27. Fontenot, J.D., M.A. Gavin, and A.Y. Rudensky. 2003. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* 4:330-336.
28. Fontenot, J.D., J.P. Rasmussen, L.M. Williams, J.L. Dooley, A.G. Farr, and A.Y. Rudensky. 2005. Regulatory T cell lineage specification by the forkhead transcription factor foxp3. *Immunity* 22:329-341.
29. Francisco, L.M., V.H. Salinas, K.E. Brown, V.K. Vanguri, G.J. Freeman, V.K. Kuchroo, and A.H. Sharpe. 2009. PD-L1 regulates the development, maintenance, and function of induced regulatory T cells. *J Exp Med* 206:3015-3029.
30. Frazier, A.D., C.L. Zhang, L. Ni, C.J. Ma, Y. Zhang, X.Y. Wu, A.N. Atia, Z.Q. Yao, and J.P. Moorman. 2010. Programmed death-1 affects suppressor of cytokine signaling-1 expression in T cells during hepatitis C infection. *Viral Immunol* 23:487-495.

31. Freeman, G.J., A.J. Long, Y. Iwai, K. Bourque, T. Chernova, H. Nishimura, L.J. Fitz, N. Malenkovich, T. Okazaki, M.C. Byrne, H.F. Horton, L. Fouser, L. Carter, V. Ling, M.R. Bowman, B.M. Carreno, M. Collins, C.R. Wood, and T. Honjo. 2000. Engagement of the PD-1 Immunoinhibitory Receptor by a Novel B7 Family Member Leads to Negative Regulation of Lymphocyte Activation. *J Exp Med* 192:1027-1034.

32. Gabrysova, L., and D.C. Wraith. 2010. Antigenic strength controls the generation of antigen-specific IL-10-secreting T regulatory cells. *Eur J Immunol* 40:1386-1395.
33. Gergely, J., I. Pecht, and G. Sármay. 1999. mmunoreceptor tyrosine-based inhibition motif-bearing receptors regulate the immunoreceptor tyrosine-based activation motif-induced activation of immune competent cells. *immunology Letters* 68:3-15.
34. Gladyshev, V.N., T.C. Stadtman, D.L. Hatfield, and K. Jeang. 1999. Levels of major selenoproteins in T cells decrease during HIV infection and low molecular mass selenium compounds increase. *Proc. Natl. Acad. Sci. USA* 96:835-839.
35. Green, D.R. 2012. Metabolism and immunity: the old and the new. *Semin Immunol* 24:383.
36. Greenwald, R.J., Y.E. Latchman, and A.H. Sharpe. 2002. Negative co-receptors on lymphocytes. *Current Opinion in Immunology* 14:391-396.
37. Grundner-Culemann, E., G.W. Martin III, J.W. Harney, and M.J. Berry. 1999. Two distinct SECIS structures capable of directing selenocysteine incorporation in eukaryotes. *RNA* 5:625-635.

38. Hall, M.D., T.S. Marshall, A.D. Kwit, L.M. Miller Jenkins, A.E. Dulcey, J.P. Madigan, K.M. Pluchino, A.S. Goldsborough, K.R. Brimacombe, G.L. Griffiths, and M.M. Gottesman. 2014. Inhibition of glutathione peroxidase mediates the collateral sensitivity of multidrug-resistant cells to tiopronin. *J Biol Chem* 289:21473-21489.
39. Hildeman, D.A. 2004. Regulation of T-cell apoptosis by reactive oxygen species. *Free Radic Biol Med* 36:1496-1504.
40. Hirahara, K., and T. Nakayama. 2016. CD4+ T-cell subsets in inflammatory diseases: beyond the Th1/Th2 paradigm. *Int Immunol*
41. Hou, T.Z., O.S. Qureshi, C.J. Wang, J. Baker, S.P. Young, L.S. Walker, and D.M. Sansom. 2015. A transendocytosis model of CTLA-4 function predicts its suppressive behavior on regulatory T cells. *J Immunol* 194:2148-2159.
42. Huang, Z., A.H. Rose, and P.R. Hoffmann. 2012. The role of selenium in inflammation and immunity: from molecular mechanisms to therapeutic opportunities. *Antioxid Redox Signal* 16:705-743.
43. Jain, M., S. Rivera, E.A. Monclus, L. Synenki, A. Zirk, J. Eisenbart, C. Feghali-Bostwick, G.M. Mutlu, G.R. Budinger, and N.S. Chandel. 2013. Mitochondrial reactive oxygen species regulate transforming growth factor-beta signaling. *J Biol Chem* 288:770-777.
44. Jana, S., P. Jailwala, D. Haribhai, J. Waukau, S. Glisic, W. Grossman, M. Mishra, R. Wen, D. Wang, C.B. Williams, and S. Ghosh. 2009. The role of NF-kappaB and Smad3 in TGF-beta-mediated Foxp3 expression. *Eur J Immunol* 39:2571-2583.
45. Jang, J.Y., J.H. Min, Y.H. Chae, J.Y. Baek, S.B. Wang, S.J. Park, G.T. Oh, S.H. Lee, Y.S. Ho, and T.S. Chang. 2014. Reactive oxygen species play a critical role in collagen-induced platelet activation via SHP-2 oxidation. *Antioxid Redox Signal* 20:2528-2540.

46. Khayyamian, S., A. Hutloff, K. Büchner, M. Gräfe, V. Henn, R.A. Kroczek, and H.W. Mages. 2002. ICOS-ligand, expressed on human endothelial cells, costimulates Th1 and Th2 cytokine secretion by memory CD4 T cells. *Proc Natl Acad Sci U S A* 99:6198-6203.
47. Kim, H.R., A. Lee, E.J. Choi, M.P. Hong, J.H. Kie, W. Lim, H.K. Lee, B.I. Moon, and J.Y. Seoh. 2014a. Reactive oxygen species prevent imiquimod-induced psoriatic dermatitis through enhancing regulatory T cell function. *PLoS One* 9:e91146.
48. Kim, H.R., A. Lee, E.J. Choi, J.H. Kie, W. Lim, H.K. Lee, B.I. Moon, and J.Y. Seoh. 2014b. Attenuation of experimental colitis in glutathione peroxidase 1 and catalase double knockout mice through enhancing regulatory T cell function. *PLoS One* 9:e95332.
49. Kohrle, J. 2000. The deiodinase family: selenoenzymes regulating thyroid hormone availability and action. *Cell Mol Life Sci* 57:1853-1863.
50. Kwon, J., S. Devadas, and M.S. Williams. 2003. T cell receptor-stimulated generation of hydrogen peroxide inhibits MEK-ERK activation and lck serine phosphorylation. *Free Radical Biology and Medicine* 35:406-417.
51. Labunskyy, V.M., D.L. Hatfield, and V.N. Gladyshev. 2014. Selenoproteins: molecular pathways and physiological roles. *Physiol Rev* 94:739-777.
52. Langhoff, E., and R.M. Steinman. 1989. Clonal expansion of human T lymphocytes initiated by dendritic cells. *J Exp Med* 169:315-320.
53. Latchman, Y., C.R. Wood, T. Chernova, D. Chaudhary, M. Borde, I. Chernova, Y. Iwai, A.J. Long, J.A. Brown, R. Nunes, E.A. Greenfield, K. Bourque, V.A. Boussiotis, L.L. Carter, B.M. Carreno, N. Malenkovich, H. Nishimura, T. Okazaki, T. Honjo, A.H. Sharpe, and G.J. Freeman. 2001. PD-L2 is a second ligand for PD-1 and inhibits T cell activation. *Nature immunology* 2:261-268.
54. Leibson, P.J. 2004. The regulation of lymphocyte activation by inhibitory receptors. *Curr Opin Immunol* 16:328-336.
55. Liu, R.M., and K.A. Gaston Pravia. 2010. Oxidative stress and glutathione in TGF-beta-mediated fibrogenesis. *Free Radic Biol Med* 48:1-15.
56. Liu, Z., M.Y. Gerner, N. Van Panhuys, A.G. Levine, A.Y. Rudensky, and R.N. Germain. 2015. Immune homeostasis enforced by co-localized effector and regulatory T cells. *Nature* 528:225-230.
57. Lobanov, A.V., D.L. Hatfield, and V.N. Gladyshev. 2008. Selenoproteinless animals: selenophosphate synthetase SPS1 functions in a pathway unrelated to selenocysteine biosynthesis. *Protein Sci* 17:176-182.
58. Lubos, E., J. Loscalzo, and D.E. Handy. 2011. Glutathione peroxidase-1 in health and disease: from molecular mechanisms to therapeutic opportunities. *Antioxid Redox Signal* 15:1957-1997.
59. Matzinger, P. 2002. The danger model: A renewed sense of self. *Science* 296:
60. Mayne, S.T., M.C. Playdon, and C.L. Rock. 2016. Diet, nutrition, and cancer: past, present and future. *Nat Rev Clin Oncol*
61. Medzhitov, R., and C.A. Janeway, Jr. 2000. How does the immune system distinguish self from nonself? *Semin Immunol* 12:185-188; discussion 257-344.
62. Nishimura, H., T. Okazaki, Y. Tanaka, K. Nakatani, M. Hara, A. Matsumori, S. Sasayama, A. Mizoguchi, H. Hiai, N. Minato, and T. Honjo. 2001. Autoimmune dilated cardiomyopathy in PD-1 receptor-deficient mice. *Science* 291:319-322.

63. Okoye, A.A., and L.J. Picker. 2013. C4+ T-cell depletion in HIV infection: mechanisms of immunological failure. *Immunological Reviews* 254:54-64.
64. Parry, R.V., J.M. Chemnitz, K.A. Frauwirth, A.R. Lanfranco, I. Braunstein, S.V. Kobayashi, P.S. Linsley, C.B. Thompson, and J.L. Riley. 2005. CTLA-4 and PD-1 receptors inhibit T-cell activation by distinct mechanisms. *Mol Cell Biol* 25:9543-9553.
65. Patsoukis, N., K. Bardhan, P. Chatterjee, D. Sari, B. Liu, L.N. Bell, E.D. Karoly, G.J. Freeman, V. Petkova, P. Seth, L. Li, and V.A. Boussiotis. 2015. PD-1 alters T-cell metabolic reprogramming by inhibiting glycolysis and promoting lipolysis and fatty acid oxidation. *Nature communications* 6:6692.
66. Policastro, L.L., I.L. Ibanez, C. Notcovich, H.A. Duran, and O.L. Podhajcer. 2013. The tumor microenvironment: characterization, redox considerations, and novel approaches for reactive oxygen species-targeted gene therapy. *Antioxid Redox Signal* 19:854-895.
67. Reeves, M.A., and P.R. Hoffmann. 2009. The human selenoproteome: recent insights into functions and regulation. *Cell Mol Life Sci* 66:2457-2478.
68. Reinke, E.N., D.N. Ekoue, S. Bera, N. Mahmud, and A.M. Diamond. 2014. Translational regulation of GPx-1 and GPx-4 by the mTOR pathway. *PLoS One* 9:e93472.
69. Rubtsov, Y.P., J.P. Rasmussen, E.Y. Chi, J. Fontenot, L. Castelli, X. Ye, P. Treuting, L. Siewe, A. Roers, W.R. Henderson, Jr., W. Muller, and A.Y. Rudensky. 2008. Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. *Immunity* 28:546-558.
70. Schwartz, R.H. 2003. T cell anergy. *Annu Rev Immunol* 21:305-334.
71. Sharpe, A.H., E.J. Wherry, R. Ahmed, and G.J. Freeman. 2007. The function of programmed cell death 1 and its ligands in regulating autoimmunity and infection. *Nat Immunol* 8:239-245.
72. Shimizu, Y., A. Van Seventer, R. Siraganian, L. Wahl, and S. Shaw. 1989. Dual role of the CD44 molecule in T cell adhesion and activation. *Journal of immunology* 143:2457-2463.
73. Shrimali, R.K., R.D. Irons, B.A. Carlson, Y. Sano, V.N. Gladyshev, J.M. Park, and D.L. Hatfield. 2008. Selenoproteins mediate T cell immunity through an antioxidant mechanism. *J Biol Chem* 283:20181-20185.
74. Singh, A.K., G.S. Dhaunsi, M.P. Gupta, J.K. Orak, K. Asayama, and I. Singh. 1994. Demonstration of Glutathione Peroxidase in Rat Liver Peroxisomes and Its Intraorganellar Distribution. *Archives of Biochemistry and Biophysics* 315:331-338.
75. Spranger, S. 2016. Mechanisms of tumor escape in the context of the T-cell-inflamed and the non-T-cell-inflamed tumor microenvironment. *Int Immunol*
76. Tato, C.M., and J.J. O'Shea. 2006. What does it mean to be just 17? *Nature* 441:166-168.
77. Tkachev, V., S. Goodell, A.W. Opipari, L.Y. Hao, L. Franchi, G.D. Glick, J.L. Ferrara, and C.A. Byersdorfer. 2015. Programmed death-1 controls T cell survival by regulating oxidative metabolism. *J Immunol* 194:5789-5800.
78. Tone, Y., K. Furuuchi, Y. Kojima, M.L. Tykocinski, M.I. Greene, and M. Tone. 2008. Smad3 and NFAT cooperate to induce Foxp3 expression through its enhancer. *Nat Immunol* 9:194-202.
79. Unkeless, J.C., and J. Jin. 1997. Inhibitory receptors, ITIM sequences and phosphatases. *Current Opinion in Immunology* 9:338-343.
80. Veal, E.A., A.M. Day, and B.A. Morgan. 2007. Hydrogen peroxide sensing and signaling. *Mol Cell* 26:1-14.

81. Verbist, K.C., R. Wang, and D.R. Green. 2012. T cell metabolism and the immune response. *Semin Immunol* 24:399-404.
82. Wang, S., and L. Chen. 2004. Co-signaling molecules of the B7-CD28 family in positive and negative regulation of T lymphocyte responses. *Microbes Infect* 6:759-766.
83. Wildin, R.S., S. Smyk-Pearson, and A.H. Filipovich. 2002. Clinical and molecular features of the immunodysregulation, polyendocrinopathy, enteropathy, X linked (IPEX) syndrome. *J Med Genet* 39:537-545.
84. Williams, M.S., and J. Kwon. 2004. T cell receptor stimulation, reactive oxygen species, and cell signaling. *Free Radic Biol Med* 37:1144-1151.
85. Won, H.Y., J.H. Sohn, H.J. Min, K. Lee, H.A. Woo, Y.S. Ho, J.W. Park, S.G. Rhee, and E.S. Hwang. 2010. Glutathione peroxidase 1 deficiency attenuates allergen-induced airway inflammation by suppressing Th2 and Th17 cell development. *Antioxid Redox Signal* 13:575-587.
86. Wrobel, J.K., R. Power, and M. Toborek. 2016. Biological activity of selenium: Revisited. *IUBMB Life* 68:97-105.
87. Xu, X., B.A. Carlson, H. Mix, Y. Zhang, K. Saira, R.S. Glass, M.J. Berry, V.N. Gladyshev, and D.L. Hatfield. 2007. Biosynthesis of Selenocysteine on Its tRNA in Eukaryotes. *Plos Biology* 5:0096-0105.
88. Yamazaki, T., H. Akiba, H. Iwai, H. Matsuda, M. Aoki, Y. Tanno, T. Shin, H. Tsuchiya, D.M. Pardoll, K. Okumura, M. Azuma, and H. Yagita. 2002. Expression of Programmed Death 1 Ligands by Murine T Cells and APC. *The Journal of Immunology* 169:5538-5545.
89. Ye, B., X. Liu, X. Li, H. Kong, L. Tian, and Y. Chen. 2015. T-cell exhaustion in chronic hepatitis B infection: current knowledge and clinical significance. *Cell Death Dis* 6:e1694.
90. Yuan, J., S. Palioura, J.C. Salazar, D. Su, P. O'Donoghue, M.J. Hohn, A.M. Cardoso, W.B. Whitman, and D. Söll. 2006. RNA-dependent conversion of phosphoserine forms selenocysteine in eukaryotes and archaea. *PNAS* 103:18923-189277.
91. Zhang, Y., D.E. Handy, and J. Loscalzo. 2005. Adenosine-dependent induction of glutathione peroxidase 1 in human primary endothelial cells and protection against oxidative stress. *Circ Res* 96:831-837.
92. Zhou, L., J.E. Lopes, M.M. Chong, Ivanov, II, R. Min, G.D. Victora, Y. Shen, J. Du, Y.P. Rubtsov, A.Y. Rudensky, S.F. Ziegler, and D.R. Littman. 2008. TGF-beta-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing RORgammat function. *Nature* 453:236-240.
93. Zhou, L.Z.-H., A.P. Johnson, and T.A. Rando. 2001. NF kappaB and AP-1 mediate transcriptional responses to oxidative stress in skeletal muscle cells. *Free Radical Biology & Medicine* 31:1405-1416.
94. Zúñiga-Pflücker, J.C. 2004. T-cell development made simple. *Nature Reviews Immunology* 4:67-72.

Appendix

Table A: Primer list for qPCR			
Name	**Abbreviation**	**Sense**	**Anti-Sense**
Cystathionine gamma-lyase	CTH	GGCTTCCTGCCTAGTTTCCAG	AGTCCTGCTTAAATGTGGTGG
Selenophosphate Synthetase 1	SPS1	GAGAGTCCTTTAACCCGGAGA	CATCCCAATGCCAAGTCGTG
Selenophosphate Synthetase 2	SPS2	GATAGTGCCGTGGTAGGAGA	CTCTGGAAACCACCATCTTG
SECIS Binding Protein 2	SBP2	GCTGATGTCAAACCATTCGTCC	GCCATGTCTTCGGGATACATTT
glutathione peroxidase 1	GPX1	CAGGAGAATGGCAAGAATGA	GAAGGTAAAGAGCGGGTGAG
glutathione peroxidase 4	GPX4	GCAGGAGCCAGGAAGTAATC	GGCTGGACTTTCATCCATTTT
thioredoxin reductase 1	TR1	CTACAGACCATTGCCTTGCT	ACCTCCTACCCACAAGATCC
thioredoxin reductase 3	TR3	TCACTGGAATTGGACTGGAT	ACACAGCCTTTCAGGAACTG
15 kDa selenoprotein	Sep15	CTGGCGACTGCGTTTCAAG	CTGTCCAAGAAGATCGCAAGAG