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Glycerol-3-Phosphate Acyltransferase Regulates T Cell Effector Function and Metabolism

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Glycerol-3-Phosphate Acyltransferase Regulates T Cell Effector Function and Metabolism

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Dedication

This dissertation is dedicated to my mother, Judy Faris who never doubted my success even in the face of the utmost adversity.

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Glycerol-3-Phosphate Acyltransferase Regulates T Cell Effector Function and Metabolism

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The aged T cell is characterized by decreased responsiveness to stimulation. Aging is associated with reduced membrane glycerophospholipid (GPL) to cholesterol ratios so it is interesting that deletion of mitochondrial glycerol-3-phosphate acyltransferase-1 which catalyzes the first step in de novo GPL synthesis induces an aged T cell phenotype in otherwise healthy mice. GPAT-1 could regulate T cell function through three possible mechanisms: maintenance of membrane GPL ratios and membrane based signaling, providing a specific substrate for downstream signaling, or direct regulation of cellular metabolism. Therefore, the goal of this project was to determine whether these mechanisms contribute to the dysfunctional T cell phenotype observed with decreased GPAT-1 activity. T cell stimulation requires significant upregulation of metabolic processes to drive clonal expansion and cytokine production. T cell dysfunction in GPAT-1 knockout mice may be partially explained by altered metabolic function. We found that GPAT-1 KO T cells have significantly reduced basal respiration rates and spare respiratory capacity which is not compensated for by increased glycolytic metabolism suggesting an inherent metabolic defect in GPAT-1 KO T cells. To better understand mechanistically how GPAT-1 regulates T cell function we moved into the Jurkat T cell line and found that shRNA mediated knockdown of the human isoform of

GPAT-1 (*GPAM*) recapitulated key aspects of the dysfunctional T cell phenotype we observed in the mouse including highly significant reductions in IL-2 production and altered membrane GPL to cholesterol ratios. Phosphatidic acid addition was not capable of rescuing these deficiencies suggesting that GPAT-1/GPAM activity is required for proper T cell function. This was the first time that GPAT-1 activity has been shown to be important for T cell function in a non-murine model system and strongly suggests that GPAT-1/GPAM deficiency regulates T cell function at the cellular level. We further demonstrate that phosphorylation of ZAP-70 a proximal effector of T cell activation is significantly reduced in GPAM knock down Jurkat T cells, suggesting that membrane based signaling is dysfunctional. Taken together these data suggest that GPAT-1 is necessary for regulating cellular energy demands in T cells and essential for optimal T cell activation following stimulation.

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Chapter 1

Review of Literature

1.1 Overview

A key role is emerging for cellular metabolism in the regulation of T cell survival, differentiation, memory, and effector function [1,2,3,4,5]. The classical paradigm of T cell activation revolves around an activation induced metabolic switch that enables the stimulated cell to preferentially engage aerobic glycolytic metabolic pathways to meet energy needs in a process similar to the Warburg effect observed in cancer cells [1,6]. Current studies are starting to reveal that this paradigm is only part of the story and the resting energy state of the cell and factors of the metabolic machinery itself play an important role in determining T cell fate and effector function. Within the current paradigm, the role of lipid metabolism is largely understudied but recent developments are beginning to show that an important determining factor of T cell subset differentiation and effector function is the extent to which lipid or glycolytic pathways are engaged in the T cell [3,5,4,2].

New studies are revealing a pivotal role for lipid metabolism in T cell fate and effector function. It is very interesting then, that ablation of one of the rate limiting enzymes of *de novo* triacylglyceride (TAG) and gycerophospholipid (GPL) synthesis, GPAT-1, appears to play a central role in T cell biology. Specifically, T cells isolated from *Gpat-1* $^{-/-}$ mice are deficient in proliferation and interleukin-2 (IL-2) production while mice lacking GPAT-1 are more susceptible to virulence associated with Coxsackievirus B3 infection as demonstrated by higher incidences of mortality and 100 fold increases of viral titer in the heart [7,8]. These dysfunctions

and pathologies are of physiological relevance as they mirror defects observed in the aging immune system. *Gpat-1* $^{-/-}$ mice are deficient in nearly all major classes of membrane glycerophospholipid [7]. We hypothesize that these deficiencies in GPL result in changes to membrane fluid dynamics similar to those observed with aging. It is also of interest that GPAT-1 activity is significantly down regulated in T cells isolated from old rats, implying that down regulation of GPAT-1 with age may be a major contributor to altered membrane composition coincident with aging [9]. These data suggest that GPAT-1 may play a key role in the decline of the adaptive immune response with aging. Engagement of membrane based receptors such as the T cell receptor (TCR) initiate internal signal transduction cascades necessary for T cell activation. The Membrane Gate Theory postulates that age related changes in membrane structure perturb engagement of membrane based receptors which results in an insensitivity to antigenic stimuli and subsequent deficiencies in receptor tyrosine kinase and G protein coupled receptor signaling[10]. It is therefore possible that GPAT-1 regulates T cell function through modulation of membrane phospholipid composition and membrane based receptor signaling. Additionally, dysregulation of lipid metabolic pathways have specifically been shown to enhance T cell phenotypes that may work against an effective T cell driven immune response. It has been demonstrated that encouraging lipid oxidation over glycolysis can increase the expression of FOXP3, a transcription factor essential for the function and development of regulatory T cells (Tregs) which act to suppress effector T cell function [4]. Expression of this transcription factor in Jurkat T cells has been shown to be sufficient to induce a Treg-like phenotype [11]. Furthermore, studies in elderly humans have revealed a significant increase in FOXP3+ T cells and shown that in vitro depletion of this population improves effector T cell function[12]. We have also observed that FOXP3+ T cells are elevated in the *Gpat-1* $^{-/-}$ mouse (unpublished observation, Faris, Jolly).

Findings that increased lipid oxidation can alter T cell subsets is significant as it has been suggested that in the absence of GPAT-1 lipid oxidation is elevated. This is thought to be the result of fatty acyl-CoA being shunted away from TAG and GPL synthesis in the absence of GPAT-1 and toward mitochondrial β-oxidation via carnitine palmitoyl transferase 1 (CPT-1a). CPT-1a is located on the mitochondrial membrane and is believed to compete with GPAT-1for fatty acyl-CoA substrate. Interestingly, we have recently shown that CPT-1a expression is elevated in thymic T cells from *Gpat-1* $^{-/-}$ mice [13].

Our work in the *Gpat-1* $^{-/-}$ mouse has helped us to develop a model in which to observe the effects of altered membrane lipid composition on T cell function. To date this is the only known model of reduced cellular glycerophospholipid. However, a question that often arises in the study of a specific cell type isolated from a whole animal knockout is to what extent the macro environment of the model influences the observed cellular phenotype. Therefore, one goal of this project is to determine whether the dysfunctional T cell phenotype we observe in murine *Gpat-1* $^{-/-}$ T cells is due to an inherent loss of GPAT-1 resulting in a cell intrinsic defect rather than being the consequence of altered thymic development, epigenetic changes, or other mechanisms in the periphery of the *Gpat* $^{-/-}$ mouse. To accomplish this goal we generated a Jurkat T cell with GPAM, the human homologue of GPAT-1, knock down line (GPAMKD). This cell line will be used to determine whether the phenotype of GPAT-1 deficient T cells is due to developmental processes or a cell intrinsic effect. This will also be the first time that the effects of GPAM deletion are observed in a human T cell line. Furthermore, this line will be used to better our understanding of how GPAT-1/GPAM mechanistically regulates T cell function and fate.

It has long been speculated that GPAT-1 deficiency results in elevated lipid oxidation. Previous studies revealed a significant increase in radical oxygen species (ROS) from hepatocytes of *Gpat-1* $^{-/-}$ mice which was suggested to be a result of increased fatty acid metabolism[14]. However, to date, direct metabolic analysis of *Gpat-1* $^{-/-}$ cells has not been conducted. It is possible to determine the precise contributions of respiratory and glycolytic metabolism by measuring the oxygen consumption rate (OCR) and extra cellular acidification rate (ECAR) using special instrumentation such as the Seahorse XF analyzer. Therefore, the second major goal of this project is to determine whether primary murine T cells from *Gpat-1* $^{-/-}$ mice exhibit altered metabolic profiles at rest and in the presence of various modes of stimulation. This information combined with our previous observations will help us to gain insight as to whether GPAT-1 plays a role in regulating the metabolic switch in T cells and if so, how T cell effector function is modulated by these processes.

The overall goal of this project is to gain mechanistic insight as to how GPAT-1/GPAM regulates T cell fate and function. Understanding how GPAT-1 regulates T cell function will better our understanding of how metabolism influences T cell fate and effector function.

1.2 Objectives

The objectives of this project are to:

1) Develop and phenotypically characterize a stable GPAM knockdown Jurkat T cell line to determine whether GPAT-1/GPAM deficient phenotypes are the result of a cell intrinsic mechanism

Hypothesis: GPAT-1/GPAM deficiency results in cell intrinsic defects in the absence of developmental processes and GPAMKD Jurkat T cells recapitulate key aspects of T cell dysfunction observed in GPAT-1 knockout mice.

2) Identify the mechanism(s) by which GPAT-1/GPAM regulates T cell function and fate

Hypothesis: GPAT-1/GPAM regulates T cell function and fate through either, or a combination of a) Altering T cell metabolism, b) modulation of membrane phospholipids and membrane based receptor signaling, c) regulation of PA dependent signaling

1.3 Background

GPAT-1 is an 828 amino acid multi-pass integral outer mitochondrial membrane protein that catalyzes the conversion of glycerol-3 phosphate and acyl-CoA to lyosphosphatidic acid (LPA) which is then further acylated to produce phosphatidic acid (PA) presumably for phospholipid and triglyceride synthesis (reviewed by Coleman in [15]). Fatty acids are activated to acyl-CoAs on the outer mitochondrial membrane before entering either the glycerolipid biosynthetic pathway via GPAT-1 or the β-oxidation pathway via carnitine palmitoyltransferase-1 (CPT-1a). CPT-1a is believed to compete with GPAT-1 for fatty acyl-CoA at the outer mitochondrial membrane. Both CPT-1a and GPAT-1 are sensitive to nutrient levels within the cell, specifically the ATP/AMP ratio. GPAT-1 is the only GPAT isoform responsive to these nutrient signals. AMP activated protein kinase (AMPK) activity increases when there is an abundance of AMP within the cell, signaling that ATP levels are low. Consequently, activated AMPK regulates both CPT-1a and GPAT-1 reciprocally. When cellular energy stores are low AMPK is activated and downregulates GPAT-1 activity, while promoting CPT-1a activity [16]. The nutrient sensitivity of GPAT-1 enzymatic activity is due to post translational regulation via phosporylation by both PKC-θ and Casein Kinase II (CKII). Phosphorylation by either of these kinases increases GPAT-1 activity *in vitro*, as we, and others have shown [17], [18].

Four isoforms of the GPAT enzyme have been identified; 2 microsomal isoforms (GPAT-3 and GPAT-4) and 2 mitochondrial isoforms (GPAT-1 and GPAT-2)[19]. We have shown a global decrease in glycerophospholipid mass in murine *Gpat-1* $^{-/-}$ T cells [20]. It is of interest that murine T cells have only one mitochondrial isoform of GPAT, GPAT-1, while others have shown that murine hepatocytes actually have two distinct GPAT isoforms on the mitochondria (GPAT-1 and GPAT-2) [18],[21],[19]. In rats, GPAT-1 mRNA is found predominantly in liver, skeletal

muscle, and adipose tissue[22,23]. It is also found at lower levels in brain, kidney, heart, and adrenal gland. In mice, GPAT-1 mRNA is found predominantly in lung, liver, heart, and white adipose tissue[22,24]. In humans, GPAT-1 mRNA is found primarily in adipose tissue [22,24]. This is very interesting as, it suggests that GPAT family enzymes regulate glycerophospholipid mass differentially in tissues. The mammalian liver is the primary source of triglyceride and lipid production for the body, so it follows that a redundancy in mitochondrial GPAT may have evolved to provide a backup system for TAG and GPL synthesis. Regulation of GPAT-1 is controlled at multiple levels including nutritionally, developmentally, and hormonally through regulation of its transcription and through posttranslational modification. Studies have shown that treatment with insulin or administration of glucose can increase GPAT-1 expression 2-4 fold under the control of SREBP, Sp1, and insulin while polyunsaturated fatty acids and glucagon are strong inhibitors[25]. The orientation of GPAT-1 on the outer mitochondrial membrane puts its active site in a position facing the cytosol where it can be regulated by cytosolic effector kinases. Direct phosphorylation of GPAT-1 on mitochondria isolated from rat liver has been demonstrated by CK2 and PKCθ in vitro [26,27]. PKCθ is the predominant PKC isoform activated downstream of TCR/CD3 stimulation and has been shown to be dysregulated with age[28]. This is very interesting as we have shown that GPAT-1 activity is also decreased with age suggesting that GPAT-1 dysfunction may be partially attributed to decreased PKCθ activity. A positive regulatory loop model in which GPAT-1 regulates phospholipid mass at the cell membrane and in turn promotes successful TCR engagement and PKCθ activation that works to positively reinforce GPAT-1 activity is possible.

The T cell is the principle regulator of the type and extent of an immune response. At rest, and in the absence of foreign challenge, the T cell resides in a state of relative dormancy;

however, upon encountering foreign antigen presented on the surface of ancillary antigen presenting cells (APCs) the T cell can be stimulated to aggressively divide, producing numerous clones of itself, which specifically recognize the triggering antigen, in a process called clonal expansion. TCR engagement culminates in the activation of numerous signal enhancing and signal attenuating cascades which ultimately result in T cell proliferation and IL-2 production. The ultimate target of these signal cascades are transcription factors (NFAT, AP-1, NF-κB) that work synergistically to bind the IL-2 promoter and initiate transcription of IL-2 (interleukin-2). IL-2 is the key cytokine involved in T cell activation and proliferation. T cells are dependent upon IL-2 through autocrine and paracrine signaling for maintenance of activation and to reinforce pro-proliferative signaling in surrounding cells.

Successful TCR engagement results in phosphorylation of ITAM motifs on CD3-ζ subunits [29]. A recent study by Gagnon, et. al. has demonstrated that TCR engagement induces dissociation of the CD3ε cytoplasmic domain due to a reduction in negative charge and phosphatidyl serine in membrane TCR microclusters, thus inducing a conformational change that exposes the cytoplasmic ITAM domains of the subunit[30]. ITAM phosphorylation is initiated by the Src-related protein tyrosine kinases: Lck and Fyn [31], [32]. Lck and Fyn are thought to associate with the cytosolic tails of the CD4 and CD8 co-receptors on helper and cytotoxic T cells while Lck association with CD28 has also been demonstrated [33],[34], [35], [36], [37]. Lck is negatively regulated through phosphorylation of inhibitory tyrosine residues by Csk and positively regulated through the protein phosphatase CD45, however it has been recently demonstrated that Lck exists in a constitutively active form and its activity is spatially regulated through its association and co-recruitment to the immunological synapse via its association with CD4 and/or CD28 [33], [38],[39], [40]. A recent study by Rossy, et. al. has gone further to show that Lck clustering is driven by

conformational changes changes in the protein itself[41]. Lck clustered at the TCR/CD3 complex can phosphorylate tyrosine residues on CD3-ζ subunits which serve as recruitment sites for the tyrosine kinases: Fyn and ZAP-70 [32],[31],[42]. ZAP-70 binds and engages the dual phosphorylated ITAMs on the CD3-ζ chain via its tandem SH-2 domains [42], [32]. ZAP-70 localization to the immunological synapse is highly dependent on co stimulatory signals through coreceptor complexes[43]. Once docked on the ITAM, Lck can phosphorylate and activate ZAP-70 placing it in a position to phosphorylate the transmembrane adaptor protein; LAT. Phosphorylated LAT in turn serves as a docking site for a myriad of SH2 domain containing signaling proteins including: SHB, PLCG1, GRAP2, GRAP, Grb2, PIK3R1, ITK, MAP4K1 and VAV1 [44]. Once VAV, SLP76 and Grb2 have docked on LAT they can act to recruit PI3K and PLCγ which are both necessary for the activation of Protein Kinase Cs (PKCs) [45]. ZAP-70 and SLP-76 regulate PKC-θ and NF-κB activity in response to TCR activation [46]. Following activation of the LAT scaffolding complex, PLCγ1 is found in the signaling complex bound to SLP-76, Vav1, and LAT, where it is phosphorylated and activated by Itk [47], [48], [49], [50]. PLC-γ1, goes on to cleave phosphatidylinositol 4,5-bisphosphate generating the second messenger's inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 induces Ca^{2+} influx and DAG activates PKCs, with PKC-θ being the isoform most prevalent to T cell activation [51]. Ca^{2+} plays a key role in the activation of calmodulin which in turn activates calcineurin that can subsequently induce a conformational change in the nuclear factor for activated T cells (NFAT) exposing a nuclear localization sequence. NFAT family mediated gene transcription is necessary for the propagation of pro-proliferative signaling in T cells following sufficient activation of the TCR/CD3/CD28 complex, so NFAT activation is a reliable marker of successful TCR engagement and signal transmission. To date five isoforms of NFAT have been identified:

NFATc1, NFATc2, NFATc3, NFATc4, and NFATc5 while NFATc1 and NFATc2 are thought to be the principle isoforms involved in IL-2 induction [52],[53]. NFAT alone is not sufficient to activate IL-2 transcription or promote proliferation; in fact NFAT binding of target sequences alone will ultimately initiate a program of transcription leading to T cell anergy (reviewed in[54]). However, NFAT can form a complex with AP-1(cJun/cFos subunits) integrating upstream Ca^{2+} and Ras signaling altering the transcriptional profile of the complex and promoting proliferation [55]. The NFAT/AP-1 complex is capable of propagating pro-proliferative signaling through activation of mitogenic and IL-2 related genes. NFAT can also form complexes with a number of other transcription factors key to T cell signaling including, Oct1 and NF-κB, with each complex being capable of initiating the transcription of a unique suite of genes. The end result of proximal TCR signaling is the activation of a multitude of distal transcription factors, (i.e. NFAT, NF-κB, AP-1, Erk) which regulate T cell survival and proliferation. Altered signaling through proximal effectors such as LAT, ZAP-70, Grb2, or PKC-θ can have dramatic effects with regards to transcription factor activation and subsequent mitogenic signaling and IL-2 production.

In the Fluid Mosaic Model of cell membranes put forth by Singer and Nicolson biological membranes are envisioned as two dimensional fluids in which lipid and protein diffuse freely in the membrane[56]. The idea that membranes are segregated as microdomains enriched in specific lipid species first emerged from studies on lipid trafficking in epithelial cells [57],[58],[59]. Membrane microdomains appear to be enriched in glycosphingolipids and cholesterol. Based on these studies, is was postulated that glycosphingolipids form clusters or "rafts" within the Golgi membrane which serve as sorting platforms for the inclusion of proteins destined for outer cell membranes [60],[59]. The idea that lipid rafts may serve as platforms for specific protein molecules came from studies in model membranes showing that glycosphingolipids and cholesterol

strongly associate with each other forming densely packed clusters which can sequester proteins that colocalize with the raft microdomain [61],[59]. Protein interactions with sphingomyelin form a detergent insoluble partition of the membrane which is strengthened by association with cholesterol[62]. The first proteins to be identified in detergent insoluble membrane fractions hypothesized to be lipid rafts were the glycosylphosphatidylinositol (GPI) anchored proteins[63]. These findings came from studies that were able to track transport and incorporation of these proteins into putative lipid rafts through the Golgi apparatus. Further work by other groups revealed that myristoylation at the N-termini of src family tyrosine kinases allowed them to associate with detergent insoluble membrane fractions suggesting a role for lipid rafts in localizing signaling to specific membrane compartments [64],[65]. The discovery that palmitoylated trimeric G-proteins and G-protein coupled receptors associate with raft fractions strengthened the argument that lipid rafts play an important role in localizing membrane based receptors with the internal signal transduction machinery [66].

T cell activation requires the coordinate activation of several membrane based receptor complexes that do not normally associate. Lipid raft microdomains could function as signaling platforms to bring together components of the immunological synapse. It has been hypothesized that coreceptors such as CD4, CD8, and CD28 associate with lipid raft microdomains in cell membranes. In the case of CD28, it has recently been demonstrated that CD28 interacts with filamin-A and directs lipid raft accumulation at the T cell immunological synsapse by inducing cytoskeletal reorganization[67]. This finding is highly significant as it for the first time shows that co-receptor localization to the immunological synapse requires raft mediated mobility. It is well known that disruption of sphingomyelin or cholesterol in the cell membrane disrupts raft formation and subsequent downstream signaling of raft associated receptor signaling complexes.

This is interesting in regards to GPAT-1 ablation, as a characteristic aspect of the *Gpat-1* $^{-/-}$ T cell is an increase in the cholesterol to phospholipid ratio. Although rafts are enriched in cholesterol, it is possible that in the absence of GPAT-1, the balance maintained between raft and non-raft partitions of the membrane are grossly perturbed and the size of raft fractions is increased in such a way as to prevent spatially dependent association of membrane based receptors and their intracellular signal transduction components. One of our principle hypotheses is that the observed decrease in membrane phospholipids observed in *Gpat-1* $^{-/-}$ T cells inhibits localization of CD28 to the immunological synapse resulting in a CD3 only signal through the TCR. This partial signal could potentially engage activation induced cell death in the T cells as we have shown that when stimulated with CD3/CD28 approximately 70% of *Gpat-1* $^{-/-}$ T cells undergo apoptotic cell death[7]. If the TCR/CD3/CD28 complex is not activated properly following stimulation, we should see a decrease in the phosphorylation of the tyrosine kinase ZAP-70, one of the earliest proximal effector kinases activated following T cell stimulation. Interestingly, when *Gpat-1* $^{-/-}$ T cells are stimulated with PMA/Ionomycin, which works through direct activation of PKC's and triggering of Ca^{2+} release from intracellular stores bypassing membrane based signaling, proliferation and IL-2 production do not appear altered[7]. This is highly significant as it suggests that GPAT-1 regulates T cell function through a membrane receptor based mechanism. Therefore, a primary goal of this study is to determine whether ZAP-70 activation is decreased in GPAMKD Jurkat T cells. This will offer strong support for our hypothesis that GPAT-1 regulates T cell effector function and fate through modulation of membrane phospholipids and perturbation of membrane based receptor signaling.

The hypothesis that PA is necessary for effective T cell activations was first put forth nearly two decades ago by Stewart [68]. Phosphatidic acid is a pleitropic lipid second messenger

and protein anchoring molecule essential for mitogenic signaling and membrane remodeling in mammalian cells as well as the structural precursor of all GPL's [69]. PA is the most structurally simple of all the phospholipids and is an important intermediate in glycerphospholipid synthesis and signaling. TCR engagement results in rapid upregulation of phospholipid metabolism culminating in the appearance of numerous lipid mediators of signaling. PA has recently risen to prominence as a key regulator of MAPK signaling in T cells. The classical view of MAPK signaling is that once engaged by their cognate ligand, protein tyrosine kinase receptors (PTKR) dimerize leading to cross phosphorylation of tyrosine residues in their cytosolic domains via the intrinisic kinase activity of that domain. In the case of the T cell, the CD3-ζ chain ITAM functions as classical PTKRs in response to antigen engagement of the TCR. Phosphotyrosine on the ITAM serve as docking sites for numerous adaptor proteins containing SH2 domains. One of these adaptors, Grb2 recruits the Ras specific GEF SOS through its SH3 domains where it acts to recruit Ras to the plasma membrane. Until very recently it was thought that Ras directly recruited the serine/threonine kinase Raf-1 to the cell membrane, however a seminal study by Rizzo, *et. al.* showed for the first time that Raf-1, an essential component of the MAPK signaling cascade physically binds PA in order to localize to membranes and this binding is independent of Ras association[70]. In fact, it is PA dependent membrane association of Raf-1 that actually activates its kinase activity through a poorly understood mechanism involving Ras. Further studies showed that mutation of PA binding domains in Ras completely abrogates Raf-1 dependent MAPK signaling and results in cell cycle arrest (reviewed in [71]). Once activated Raf-1 can phosphorylate and activate MEK, a tyrosine/threonine kinase which can go on to phosphorylate and activate Erk 1 and Erk 2. The Erk family is directly involved with transcription factor activation in the nucleus, and in the case of T cells drive the expression of IL-2 through NFAT, AP-1, and NF-κB.

Previous work probing PA mediated MAP kinase signaling in T cells has focused on two mechanisms of PA production; (i)PLD mediated hydrolysis of phosphatidylcholine and (ii) phosphorylation of diacylglycerol by DAGK-α . The predominant opinion in the literature is that PLD2 is the principle contributor of PA in TCR based mitogenic signaling. However, it is well known that the direct product of GPAT-1; lyso-PA is converted to PA via lyso-PA acyl transferases. The role of GPAT-1 in contributing to cellular PA has yet to be probed by any other group. Our previous work shows that GPAT-1 deficiency results in a >72% decrease in total cellular PA, thereby presenting the possibility that GPAT-1 is a key regulator of PA in T cells[7]. In T cells, PLD2 is activated through TCR dependent membrane based signaling. It is interesting then that we have shown that GPAT-1 activity is also regulated through membrane based TCR signaling[18]. Specifically, we have shown that GPAT-1 is positively regulated by PKC-θ through phosphorylation. PKC-θ activated in T cells following TCR engagement [51], and is itself activated by Ca^{2+} and DAG; both products of PLC-γ/phosphoinositide based signaling mechanisms. New evidence shows that mitochondria localize to the TCR-supra molecular activation complex (SMAC) following successful TCR engagement which would theoretically place GPAT-1 in a prime location to be activated by PKC-θ and subsequently generate large amounts of LPA for immediate conversion to PA by lyso-PA-acyl transferase. This PA generated at the SMAC would be readily available to facilitate Raf-1 binding and perpetuate Ras initiated MAPK signaling through ERK 1/2. It is also possible that GPAT-1 maintains cellular homeostatic levels of PA and PLD2 acts to raise intracellular PA in such a way as to create a backlog of PA at other glycerophospholipid anabolic enzymes thus allowing for sustained free PA for signaling. The most straight-forward way of testing the hypothesis that GPAT-1/GPAM contributes to the PA pool and the observed dysfunctional T cell phenotype is a result of deficient cellular PA is to add

PA back to culture and look for rescue of the phenotype. Preliminary experiments in the GPAMKD Jurkat T cell revealed a dramatic decrease in IL-2 secretion compared to control cells. Therefore, we will add PA at physiologically relevant concentrations to cultured GPAMKD Jurkat T cells and screen for the rescue of IL-2 activity.

Naïve TH_0 cells enter circulation following thymic selection where they subsist in a state of minimal energy expenditure consuming just enough glucose and other nutrients to maintain homeostatic processes [72,73,74,75]. Quiescent T cells retain the ability to rapidly upregulate metabolic pathways and induce a program of clonal expansion following encounter with their MHC presented cognate antigen. The program of gene regulation and protein modification following antigenic stimulation transforms the resting cell into a highly active cytokine producing effector cell within a matter of hours. This transformation requires enormous energy expenditure on the part of the cell with pathways of both proliferation and cytokine production being engaged simultaneously. To meet these energy needs the cell must make dramatic alterations in their metabolism. This metabolic switch allows T cells to heavily engage glycolytic pathways in response to stimulation while only modestly increasing oxygen consumption [76,72]. Since this switch is concomitant with stimulation, it follows that receptors involved in T cell activation must play a role in regulating metabolism. Further work in this area and current studies are revealing this to be the case. One intriguing example is CD28 mediated regulation of glucose metabolism. A seminal study by Frauwirth, *et. al.* showed that CD28 costimulation acts through PI3K and Akt to increase the glycolytic metabolism in T cells[73]. Furthermore, they showed that most of the lactate generated as a by-product of stimulation induced glycolysis is secreted indicating that increased metabolism in stimulated T cells is not simply a response to increased energy demand, but a direct result of CD28 costimulation. However, stimulation with

CD28 alone does not appear to activate PI3K and Akt, or glycolytic metabolism in general suggesting that TCR/CD3 stimulation is necessary for upregulation of glycolytic metabolism. It has been suggested that upregulation of glycolysis following T cell stimulation is due to a synergetic effect between the signaling pathways engaged by CD3, CD28, and other coreceptor molecules. As described by Frauwirth and Thompson, costimulation can be summarized in a two pronged model where the TCR/CD3 complex form one branch and costimulatory molecules such as CD28 form a second branch. Signaling through the TCR initiates gene transcription which places a significant demand for energy on the cell. In order to compensate, the cell undergoes increased levels of oxidative phosphorylation from its limited glucose pool. Costimulation through CD28 and other coreceptors induces the expression of glucose transporters to meet the growing energy needs of the cell. This synergistic effect is supported by the fact that glycolytic metabolism is posttranscriptionally regulated so glucose uptake can be dramatically increased through costimulation to levels exceeding the energy needs of the cell [74,73]. A similar phenomenon has been described in fat and muscle cells where insulin induces glucose uptake in excess of cellular needs which promotes TGL synthesis and glycogen storage.

Glucose metabolism undoubtedly plays a pivotal role in T cell survival and effector function. However, the role of fatty acid metabolism in T cells has been largely eclipsed by focus on the glycolytic switch. Until recently few groups have asked the question as to what, if any role lipid metabolism play in T cell function and fate. Current work is starting to unveil a previously unappreciated role for lipid metabolism during T cell activation and downstream function. The first convincing evidence showing that lipid ligands mediate the T cell response came from a study by Clark, *et. al*. demonstrating the immunoregulatory role of PPAR-γ in T helper cytokine secretion[77]. This study showed for the first time that the lipid responsive nuclear

receptor PPAR-γ 1 is expressed in murine splenocytes. Further analysis revealed that the PPAR-γ ligand 15d-PGJ2 or pharmacological inhibition with thiazolidinedione or ciglitazone significantly reduced IL-2 secretion, a key cytokine in the T cell driven adaptive immune response. In the context of *Gpat-1* $^{-/-}$ T cells, this is highly significant as we have demonstrated that levels of the potent PPAR activators LTB_4 and PGE_2 are significantly elevated [7]. The general role of PPAR family transcription factors is anti-inflammatory in nature as their activity has well studied in macrophages and dendritic cells[78],[79],[80]. A large variety of endogenously generated lipid metabolites originating from saturated or unsaturated fatty acid metabolism are capable of activating PPAR nuclear receptors which can act to positively or negatively regulate gene transcription[81]. These early studies suggest a key role for lipid metabolites in regulating the type and extent of both the innate and adaptive immune response.

Although the importance of lipid metabolites as PPAR activators has become well known over the past decade, a more direct role for lipid metabolism in T cell differentiation and effector function was not realized until very recently. A plethora of factors engage in the regulation of cellular metabolism including numerous hormones, growth factors, effector kinases, transcription factors and nutrients themselves such as glucose. One nutrient sensing kinase and its associated signaling partners has risen to prominence recently as a key regulator of the glycolytic and lipid oxidative switch, the mitochondrial target of rapamycin (mTOR). The functional significance of mTOR in regulating cell growth and proliferation has been well elucidated over the years[82]. However, evidence for the direct involvement of mTOR in regulating lipid metabolic pathways has only recently begun to surface. The first demonstration of a direct role for mTOR in regulating lipid metabolism came from work in primary cultures of rat hepatocytes[83]. In that study, the authors specifically inhibited mTOR with Rapamycin and observed that inhibition

increased oxidation of exogenous fatty acids 46%- 100%, respectively while de novo lipid synthesis were reduced by 60%. They also found that rapamycin decreased expression of mitochondrial glycerol-3-phosphate acyltransferase (GPAT-1), the rate limiting enzyme in *de novo* TGL and GPL synthesis. Consistent with a role for mTOR in regulating lipid metabolism, glucose utilization was unaffected by rapamycin. These results strongly suggest that mTOR regulates energy production through direct modulation of hepatic fatty acid metabolism. Other studies followed directly implicating mTOR in the regulation of regulatory T cells development and differentiation. CD4+FOXP3+ Regulatory T cells (Tregs) are a suppressor T cell subset that is thought to modulate immune self/non-self-discrimination by limiting T effector (Teff) cell function during and following an immune response. A key study by Haxhinsato, et. al. identified Akt as a repressor of Treg development both in vitro and in vivo [84]. Akt can be activated through the PI3K pathway and active Akt can go on to activate mTOR and negatively regulate lipid metabolism by upregulating glycolytic metabolism. This shows that suppression of lipid oxidation through constitutive activation of the Akt-mTOR axis negatively regulates Treg development. This was a highly significant finding as it for the first time implicates a requirement for lipid metabolism in the development of specific T cell subsets. Following these observation, a cutting edge study by Michalek, et. al. demonstrated for the first time that distinct glycolytic and lipid oxidative metabolic programs actually define specific T cell subsets[4]. This study showed that effector and regulatory T cell subsets prefer either glycolytic or lipid oxidative metabolic pathways. Intriguingly, they found that induction of either metabolic pathway was sufficient to enrich the target T cell phenotype. Specifically, T effector cells preferably engage in glycolytic metabolism while T regulatory cells primarily engaged lipid metabolic pathways to meet energy needs and induction or inhibition of either metabolic process was sufficient to

enrich T effector or T regulatory cell populations. These studies convincingly demonstrate the importance of lipid metabolism in T cell differentiation and effector function. The exact mechanism(s) by which lipid metabolism regulates these processes is still unclear but the involvement of PPAR and FOXP3 mediated gene regulation has been suggested. While the focus of T cell activation and differentiation has been on cytokines, antigenic signaling and various modes of stimulation, evidence is mounting that strongly implicates cellular metabolism at the fundamental level as the major determining factor of T cell function and thus the adaptive immune response[85]. A ground breaking study by Pearce, *et. al.* highlighted the critical role of fatty acid metabolism in CD8+ T cell memory[86]. The authors showed that tumor necrosis factor receptor associated factor 6 (TRAF6) regulates CD8+ memory T cell development through modulation of fatty acid metabolism. Specifically, microarray analysis revealed that TRAF6 is regulates many key genes involved in fatty acid metabolism and TRAF6 deficient T cells are defective in AMP-acivated kinase activation and mitochondrial fatty acid oxidation. This study for the first time demonstrated a necessity of lipid oxidation in the development of CD8+ memory T cell populations. Further studies conducted by van der Windt, *et. al.* demonstrated that CD8+ memory T cells, but not CD8+ effector cells possess substantially more spare respiratory capacity (SRC)[76]. SRC is defined as the potential for a cell to meet energy demands in response to stimulation or stress. Probing of possible mechanisms regulating increased SRC in CD8+ memory T cells revealed a key role for IL-15, which is known to play an indispensable role in CD8 memory development. The authors found that IL-15 regulates mitochondrial biogenesis and promotes fatty oxidation through up regulation of CPT-1a. CPT-1a is the rate limiting step in lipid oxidative metabolism.

An indirect consequence of elevated lipid oxidation is the generation of reactive oxygen species (ROS). ROS levels are associated with damage to cellular macromolecules such as protein, RNA, and DNA, which can potentially lead to deleterious mutations and have been hypothesized to play a role in aging and cancer development. Hepatocytes from *Gpat-1* $^{-/-}$ mice exhibit elevated ROS, apoptosis, and increased proliferation[14]. In this study, the authors did not find a direct link between ROS and DNA damage however a dramatic increase in Ca^{2+} induced opening of the mitochondrial permeability transition pore (MPTP) was observed. MPTP opening is associated with leakage of proapoptotic factors from the mitochondria and in response to oxidative stress. This strongly suggests that in the absence of GPAT-1 increased lipid oxidation results in elevated levels of ROS and associated cellular dysfunction. This finding is intriguing in terms of *Gpat-1* $^{-/-}$ thymocytes as we have demonstrated that CPT-1a protein levels are upregulated[13]. Increased CPT-1a protein provides a mechanism by which ROS could be elevated in the absence of GPAT-1. ROS has been shown to induce oxidative stress in T cells with a principle consequence being decreased T cell activation and cytokine production. A study by Chakravarti, et. al. specifically showed that ROS induced oxidative stress in T cells prevents the phosphorylation and activation of Zap-70, a critical effector kinase involved in antigen induced T cell activation[87]. Several studies have suggested that fatty acid metabolism is dysregulated in the absence of GPAT-1, however to date, no one has directly measured glycolytic and oxidative metabolism in GPAT-1 deficient cells. One group however, has shown that overexpression of GPAT-1 depresses hepatocyte fatty acid oxidation *ex vivo*[88]. In light of the large body of evidence supporting a role for lipid metabolism in T cell differentiation and effector function it is necessary to determine whether lipid metabolism is altered in the absence of GPAT-1. Therefore, a major goal of this project is to determine whether the metabolic profile of *Gpat-1* $^{-/-}$ T cells is

altered. Several metabolically relevant scenarios could explain the phenotype observed in *Gpat-1* $^{-/-}$ T cells. It is possible that increased ROS formed as a consequence of elevated CPT-1a induced fatty acid oxidation contributes to the phenotype of *Gpat-1* $^{-/-}$ T cells. If these ROS act locally at the mitochondrial level, they could act to compromise mitochondrial integrity and make the cell more prone to engaging apoptotic pathways when stressed by stimulation. Other mechanisms such as increased engagement of PPAR pathways or induction of a Treg-like phenotype could functionally contribute to the phenotype as well. We have observed significantly elevated CD4+CD25+FOXP3 positive T regulatory cell populations in the GPAT-1 knockout mouse. Increased levels of fatty acid oxidation have been demonstrated to promote a Treg phenotype as described above. An important point to make is that many key aspects of GPAT-1 deficiency in T cells correlate not only with age, but fit the phenotype of Treg cells as well, i.e. reduced IL-2 secretion. It is therefore possible that GPAT-1 regulates T cell function through modulating the balance between fatty acid oxidation and lipid synthesis thereby influencing effector differentiation following stimulation.

The preceding findings from the literature are very interesting in the context of *Gpat-1* $^{-/-}$ T cells, where the rate limiting enzyme of *de novo* TGL and GPL synthesis is absent. The phenotype of *Gpat-1* $^{-/-}$ T cells is characterized by decreased IL-2 secretion, increased apoptosis in response to CD3/CD28 stimulation and significantly altered phospholipid compostion[7]. We hypothesize that the dysfunctional *Gpat-1* $^{-/-}$ T cell phenotype is likely attributed to one, or combination of three biological processes. The first possibility is that alterations in membrane phospholipid content perturb signaling through membrane based receptors such as the TCR/CD3/CD28 complex. The second possibility is that GPAT-1 regulates the intracellular phosphatidic acid pool and GPAT/GPAM deficiency results in a sink within the cellular PA pool

and subsequent inhibition of PA dependent signaling pathways. The third possibility is that in the absence of GPAT-1/GPAM lipid oxidation is elevated with negative consequences for T cell effector function and specifically IL-2 production. This dissertation explores these three possibilities.

Chapter 2

Glycerol-3-phosphate Acyltransferase, Mitochondrial Regulates IL-2 Production, Phospholipid Mass and Apoptosis in Jurkat T cells

2.1 Abstract

We have previously demonstrated that Glycerol-3-phosphate acyltransferase-1, the first and rate limiting step in *de novo* glycerophospholipid synthesis regulates murine T cell function. The resultant phenotype is characterized by decreased IL-2 production, increased propensity toward apoptosis, and altered membrane glycerophospholipid mass similar to that of an old T cell. Since T cells in previous experiments were from whole animal GPAT-1 knockout mice, questions remained as to what extent the macro environment of the model influenced the observed cellular phenotype. Therefore, one goal of this study was to determine whether the GPAT-1 knock out phenotype is the result of a cell intrinsic defect rather than being the consequence of altered thymic development, epigenetic changes, or other mechanisms in the periphery of the GPAT-1$^{-/-}$ mouse. In addition, the role of GPAT-1 in T cells from human lineage is completely unknown, so we sought to generate and phenotypically characterize a mitochondrial glycerol-3-phosphate acyltransferase (GPAM) deficient Jurkat T cell. Furthermore, this line was used to probe possible mechanisms by which GPAT-1/GPAM regulates T cell function. We report here that many the key characteristics of the dysfunctional murine GPAT-1$^{-/-}$ T cell recapitulate in the GPAMKD Jurkat T cell. We found striking decreases in IL-2 production along with altered phospholipid mass and increased apoptosis. We next sought identify mechanisms by which GPAM regulates this phenotype. Since phosphatidic acid

(PA) serves as the precursor to all glycerophospholipids and this mass is decreased in both GPAT-1$^{-/-}$ T cells and GPAMKD Jurkat T cells, we asked whether PA addition to culture was capable of rescuing IL-2 production in the GPAMKD and subsequently found no effect under all of the conditions tested. We next asked whether changes in phospholipid mass and membrane composition were interfering with T cell activation. GPAMKD T cells were found to have significantly decreased Zap-70 phosphorylation following stimulation, implying that GPAM deficiency results in altered membrane based signaling possibly as the result of loss of the co stimulatory CD28 signal. These data show for the first time that GPAM deficiency results in an inherent defect in T cell functionality and that this defect is likely the result of altered membrane based signaling.

2.2 Introduction

Glycerol-3-phosphate acyltransferases (GPATs) catalyze the initial and rate limiting step in the de novo biosynthesis of triacylglycerol (TAG) and glycerophospholipids (GPL). Utilizing glycerol-3-phosphate and fatty acylCoA as substrates, GPATs generate lysophosphatidic acid which can serve as a signaling molecule, be further acylated to phosphatidic acid (PA) which can also act as a signaling molecule and docking site for mitogenic factors such as Raf-1 or can enter lipid biosynthetic pathways involved in the production of TAG and GPL. Four GPAT isoforms (GPAT 1-4) have been identified in mammalian cells with the distribution and activity of each varying between tissue and cell type (reviewed in [89]). Of these four isoforms, GPAT-1 (GPAM in humans) and GPAT-2 are integral mitochondrial membrane proteins while GPAT-3 and GPAT-4 are typically isolated in microsomal fractions. The role of GPAT-1 in generating liver derived GPL, regulating radical oxygen species (ROS) generated as a by-product of β-oxidation, and regulation of hepatocyte proliferation has been extensively studied in murine

models [14]. We published the first evidence supporting an immunoregulatory role for GPAT-1 in murine T cells [7], however little is known of the underlying mechanisms by which GPAT-1 regulates T cell function. Additionally, to the best of our knowledge it remains unknown as to whether GPAM, the human homologue of GPAT-1, acts in a similar immunoregulatory capacity in T cells.

The T cell is the principle regulator of the type and extent of an immune response. At rest, and in the absence of foreign challenge, T cells reside in a state of relative dormancy; however, upon encountering foreign antigen presented on the surface of ancillary antigen presenting cells (APCs) the T cell can be stimulated to clonally expand with clones specifically recognizing the triggering antigen. T cell receptor engagement culminates in the activation of numerous signal enhancing and signal attenuating cascades which ultimately result in T cell proliferation and cytokine production. The ultimate targets of many these signal cascades are transcription factors (NFAT, AP-1, NF-κB) that work synergistically to bind the IL-2 promoter and initiate its transcription. IL-2 is the key cytokine involved in T cell activation and proliferation. T cells are dependent upon IL-2 through autocrine and paracrine signaling for maintenance of activation and to reinforce pro-proliferative signaling in surrounding cells. Reduced IL-2 secretion is a hallmark of aging in both humans and rodents. Although the overall number of T cells does not change with age, it has been demonstrated that T cell function is comparatively impaired relative to young controls (reviewed in [90]).

Changes in membrane phospholipid composition are characteristic of aged cells belonging to various tissues and lineages. The observation that membrane fluidity is significantly decreased with age has led to the "membrane gate theory" which postulates that the cellular dysfunction observed with aging may be attributed to alterations in membrane phospholipid

composition [10]. Models in which to study the effects of altered membrane lipid composition in T cells are largely lacking, making the study of the molecular mechanisms underlying age related cellular dysfunction difficult. There are very few methods with which to artificially alter membrane phospholipid composition. One such method that has been attempted is the removal of cholesterol by treatment with cyclodextrin but this has been reported to induce off target changes in the cell. Therefore, one goal of this study was to establish a stable Jurkat T cell line deficient in GPAM in which to study the effects of altered phospholipid composition on T cell function. Jurkat cells are the most well studied model of T cell activation and signaling in which a multitude of now well-known mechanisms were first identified [91]. Due to the vast amount of data in the literature describing precise molecular mechanisms of T cell activation, proliferation and cytokine production in Jurkat T cells we selected this cell line to further elucidate the role of GPAM in T cell function.

We have previously shown that primary T cells from GPAT-1 $^{-/-}$ mice have an altered phospholipid profile and secrete reduced levels of IL-2 in response to stimulation [7]. One question that often arises in the study of a specific cell type isolated from a whole animal knockout is to what extent the macro environment of the model influences the observed cellular phenotype. Therefore, another goal of this study was to determine whether the dysfunctional T cell phenotype we observe in murine GPAT-1 $^{-/-}$ T cells is due to an inherent loss of GPAT-1 resulting in a cell intrinsic defect rather than being the consequence of altered thymic development, epigenetic changes, or other mechanisms in the periphery of the GPAT-1$^{-/-}$ mouse. In this manuscript we present evidence that GPAM itself plays a critical role in the regulation of IL-2 production in Jurkat T cells similar to that of GPAT-1 in primary murine T cells. We further show that this effect is not rescued by addition of PA and that ZAP-70 phosphorylation, a very

early event in T cell activation is significantly decreased following stimulation, suggesting that GPAM regulates IL-2 production through a membrane based mechanism.

2.3 Methods and materials

Generation of a stable GPAM knock down Jurkat T cell

Jurkat T cells were plated 24 hours prior to lentiviral infection in 12 well plates (1 ml/well at 10^5cells/well) in complete media (10% heat-inactivated fetal bovine serum plus 100 U/ml penicillin, 100 μg/ml streptomycin, 10 μM β-mercaptoethanol, and 100 mM L-glutamine). Cells were washed and media replaced with complete media supplemented with 5μg/ml polybrene (for neutralizing charge interactions between viral capsid and cell membrane). 10^6 GPAM shRNA lentiviral particles (Santa Cruz Biotechnology) or control scrambled shRNA (Santa Cruz Biotechnology) were added to each well. Culture medium was replaced with 1 ml complete medium without polybrene. For selection of stable clones expressing GPAM shRNA cells were split 1:5 and incubation continued for 48 additional hours. Media was changed and cells re-plated in media containing 10μg/ml Puromycin dihydrochloride for selection of transduced clones. Medium was replaced with medium containing fresh puromcyin every 3-4 days until resistant cells were identified and expanded.

Quantitative real time PCR

Total RNA was extracted from ~10^6 cells using the Qiagen RNeasy Plus Mini Kit. cDNA for GPAM and GAPDH were amplified from 100 nanograms of total RNA from GPAMKD or

ScshRNA Jurkat T cells using Qiagen QuantiTect Primer Assays (commercially available proprietary primer sets, Qiagen) in a one-step qRT-PCR reaction with the Quantitect SYBR Green RT-PCR kit. One-step qRT-PCR was performed using an Eppendorf RealPlex Master Gradient Thermocycler. The one-step RT-PCR reaction was performed in at least triplicate under the following conditions: reverse transcription: 30 minutes at 50°C, PCR initial activation: 15 minutes at 95°C, followed by PCR amplification: 15 seconds at 94°C, 30 seconds at 55°C, and 30 seconds at 72°C X 40 cycles. The $\Delta\Delta C^T$ method was used in the evaluation of qPCR data. Samples were run in triplicate and the following formula was used to calculate the fold-change in GPAM mRNA expression. $\frac{X_{test}}{X_{control}} = 2^{\Delta\Delta C_T} = 2^{(C_{T,X} - C_{T,R})_{control} - (C_{T,X} - C_{T,R})_{test}}$ Results are normalized to control ScshRNA set at a value of 1 for GPAM mRNA expression.

Western blotting

One million cells were washed twice with cold PBS and lysed with 30µl of Laemmli's sample buffer and homogenized under denaturing conditions. Cell lysates were centrifuged at 13,000 X g and supernatant containing protein collected. Samples were rapidly were heated at 95°C under denaturing conditions. 20µl of lysate (approximately 1×10^6 cell equivalents) was loaded onto a 10% polyacrylamide gel (Tris-HEPES-HCl), resolved by electrophoresis (90mA/gel) and transferred onto PVDF membrane. PVDF membranes were blocked with 5% BSA in TBS and incubated overnight at 4°C with rabbit anti human polyclonal GPAM antibody (Santa Cruz). Membranes were incubated for 1h at room temperature with anti rabiit HRP conjugated antibody; immunoreactive proteins are visualized with ECL reagent. The Syngene Image Documentation system was used to capture ECL signal and qualitatively quantify protein

following Western blot. Densitometric analysis was performed using Syngene Genesnap software.

Proliferation and apoptosis

Proliferation was measured using the BD biosciences FITC BrdU Flow Kit. For stimulated conditions, 96 well plates were coated with 10 µg/ml of CD3 antibody in NaHCO3 buffer and incubated for 4 hours at 37°C. Then 1 mM BrdU diluted in PBS and 1 µg/mL of CD28 antibody was added to GPAMKD or ScshRNA cells in complete media at a density of 10^6 cells/mL and incubated at 37°C for 24 hours. For unstimulated conditions, non CD3 coated plates were used and CD28 antibody was not added. Following incubation cells were fixed and permeabilized according to the manufacturer's protocol and FITC fluorescence was measured by flow cytometry on an Accuri C6 flow cytometer. For flow cytometry, each sample was replicated 3 times, with each replicate representing an independently stimulated or unstimulated culture. 100,000 events were analyzed for each sample.

For apoptosis assays, cells were stimulated or not as described above for 24 hours and the Metabolic Activity Dead Cell Apoptosis Kit (Molecular Probes) was used to determine live, dead, and apoptotic populations according to the manufacturer's instructions. Briefly, a combination of APC labeled Annexin V to detect exposed phosphatidyl serine, C12 resazurin to detect mitochondrial membrane potential, and SYTOX Green nucleic acid stain for determining membrane permeability were used to identify characteristics associated with living, dead, and apoptotic cells by flow cytometry as described above.

Enzyme linked immunosorbent assay for IL-2 and ZAP-70

To measure secreted IL-2 in culture supernatants, we used the Human IL-2 Ready-SET-Go! ELISA kit (eBiosciences). GPAMKD and ScshRNA control cells were stimulated or not with plate bound CD3 and soluble CD28 or PMA/Ionomycin as described above and plated at a density of 10 X 10^6 cells/mL. Culture supernatants were collected after either 6 or 24 hours and ELISA was performed according to the manufacturer's instructions. Briefly, supernatants were collected and incubated in 96 well plates precoated with anti-IL-2 capture antibody (and blocked with 5% BSA for 1 hour) for 16 hours at 4°C. Next, biotin conjugated anti-IL-2 detection antibody was added to each well and plates were incubated for 60 minutes at 25°C followed by addition of avidin conjugated HRP and additional 30 minute incubation. TMB substrate was added followed by quenching of the reaction with 2N H_2SO_4 and absorbance was read at 450 nm. Standards containing known amounts of IL-2 were used with each ELISA to calculate IL-2 concentration relative to sample absorbance. Each IL-2 assay was carried out with at least six replicates.

To measure ZAP-70 phosphorylation, we used a commercially available rabbit anti-human Zap-70 capture antibody (Cell Signaling, supplied at 100X) in combination with a rabbit detection antibody directed against phospho Zap-70 (Y319)(Cell Signaling, supplied at 100X). GPAMKD and ScshRNA control cells were stimulated or not with soluble CD3/CD28 in tubes at a density of 10 X 10^6 cells/mL for 2 minutes immediately followed by the addition of a 10 fold excess of ice cold complete media. Samples were then centrifuged at 14,000 x *g* for 2 minutes followed by aspiration of the supernatant and addition of 100 µL of lysis buffer (20 mM Tris; pH 7.5, 150 mM NaCl, 20 mM DTT, 1 mM EDTA, 1 mM EGTA, 1% Triton, 1 mM Na3VO4 , 2% SDS, and 1 mM beta-glycerophosphate, 2.5 mM sodium pyrophosphate, and 1µg/mL leupeptin

supplemented with 1mM PMSF immediately prior to use). Lysates were centrifuged at 4°C for 10 minutes and supernatants collected. Debris-free lysates were then loaded into 96 well plate wells precoated with Zap-70 capture antibody (and blocked with PBS+5% BSA for 1 hour) and incubated at 37°C for 2 hours. Wells were emptied and washed with PBS supplemented with 0 .05% Tween-20. Detection antibody diluted in blocking buffer was added to each well at 1X and plates were incubated at 37°C for 1 hour. Plates were washed and 1X Anti-rabbit IgG, HRP-linked Antibody was added to each well and plates were incubated at 37°C for 30 minutes. Plates were then washed and TMB substrate was added to each well followed by quenching of the reaction with 2N H_2SO_4 and absorbance was read at 450 nm. No standards are currently available for quantitative determination of phospho Zap-70 (Y319), therefore results are presented as the mean raw absorbance values for samples in quadruplet wells.

Phospholipid extraction and mass analysis

Frozen ScshRNA and GPAMKD Jurkat T cells were thawed, and lipids extracted by the addition of 1 ml of 2-propanol to the tube containing the cells. These contents were then transferred to a test tube containing 3 ml hexane, and the original tube containing the cells was rinsed with another aliquot of 2-propanol (12, 25). The single-phase extract was thoroughly mixed, and the residue was pelleted by centrifugation at 1,000 g. The lipid containing organic fraction was removed, and dried under nitrogen, and resuspended in organic solvent solvent. The pellet was kept for protein quantitation. The lipid sam- ples were quantitatively spotted on a heat-activated Whatman LK6 thin-layer chromatography plate and developed in a solvent system containing chloroform:methanol:acetic acid:water (60:30:3:1 by vol- ume). Individual bands

corresponding to commercially prepared standards were scraped into acid-washed tubes, and the phosphorus content was analyzed. The protein pellet was air-dried overnight and then incubated with 0.2 M KOH at 65°C overnight to resolubilize the proteins. Protein concentration was determined using the Bradford method.

Phosphatidic acid addition

18:1 PA (1, 2-dioleoyl-*sn*-glycero-3-phosphate # 840875C) and 12:0 PA (1,2-dilauroyl-*sn*-glycero-3-phosphate product # 840635) were purchased from Avanti Polar Lipids, Inc. (AL, USA) and resuspended in choloroform. Chloroform suspended PA was dried under nitrogen gas and then suspended to a concentration of 10 mM in 150 mM NaCl and 10 mM Tris·HCl, pH 8. The solution was then mixed vigorously for 2 min, incubated at 37°C for 10 min, and mixed again for 2 min as previously described. Lipid micelles were generated by sonicating the lipid emulsion until clear. PA micelles were added to stimulated or unstimulated culture dishes of ScshRNA or GPAMKD cells at a final concentration of 20 μM. Cells were incubated with PA for 24 hours after which point supernatants were assayed for IL-2 by ELISA.

Statistical analysis

Statistical significance was determined using either one-way or when appropriate two-way ANOVA. Data were considered significant relative to control values when $P < 0.05$. Post hoc analysis was conducted using Tukey's or Bonferroni's multiple-comparison test with GraphPad Prism software (San Diego, CA, USA)

2.4 Results

We chose a lentiviral mediated strategy for shRNA delivery to generate a stable knockdown cell line based on the prediction that GPAM protein turnover could be such that transient knockdown with plasmid vectors may be insufficient to knock down protein expression before loss of the plasmid. Transduction of Jurkat T cells with GPAM shRNA encoding lentiviral particles resulted in a 7.3 fold decrease in GPAM mRNA after 4 passages in culture under puromycin selection relative to the ScshRNA control (Fig. 2.1). After 11 passages in culture GPAM protein was decreasd 2.8 fold by Western blot and densitometry analysis relative to the ScshRNA control (Fig. 2.2).

Proliferation was measured by BrdU incorporation assay and samples were analyzed by flow cytometry. Measurement of BrdU incorporation following 24 hours with or without CD3/CD28 stimulation revealed that proliferation rates of GPAMKD and ScshRNA control cells were not significantly different although nor was any difference in proliferative capacity observed between stimulated and unstimulated conditions, with the exception of their being a significant decrease in stimulated ScshRNA cells compared to unstimulated GPAMKD T cells (Fig. 2.3). Next, apoptosis and cell death were measured in stimulated and unstimulated GPAMKD and ScshRNA control cells using a combination of annexin V C12-Resazurin and SYTOX Green by flow cytometry. Significantly higher occurrences of cell death and apoptosis were observed in GPAMKD cells compared to ScshRNA controls (Fig. 2.3).

IL-2 induction is a well-studied and characteristic event indicative of healthy T cell function [92]. Based on previous observations that cytokine production is altered in primary T cells from the GPAT-1 $^{-/-}$ mouse we predicted that these effects may be more pronounced in the Jurkat

T cell line, one known for secretion of large amounts of IL-2 in response to stimulation. ELISA for IL-2 secretion was performed following 6 and 24 hours with or without CD3/CD28 stimulation in GPAMKD and ScshRNA control cells. We observed nearly a 9 fold decrease in IL-2 production in the GPAMKD compared to ScshRNA at both 6 hour and 24 hour (Fig. 2.4) time points. We chose these time points based on the earliest observed maximal IL-2 production (6 hours) and proliferative patterns of our Jurkat cell line (~24 hour doubling time). IL-2 production in unstimulated cells was not significantly different between the GPAMKD and ScshRNA control. PMA/Ionomycin stimulation resulted in a 4 fold increase of secreted IL-2 in the ScshRNA control relative to CD3/CD28 stimulation while the GPAMKD showed nearly a 20 fold increase. Stimulation with CD3 alone resulted in similar amounts of IL-2 secretion between the GPAMKD and ScshRNA control although notably less IL-2 when compared to CD3/CD28 costimulation.

Based on our previous observations that phospholipid mass is decreased in primary T cells from the GPAT $^{-/-}$ mouse we predicted that GPAM may similarly regulate phospholipid mass in Jurkat T cells. Total lipids were extracted from GPAMKD and ScshRNA control cells and phosphorous assay was performed to determine the mass and mole percentage of Ptd2Gro (cardiolipin), PtdOh (phosphatidic acid) EtnGpl (phosphatidylethanolamine), PtdIns (phosphatidylinositol), PtdSer (phosphatidylserine), ChoGpl (phosphatidylcholine), CerPCho (sphingomyelin), and LysoPtdCho (lysophosphatidylcholine) (Table 2.1). Ptd2Gro, EtnGpl, PtdIns, and LysoPtdCho mass were not significantly altered in GPAMKD cells relative to the ScshRNA control. PtdSer mass was on average decreased in GPAMKD cells while ChoGPl mass was greater compared to ScshRNA control cells, although not significantly (P=.053, and P=.055

respectively). PtdOH, the closest direct downstream product of GPAM and CerPCho a key component of lipid raft microdomains were significantly decreased in GPAMKD T cells.

Due to the critical role of phosphatidic acid in T cell signaling [93],[94] and as a substrate for further GPL biosynthesis we hypothesized that the reduced PA mass of GPAMKD T cells may be a major contributing factor to their dysfunction and that introduction of exogenous PA may rescue the IL-2 deficient phenotype. Lipid micelles of dioleoyl (18:1) and dilauroyl (12:0) PA were generated and added to cultures of GPAMKD and ScshRNA cells followed by measurement of secreted IL-2. Addition of exogenous PA to cell cultures had a generally negative effect on IL-2 secretion uniformly across all samples and conditions examined (Fig. 2.5). Most importantly, the addition of PA did not appear to increase IL-2 secretion in the GPAMKD under any conditions tested. The effects of PA addition appeared to be modestly influenced by dosage with less IL-2 inhibition observed at lower PA concentrations (data not shown).

Co stimulation of Jurkat T cells with CD3 and CD28 antibody results in the rapid recruitment and phosphorylation of a number of key signal transduction effectors including Zap-70 [91]. Changes in membrane phospholipid composition have been reported to disrupt signaling from lipid raft based receptor complexes [95],[96] . Our observation that overall phospholipid mass is decreased in GPAMKD T cells led us to hypothesize that these changes may be interfering with T cell receptor (TCR) activation and subsequent phosphorylation of Zap-70, one of the earliest events in the IL-2 signal transduction pathway. A ~50% decrease in phospho Zap-70 (Y319) was observed in stimulated GPAMKD cells compared to ScshRNA controls. Stimulated GPAMKD cells showed a ~25% increase in Zap-70 phosphorylation compared to their unstimulated

counterparts while stimulation induced a ~55% increase in ScshRNA cells compared to their unstimulated cells (Fig. 2.6).

2.5 Discussion

We have previously demonstrated that GPAT-1 plays a key role in regulating murine T cell cytokine production, subset polarization, and proliferation [7]. That data was obtained using primary T cells isolated from GPAT-1 knockout mice. One question that often arises when experimenting with specific subsets of cells from knockout animals is to what extent developmental or peripheral processes influence the phenotypic anomaly in question. To address this, we used a lentiviral delivered shRNA strategy to knock down GPAM expression in Jurkat T cells. With this approach, we were able to achieve a 60% reduction in GPAM protein maintained stably in culture. We next sought to determine whether our GPAMKD line recapitulated key aspects of the murine GPAT KO T cell phenotype with regards to proliferation, apoptosis, and phospholipid composition.

Primary T cells from GPAT KO mice show reduced proliferative capacity at 20 hours by MTT assay, while no difference is evident at 24 hours compared to WT controls [7]. We show here that GPAMKD Jurkat T cells do not appear to have a proliferative defect relative to ScshRNA control cells and in one instance (ScshRNA stimulated vs. GPAMKD unstimulated), actually showed a modestly significant increase in BrdU incorporation. There are several feasible explanations for this disparity. One possibility is that Jurkat T cells are simply more resilient to the effects of GPAM deficiency than primary T cells in terms of proliferative capacity. Immortal cell lines such as Jurkat may have compensatory mechanisms by which to overcome alterations

in glycerolipid metabolism. Further experiments are needed to determine whether this is the case here. Another possibility has to do with the fact that there is a fundamental difference between what the two assays measure and the data cannot be interpreted on the same grounds. MTT and BrdU incorporation measure two different indicators of cellular health: metabolic activity and DNA replication, respectively. Further proliferation experiments in primary murine T cells by our group have revealed no significant difference in BrdU incorporation between T cells from WT and GPAT KO mice (data not shown).

Following antigenic stimulation T cells are triggered to rapidly proliferate and expand to combat infectious microorganisms. This rapid expansion is usually followed by a reprogramming of these T cells clones with a select few becoming memory cells and the rest succumbing to apoptosis [97,98]. Very precise molecular mechanisms have evolved to control this expansion-deletion dichotomy so as to prevent autoimmunity and tissue damage due to long term occupation by cytotoxic T cell subsets. However, improper or early induction of apoptosis can actually hamper a proper immune response and leave an organism susceptible to infection. Karlsson, et. al. showed that infection of GPAT-1 KO mice with coxsackievirus B3 resulted in significantly increased mortality and significantly higher liver and heart viral titers compared to WT infected mice [8]. This strongly suggests that GPAT-1 is necessary for mounting a proper immune response. We have previously demonstrated that T cells from GPAT-1 KO mice are highly prone to apoptosis following stimulation with CD3 and CD28 antibodies. We similarly show here that GPAM knock down in Jurkat T cells significantly increases the occurrence of apoptosis in cultured cells under both stimulated and unstimulated conditions. This is very intriguing as GPAM deficiency does not appear to alter proliferative tendencies. This result is however not without precedent as Hammond et. al. have demonstrated similar phenomena;

increased apoptosis balanced by increased proliferation in hepatocytes from GPAT-1 KO mice [14]. Findings in that paper suggested that radical oxygen species (ROS) generated as a result of increased lipid oxidation may be responsible for increased occurrence of apoptosis in GPAT-1 KO hepatocytes, however no direct link between ROS and DNA damage was observed nor was elevated active caspase 3 detected. It has been suggested that GPAT-1 plays a central role in the regulation of metabolic processes by competing with CPT-1 on the mitochondrial membrane for fatty acyl CoA substrate [99]. We have recently shown that CPT-1 protein is significantly elevated in thymocytes from young GPAT KO mice which may indicate that lipid oxidation is favored over glycolytic energy production. In the context of T cells, this is an important observation as the classic paradigm is that T cells become glucose addicted following stimulation and shifting energy production toward lipid oxidation can induce dramatic changes in cytokine production and T cell phenotype. Mitochondria isolated from livers of GPAT-1 KO mice are reported to have an increased sensitivity to Ca^{2+} induced opening of the mitochondrial permeability transition pore (MPT). Increased levels of cytosolic Ca^{2+} can aid in the opening of the permeability transition pore which is thought to play a key role in mitochondrial mediated apoptosis by inducing a loss of mitochondrial membrane potential and leakage of pro apoptotic components such as cytochrome c and procaspases into the cytosol. The observation that elevated Ca^{2+} induces MPT formation at lower concentrations in the absence of GPAT-1 is very important in the context of the T cell. Stimulation of T cells with CD3/CD28 antibodies results in the activation of PLCγ, subsequent generation of IP3 and Ca^{2+} release from the ER. Ca^{2+} release is a critical event during T cell activation as it is necessary for Calm induced activation of the transcription factor NFAT, which regulates IL-2 gene expression. In T cells then, there is an

inherent mechanism by which cytosolic increases in Ca^{2+} could physiologically contribute to MPT opening and subsequent apoptotic processes.

IL-2 is a key cytokine produced primarily by the T_h1 T cell subset that is necessary for the maturation of CD4+ and CD8+ effector T cells and serves to sustain an immune response by reinforcing pro proliferative pathways in many subsets. Activation of the TCR-CD3 complex followed by coactivation of CD28 via APC presented antigen and CD80/86 respectively, comprise the canonical Signal 1 and Signal 2 in the activation of naïve T_h cells [100]. These events result in a signal transduction cascade ultimately resulting in the activation of mitogenic factors involved in proliferation and activation of genes encoding cytokines critical in regulating the immune response. Primary targets of the signal transduction pathways activated following engagement of the TCR/CD3 and CD28 signaling complexes are the IL-2 locus and genes encoding the IL-2 receptor subunits. Secreted IL-2 can then act locally through paracrine and autocrine signaling to activate other T cells or reinforce signaling through the IL-2 receptor complex by activating MAP kinase, PI3 kinase, and JAK-STAT pathways involved in proliferation and survival. We have previously demonstrated that splenic T cells isolated from GPAT KO mice produce less IL-2 when stimulated with CD3/CD28 antibodies than WT control mice. We show here that Jurkat T cells deficient in GPAM secrete ~40 fold less IL-2 in response to CD3/CD28 stimulation than their ScshRNA counterparts. Stimulation of primary T cells from GPAT-1 KO mice with the diacylglycerol mimic phorbol 12-myristate 13-acetate (PMA) and calcium ionophore, ionomycin, resulted in no significant decrease in IL-2 secretion compared to WT controls suggesting that the defect responsible for reduced IL-2 output in GPAT-1 deficient cells results from insufficient signaling or activation of the TCR-CD3 or CD28 signaling complex. When we stimulated GPAMKD Jurkat T cells with PMA/ionomycin we observed a

~20 fold increase in IL-2 secretion compared to CD3/CD28 stimulation while ScshRNA cells only showed a ~6 fold increase. This data suggests that PMA/ionomycin partially rescues IL-2 production in GPAM deficient Jurkat T cells and supports our hypothesis of a defect in membrane based signaling. However, there is still some issue as to why we do not observe a complete rescue of IL-2 output as observed in primary T cells. Further experiments possibly looking at the role of signaling through the IL-2 receptor or regulation of the IL-2 receptor subunits are needed to more thoroughly examine these phenomena. According to the membrane gate theory of aging, cell membranes become more rigid and lose fluidity with age [10]. This loss in membrane fluidity is thought to perturb signal transduction through membrane activated receptors. This hypothesis is has gained special attention in the aging immunology field as a key characteristic of aged T cells is reduced IL-2 output and proliferative capacity. We have previously shown GPAT-1 activity in aged rats is significantly reduced in response to stimulation [9] suggesting that decreased GPAT-1 activity with age may contribute to a loss in membrane fluidity. Stimulation with CD3 alone results in modest amounts of IL-2 production and secretion by T cells. Co stimulation with CD28 antibody is needed to provide "signal 2" and fully engage signaling pathways that drive expansion processes and cytokine production. The CD28 receptor is localized to lipid raft microdomains and plays a key role in recruiting Lck tyrosine kinase and other lipid raft localized factors to the immunological synapse, a key event in T cell activation and induction of signaling pathways involved in IL-2 production [67,34,101]. If GPAM deficiency alters membrane fluidity it is possible that lipid raft dynamics may also be perturbed and that CD28 dependent raft recruitment and localization is dysfunctional, thereby hampering the co stimulatory signal. To test this hypothesis in GPAMKD and ScshRNA Jurkat T cells we stimulated with CD3 or CD28 alone and found that IL-2 secretion was unaltered in

GPAMKD Jurkat cells. One interpretation of this data is that TCR-CD3 based signaling alone and C28 signaling alone are intact when stimulated independently of each other and GPAM deficiency leads to a loss of the CD3/CD28 co stimulatory signal. This data therefore suggests that GPAM regulates IL-2 production in Jurkat T cells by interfering with co stimulatory signals necessary for full induction of IL-2 expression through a mechanism possibly involving perturbation of membrane fluid dynamics.

The direct downstream product of GPAT-1 is lysophosphatidic acid which can be further acylated to phosphatidic acid by AGPAT enzymes on the endoplasmic reticulum. The exact contribution of GPAT-1 to the generation of triglyceride and glycerophospholipid is not completely known but several studies have indicated that it may vary depending on tissue and cell type [89]. Glycerophospholipids play a key role in the regulation of membrane fluid dynamics and it is possible that loss of GPAT activity with age contributes to reduced membrane fluidity by reducing gycerophospholipid output. It is interesting then that primary T cells from GPAT-1 KO mice have significantly decreased membrane glycerophospholipid compared to their WT counterparts. The most striking decrease however was observed in phosphatidic acid, the precursor of all glycerophospholipids which suggested that in T cells, GPAT-1 is a principle contributor to the phosphatidic acid pool and may play an essential role in providing PA for the bulk production of all glycerophospholipid. Phosphlipid mass analysis in GPAMKD Jurkat T cells revealed significant decreases in phosphatidic acid and sphingomyelin. However, other phospholipid levels remained unaltered. Sphingomyelin is a key component of lipid rafts and is thought to contribute to their structure through interaction with cholesterol in the cell membrane. Decreased sphingomyelin could interfere with lipid raft dependent signal transduction such as in the case of TCR based activation. The decrease in phosphatidic acid observed first in primary T

cells and then in GPAMKD Jurkat cells led us to hypothesize that T cell dysfunction observed in old and GPAT-1 KO cells may be due to decreases in the cellular phosphatidic acid pool. Until very recently it was thought that Ras directly recruited the serine/threonine kinase Raf-1 to the cell membrane, however a seminal study by Rizzo, et. al. showed for the first time that Raf-1, an essential component of the MAPK signaling cascade physically binds PA in order to localize to membranes and this binding is independent of Ras association [102]. In fact, it is PA dependent membrane association of Raf-1 that actually activates its kinase activity through a poorly understood mechanism involving Ras. Further studies showed that mutation of PA binding domains in Ras completely abrogates Raf-1 dependent MAPK signaling and results in cell cycle arrest [103]. Once activated Raf-1 can phophorylate and activate MEK, a tyrosine/threonine kinase which can go on to phosphorylate and activate the pluripotent serine/threonine kinases Erk 1 and Erk 2. The Erk family is directly involved with transcription factor activation in the nucleus, and in the case of T cells drive the expression of IL-2 through NFAT, AP-1, and NF-κB transcription factors. Previous work probing PA mediated MAP kinase signaling in T cells has focused on two mechanisms of PA production; (i)PLD mediated hydrolysis of phosphatidylcholine and (ii) phosphorylation of diacylglycerol by DAGK-α . The predominant opinion in the literature is that PLD2 and DAGK-α are the principle contributors of PA in TCR based signal transduction cascades [94]. We hypothesized that GPAM may be a significant contributor to the phsophatidic acid pool and in its absence; PLD2 and DAGK may be incapable of overcoming this sink and thusly hindering T cell activation. To test this hypothesis we attempted to rescue IL-2 production in GPAMKD Jurkat T cells by the addition of exogenous PA to culture. We used two types of PA, 12:0 which has been reported in the literature to have a mitogenic effect on T cells and 18:1 which is more physiologically relevant in terms of PA found in most cells types. We did not

observe any increase in IL-2 production with PA addition under any of the conditions tested. In fact, we report here an inhibitory effect of PA addition under stimulated and unstimulated conditions. It is possible that the PA contributed by GPAM is actually serving as precursor for further GPL biosynthesis and not in the capacity of a signaling or anchoring molecule itself. It is likely that the time points we examined of 6 and 24 hours were insufficient to allow for global membrane remodeling. Taking this experiment out to further time points creates a challenge as lipid toxicity becomes an issue as more PA is added to culture. Another possibility is that PA is not suitably available biologically in the free and micelle form which we added it to culture. Dose responses revealed that concentration did play a role in the reduction of IL-2 production (data not shown) albeit modestly. At concentrations of 10 μM PA of both types in micelle form resulted in a nearly identical proportional decrease in IL-2 output under all conditions tested in both GPAMKD and ScshRNA control cells suggesting that this phenomenon was independent of GPAM. Further elucidation of the role of GPAM generated PA in activation of mitogenic signaling and contribution to GPL synthesis is needed before its role is completely dismissed.

Successful TCR engagement results in phosphorylation of ITAM motifs on CD3-ζ subunits initiated by Lck and Fyn which associate with the cytosolic tails of the CD4 and CD8 co-receptors on helper and cytotoxic T cells. Activated Lck can phosphorylate tyrosine residues on CD3-ζ subunits which can then serve as a recruitment site for the tyrosine kinase ZAP-70. ZAP-70 binds and engages the dual phosphorylated ITAMs on the CD3-ζ chain via its tandem SH-2 domains allowing for Lck mediated phosphorylation and activation of ZAP-70. This puts ZAP-70 in a position to phosphorylate the transmembrane adaptor protein; LAT. Phosphorylated LAT in turn serves as a docking site for a myriad of SH2 domain containing signaling proteins. If deficiencies in GPAM generated GPL are responsible for perturbations in

membrane based signaling we hypothesized that very early events in TCR activation following CD3/CD28 stimulation may be disrupted. To test this hypothesis we stimulated GPAMKD and ScshRNA cells and measured the relative amounts of phospho ZAP-70 (Y319) by ELISA, an indicator of successful Lck mediated phosphorylation and successful TCR-CD3 and CD28 activation. We observed a significant decrease in the amount of phospho ZAP-70 (Y319) in GPAMKD Jurkat cells compared to ScshRNA control cells. This data strongly suggests that GPAM regulates IL-2 production in Jurkat T cells through a mechanism dependent on very early signal transduction events following stimulation. Based on the observation that CD3 stimulation yielded equal IL-2 output in GPAMKD and ScshRNA cells, we propose that decreased IL-2 production in the absence of GPAM may be at least partially attributed to a failure in the recruitment of key raft components to the immunological synapse to provide signal 2. Lck is thought to autophosphorylate following T cell activation. The precise mechanisms involved in Lck activation are still under investigation although evidence has been presented that support a role for CD4 and CD28 induction and maintenance of Lck phosphorylation. It has also been proposed that CD28 and lipid rafts coordinate the recruitment of Lck to the immunological synapse [67]. Further experiments to determine the localization of Lck to the immunological synapse are needed to verify that GPAM regulates IL-2 production in Jurkat T cells by altering membrane phospholipid in such a way as to interfere with CD28 costimulation.

We have for the first time shown here that the human homologue of GPAT-1, GPAM plays an important role in stimulation induced IL-2 production and T cell fate. Additionally, we demonstrate that GPAM deficiency itself plays a key role in T cell dysfunction outside of the context of an animal model. Our data suggest that GPAM regulates IL-2 production through

altering membrane phospholipids in such a way as to alter proper T cell activation and that this process results in increased apoptosis and cell death.

Figure 2.1 Quantitative RT PCR for GPAM transcript.

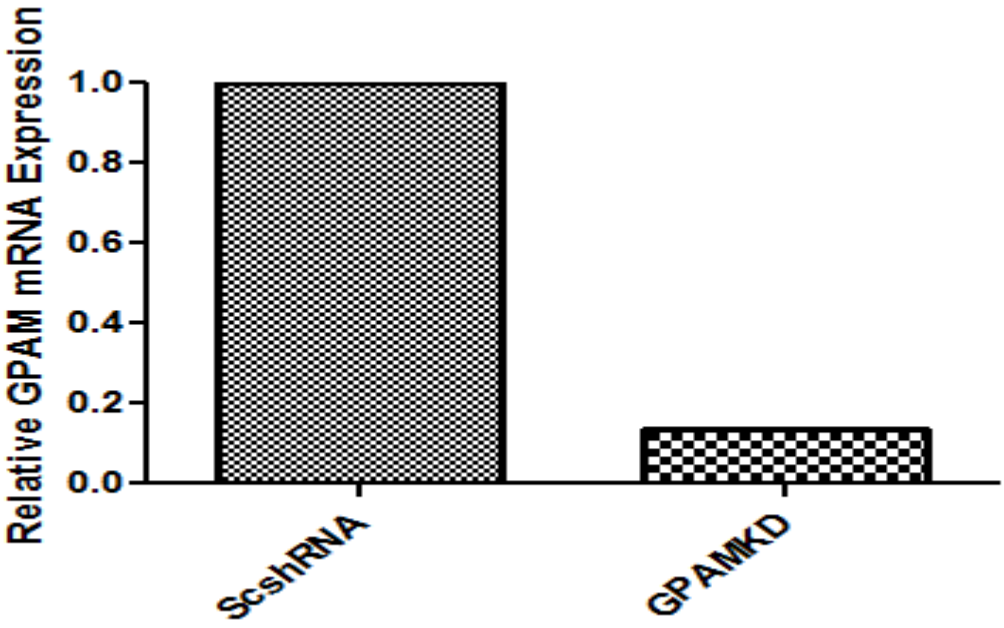

Figure 2.1. mRNA was isolated from ScshRNA and GPAMKD Jurkat T cells and quantitative reverse transcriptase PCR was performed to determine GPAM gene expression. Results were normalized to GAPDH.

Figure 2.2 GPAM Western Blot and Densitometry

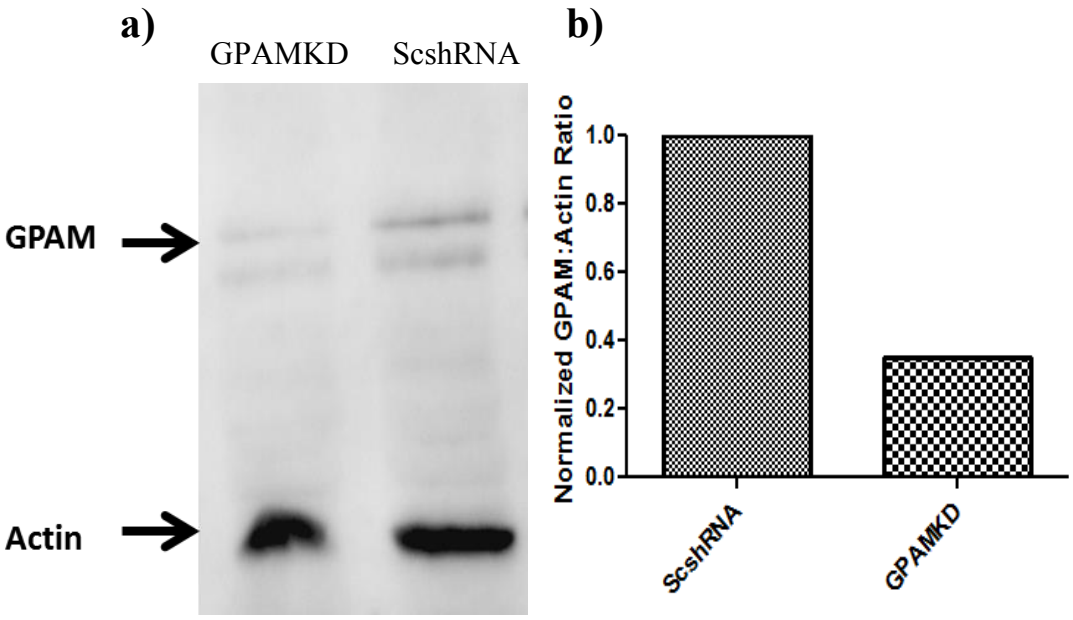

Figure 2.2. a). GPAM Western blot in ScshRNA and GPAMKD Jurkat T cells. b). Densitometry was performed to normalize relative amount of GPAM protein present against Actin loading control.

Figure 2.3 BrdU Proliferation and Apoptosis in GPAMKD and ScshRNA Jurkat T cells

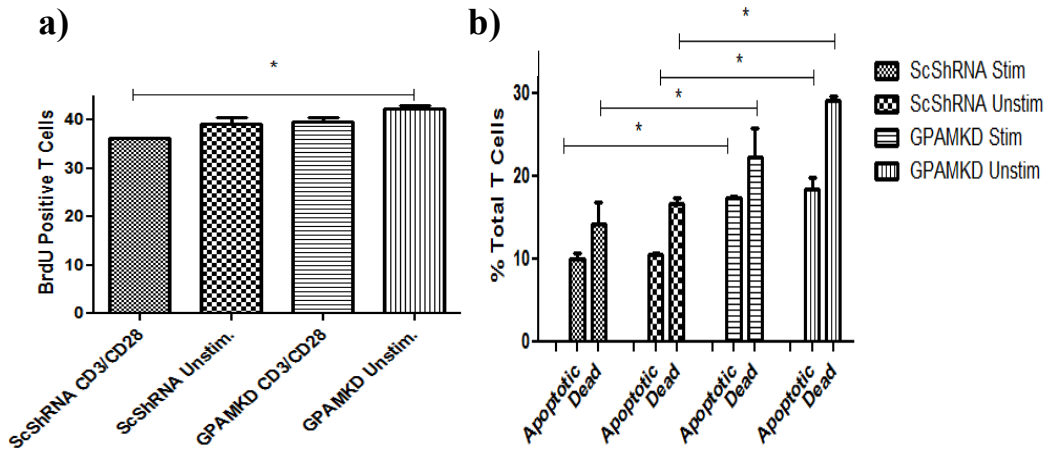

Figure 2.3 a). BrdU proliferation assay in GPAMKD and ScshRNA Jurkat T cells. T cells were stimulated or not with CD3/CD28 antibody for 24 hours in the presence of BrdU and subsequently stained with fluorophore conjugated BrdU antibody and fluorescence was read on an Accuri C6 flow cytometer. b). T cells were stimulated or not with CD3/CD28 antibody for 24 hours and apoptotic and dead cells were determined as described in materials and methods. Significance ($p>0.05$) is indicated with an asterisk. Each assay was carried out in sextuplet. Significance was determined using one-way ANOVA and post-hoc analysis carried out with Tukey's multi-comparison test ($P>0.05$).

Figure 2.4 IL-2 Production at 24 hours and CD3 only Stimulation

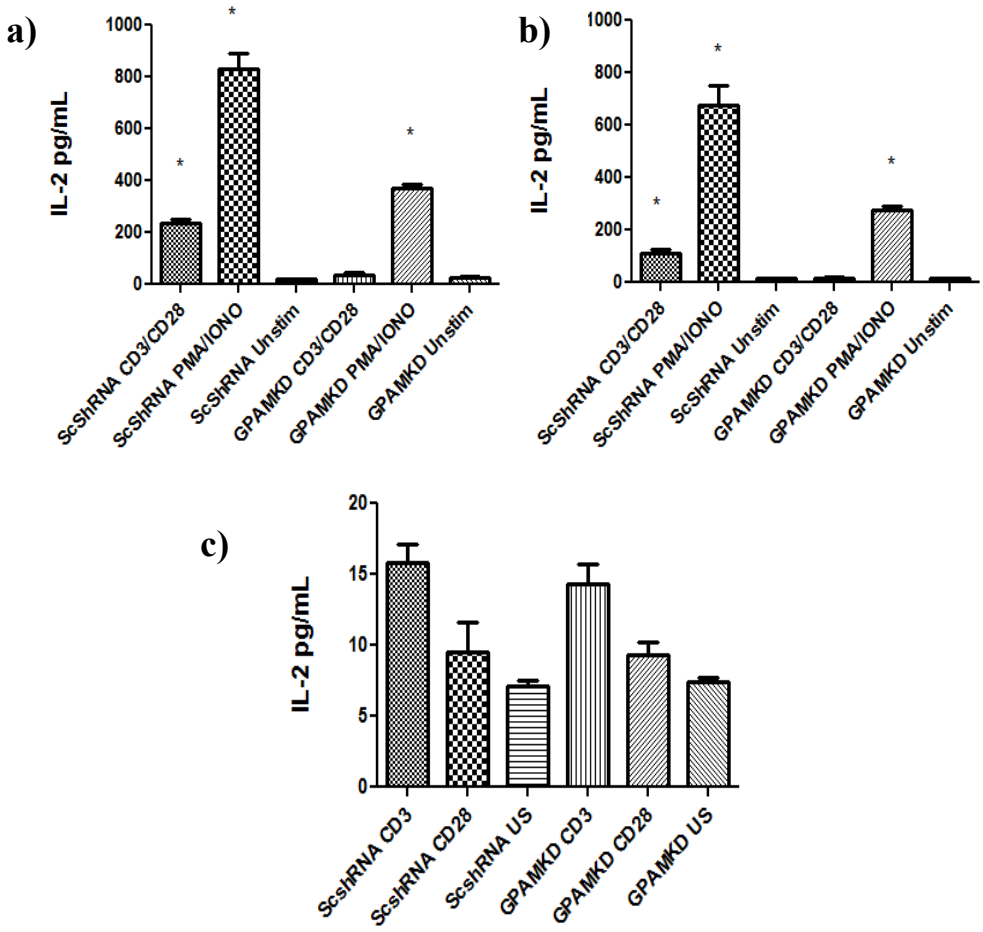

Figure 2.4 a). IL-2 secretion by ScshRNA and GPAMKD Jurkat T cells at 24 hours. T cells were stimulated or not with CD3/CD28 antibody or PMA/Ionomycin for 24 hours or 6 hours (b) and IL-2 secretion was determined by ELISA. c). T cells were stimulated or not with CD3 or CD28 antibody alone for 24 hours and IL-2 secretion was determined by ELISA. Each assay was carried out in sextuplet. Significance was determined using one-way ANOVA and post-hoc analysis carried out with Tukey's multi-comparison test ($P>0.05$).

Table 2.1. Phospholipid Mass and Mole % in ScshRNA and GPAMKD Jurkat T cells

Mole %	ScshRNA		GPAMKD			
	AVG	STD	AVG	STD	P value	sig?
Ptd2Gro	2.20	0.257	2.06	0.231	0.344	ns
PtdOH	2.79	1.368	1.94	0.613	0.195	ns
EtnGpl	17.74	2.213	16.03	3.253	0.312	ns
PtdIns	3.30	1.338	3.44	1.343	0.860	ns
PtdSer	4.23	1.293	3.05	0.863	0.093	ns
ChoGpl	58.84	4.144	64.66	3.603	0.027	sig
CerPCho	6.13	0.349	4.18	1.515	0.012	sig
LysoPtdCho	3.74	1.249	3.52	1.164	0.759	ns
Mass	ScshRNA		GPAMKD			
	AVG	STD	AVG	STD	P value	sig?
Ptd2Gro	4.05	0.526	3.66	0.218	0.124	ns
PtdOH	5.24	2.753	3.52	1.365	0.200	ns
EtnGpl	32.65	3.947	28.22	3.494	0.067	nqs
PtdIns	6.18	2.725	6.28	2.851	0.952	ns
PtdSer	7.88	2.708	5.47	1.807	0.100	nqs
ChoGpl	108.49	9.138	115.84	16.206	0.356	ns
CerPCho	11.28	0.450	7.51	2.712	0.007	sig
LysoPtdCho	6.83	2.040	6.14	1.598	0.693	ns

Table 2.1 Total lipids were extracted from ScshRNA and GPAMKD Jurkat T cells and separated via TLC. Phospholipid mass was determined by phosphorous assay. Averages (AVG) respresent 3 independent cultures. Significance ($P>0.05$) was determined by 2 tailed students T test. Significantly different is indicated by “sig” and not significant “ns”.

Figure 2.5 The Effect of Phosphatidic Acid (Micelle) Addittion on IL-2 Production in GPAMKD and ScShRNA Jurkat T Cells During 24 Hour Stimulatory Period

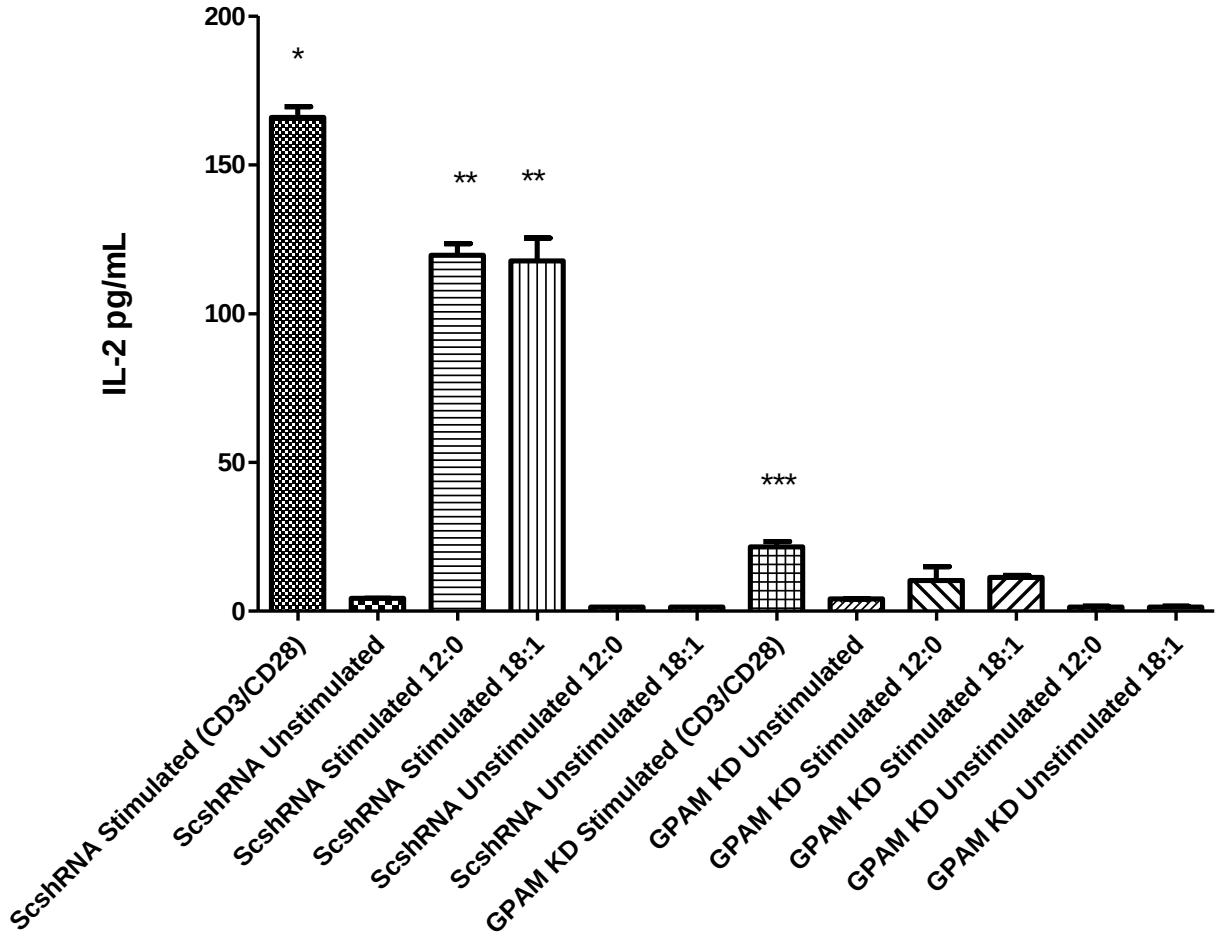

Figure 2.5. PA lipid micelles were made as described and added to cultures of ScshRNA or GPAMKD Jurkat T cells under stimulated (CD3/CD28) or unstimulated conditions and cultured for 24 hours. IL-2 secretion was then measured by ELISA. Each assay was carried out in sextuplet. Significance was determined using one-way ANOVA and post-hoc analysis carried out with Tukey's multi-comparison test ($P>0.05$).

Figure 2.6 Zap-70 Phosphorylation

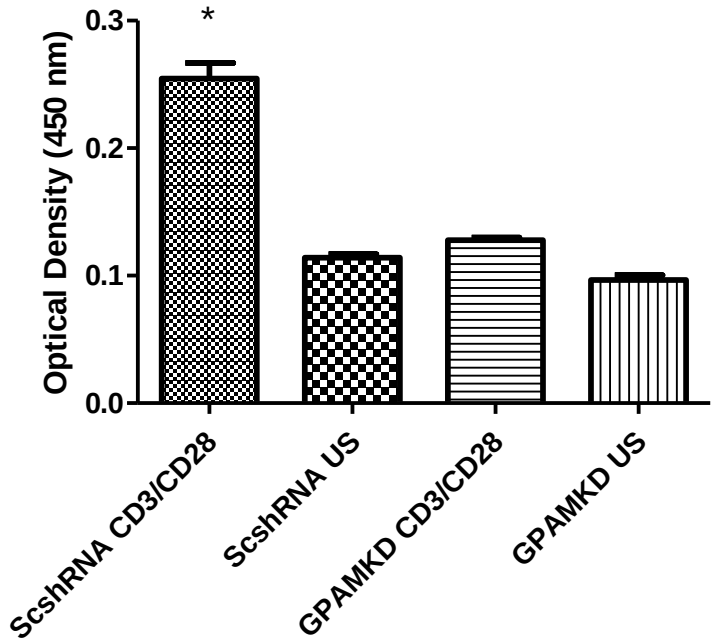

Figure 2.6. ScshRNA and GPAMKD Jurkat T cells were stimulated with soluble CD3/CD28 and rapidly lysed. Phospho Zap-70 was detected and quantified via ELISA. Each assay was carried out in sextuplet. Significance was determined using one-way ANOVA and post-hoc analysis carried out with Tukey's multi-comparison test ($P>0.05$).

Chapter 3

Glycerol-3-Phosphate Acyltransferase Is Necessary for Activation of the Stimulation Induced Metabolic Switch in Murine CD4^{+} T cells

3.1 Abstract

Glycerol-3-phosphate acyltransferase-1 is the first and rate limiting step in *de novo* glycerophospholipid synthesis. We have previously demonstrated that GPAT-1 regulates murine T cell function. The resultant phenotype is characterized by decreased IL-2 production, increased propensity toward apoptosis, and altered membrane glycerophospholipid mass similar to that of an old T cell. Recent studies have suggested that changes in the metabolic profile of T cells are responsible for defining specific effector functions and T cell subsets. So we asked the question as to whether our previous observations regarding T cell dysfunction in GPAT-1 $^{-/-}$ T cells could possibly be explained by changes in cellular metabolism. We show here for the first time that GPAT-1 $^{-/-}$ T cells suffer from several key metabolic defects likely contributing to their dysfunctional phenotype. First, we observed a significant reduction in mitochondrial mass in GPAT-1 $^{-/-}$ T cells compared to their WT counterparts indicating that GPAT-1 deficiency results in either fewer or smaller mitochondria. We next identified striking decreases in both the oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR) of GPAT-1 $^{-/-}$ T cells following CD3/CD28 stimulation indicating an inherent cellular defect in energy production. These differences were observed throughout a series of mitochondrial stress tests using oligomycin, FCCP, and rotenone to detect OCR under coupled, uncoupled and non-mitochondrial respiration respectively. Treatment with FCCP and subsequent subtraction from

basal readings allowed us to calculate the spare respiratory capacity (SRC) of GPAT-1 $^{-/-}$ T cells, a key indicator of the cells ability to cope with mitochondrial stress. We found that the proportional decreases in the metabolic profile observed basally and in the presence of inhibitors were underscored by an inherent mitochondrial defect as evidenced by significantly decreased SRC in in GPAT-1 $^{-/-}$ T cells. These data demonstrate that deletion of GPAT-1 has deleterious effects on total cellular metabolism under conditions of increased energy need. Furthermore, altered metabolic response following stimulation may be the defining mechanism underlying T cell dysfunction in GPAT-1 $^{-/-}$ T cells. Taken together these results indicate that GPAT-1 is essential for regulation of the stimulation induced metabolic switch in T cells.

3.2 Introduction

Glycerol 3-phosphate acyl transferase-1 [GPAT-1] is an integral mitochondrial membrane protein responsible for conjugating fatty acyl-CoA with glycerol-3 phosphate in the first and rate limiting step of *de novo* glycerophospholipids synthesis[104]. GPAT-1 catalyzes the conversion of glycerol-3 phosphate and acyl-CoA to lyosphosphatidic acid (LPA) which is then further acylated to phosphatidic acid which serves as a precursor for all glycerophospholipid (GPL) and triglyceride synthesis. Fatty acids are activated to acyl-CoAs on the outer mitochondrial membrane before entering either the glycerolipid biosynthetic pathway via GPAT-1 or the β-oxidation pathway via carnitine palmitoyltransferase-1 (CPT1) which is thought to compete with GPAT-1 for fatty acyl co-A substrate at the outer mitochondrial membrane. Both CPT-1 and GPAT-1 are sensitive to nutrient levels within the cell, specifically the ATP/AMP ratio. AMP activated protein kinase (AMPK) activity increases when there is an abundance of

AMP within the cell, signaling that ATP levels are low. Consequently, activated AMPK regulates both CPT-1 and GPAT-1 reciprocally. When cellular energy stores are low AMPK is activated and downregulates GPAT-1 activity, while promoting CPT-1 activity. We have previously shown that GPAT-1 activity is upregulated following stimulation by T cell specific protein kinase c theta (PKCθ) [27]. Interestingly, we also found that stimulation induced upregulation of GPAT-1 activity is significantly blunted in old T cells, suggesting that T cell dysfunction with age may be at least partly attributed to altered cellular GPL levels.

T cells from GPAT-1 $^{-/-}$ mice share many characteristics with old T cells including increased cholesterol to glycerophospholipid proportions and reduced IL-2 production following stimulation [7]. GPAT-1 activity, but not expression is reduced in T-cells from old rats implying that age related decline in GPAT-1 activity may be a primary contributor to immunosenesence through a mechanism modulated by alterations in glycerophospholipid [9]. Decreased GPL to cholesterol ratios are thought to be a principle contributor to decreased membrane based signal transduction in senescent cells as described by the "membrane gate" theory of aging [10]. Interestingly, we have also shown that GPAT-1$^{-/-}$ T cells secrete increased levels of IL-17 [105], a proinflammatory cytokine associated with increased autoimmunity in aging and have elevated levels of $CD4^+CD25^+FoxP3^+$ T cells (Treg) (unpublished observation) also observed in aging [12],[106]. Elevated IL-17 secretion in combination with elevated Treg is highly significant as it has been demonstrated that in the absence of TGF-β Treg are capable of polarizing $CD4^+$ T cells toward a Th17 phenotype [107].

Quiescent T cells must rapidly upregulate modes of energy production in response to stimulation by cognate antigen to drive clonal expansion and cytokine production. T cell metabolism is emerging as a key regulator of the type and extent of an immune response.

Canonically, it is thought that this process primarily engages glycolytic pathways of energy production. However, evidence is emerging that the preferred energy substrate depends on the T cell subset in question. It was recently shown that Treg subsets for example, have a metabolic preference for lipid oxidation while Th1 and Th2 subsets rely heavily on glycolysis and Th17 subsets engage both lipid and glycolytic pathways [4]. In that study it was also shown that rapamycin treatment or fatty acid addition alone enhanced Treg differentiation while blockade of lipid oxidation via etomoxir prevented Treg generation [4]. In another interesting study, CD8+ memory T cells have recently been shown to possess substantially more spare respiratory capacity than CD8+ T effector cells which confers the ability to handle increased stress and promote long term survival, possibly [76].

Aged T cells are more susceptible to the damaging effects of oxidative stress which has been linked to sensitization of the mitochondria and reduced spare respiratory capacity [108]. Increased oxidative stress is thought to contribute significantly to mitochondrial DNA mutations with age and has been suggested to play a role in degenerative disease and cancer development [109]. This is significant in the context of GPAT-1 for three key reasons. First, GPAT-1 is an integral mitochondrial membrane protein and its activity may serve to locally supply the mitochondria with lysophosphatidic acid for glycerophospholipid synthesis. Therefore, loss of GPAT-1 may structurally alter mitochondrial integrity and compromise mitochondrial membrane potential resulting in T cell unresponsiveness. We have previously shown that the exclusive mitochondrial membrane lipid; cardiolipin is decreased in T-cells of *Gpat-1* $^{-/-}$ mice in addition to a global decrease in most major GPL [7]. Second, we have demonstrated that *Gpat-1* $^{-/-}$ T-cells are more prone to an "apoptosis like" program of cell death following stimulation and proliferation appears to be reduced as assessed by MTT assay [7]. These observations are

supported by work from Rosalind Coleman's lab with *Gpat-1* $^{-/-}$ hepatocytes, where similar stimulation induced cell death was observed [14]. Third, that mitochondria from *Gpat-1* $^{-/-}$ hepatocytes lose mitochondrial outer membrane potential (MOMP) rapidly and form extensive mitochondrial permeability transition pores (MPTP) in response to addition of exogenous Ca^{2+} suggests that GPAT-1 plays a critical role in regulating mitochondrial integrity[14]. It is also very interesting then that MPTP formation is significantly increased following TCR stimulation in old murine T-cells [110] . These observations construct a scenario in which stimulation of the T cell increases intracellular Ca^{2+} levels inducing MPTP formation and subsequent loss of MOMP. Since both GPAT-1 activity and cardiolipin content are reduced with ageing, this mechanism may be a major contributing factor to immunosenescence.

In the current study we sought to determine how GPAT-1 deficiency alters $CD4^{+}$ T cell metabolism and whether these changes may underlie T cell dysfunction. We found a small although significant difference in mitotracker staining suggesting either fewer or smaller mitochondria in the GPAT-1 KO mouse in addition to only a modest decrease in proliferative capacity as assessed by BrdU incorporation suggesting that decrease proliferation observed by MTT assay are actually more indicative of loss a loss in mitochondrial redox capacity. Unstimulated GPAT-1 KO T cells appear to be metabolically equivalent to their WT counterparts. However, stimulation revealed gross deficiencies in both respiratory and glycolytic metabolism in GPAT-1 KO T cells. We also observed significantly reduce spare respiratory capacity, an indicator of the cells ability to cope with mitochondrial stress, in GPAT-1 KO T cells. These findings suggest that T cells deficient in GPAT-1 are unable to meet the high energy demands concomitant with stimulation and shift their available metabolic resources into cell survival.

3.3 Materials and Methods

Mice and T cell Isolation/stimulation

Male C57BL/6 GPAT-1 KO mice were obtained from Dr. Rosalind Coleman (University of North Carolina at Chapel hill and bred in our animal facilities. Age matched male C57BL/6 mice were obtained from Charles River. At 5 months of age mice were sacrificed and spleens taken. Spleens were gently homogenized and single cell suspensions obtained. Cell suspensions were stained with Miltenyi anti CD4 (L3T4) coated microbeads and magnetically separated by passing them through a Miltenyi LS column within a magnetic field. Non labeled cells were washed through the column and CD4+ T cells were collcted by removing the column from the magnetic field and forcing 5 mL of buffer through the column with a plunger. Typically, 1.3-1.5X10^7 $CD4^+$ T cells were obtained from each mouse. These numbers did not significantly differ between WT and GPAT-1 KO mice. Freshly isolated CD4+ T-cells were plated at a density of 3X10^6 cells/mL and stimulated at 37°C for 20 hours in complete RPMI 1640 culture media (10% heat-inactivated fetal bovine serum plus 100 U/ml penicillin, 100 µg/ml streptomycin, 10 µM β-mercaptoethanol, and 100 mM L-glutamine) and stimulated with 10 µg/ml plate-bound anti-CD3 and 1 µg/ml soluble anti-CD28 or left unstimulated.

Mitochondrial Quantitation

Freshly isolated T cells were incubated at 37C with 100 nM Mitotracker for 30 minutes. Cells were then washed twice and then resuspended in 1 mL of PBS. Cells were then analyzed on the Accuri C6 Flow cytometer and analysis carried out with Accuri C6 software.

BrdU Incorporation and CD4 staining

T cells were isolated and pooled from 10 WT or 10 GPAT-1 KO mice. Proliferation was measured using the BD biosciences APC BrdU Flow Kit. For stimulated conditions, 96 well plates were coated with 10 μg/ml of CD3 antibody in NaHCO3 buffer and incubated for 4 hours at 37°C. Then 1 mM BrdU diluted in PBS and 1 μg/mL of CD28 antibody was added to cultures in complete media at a density of 10^6 cells/mL and incubated at 37°C for 20 hours. For unstimulated conditions, non CD3 coated plates were used and CD28 antibody was not added. Following incubation cells were fixed and permeabilized according to the manufacturer's protocol and APC fluorescence was measured by flow cytometry on an Accuri C6 flow cytometer.

Protocol Optimization for Seahorse Extracellular Flux Analyzer

We first determined the optimal number of murine T cells needed to place oxygen consumption (OCR) and extracellular acidification rate (ECAR) readings in an interpretable range within the limitations of the Seahorse XF24 Analyzer, and at the same time provide a sufficient number of $CD4^+$T cells for all of the assays planned. We found 500,000 murine T cells were sufficient to accomplish these ends. We next sought to determine optimal concentrations of the mitochondrial inhibitors oligomycin, Carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone **(**FCCP), and rotenone. Dosage response analysis with various inhibitor concentrations revealed the optimal combination of inhibitor concentrations for our needs to be: 2μM oligomycin, 3μM FCCP, and 4μM rotenone. Prior to each assay, inhibitors were made fresh from frozen stocks and aliquoted into the microinjection ports of the XF-24 flux plate. We next

determined the optimal palmitic acid/BSA conjugate concentration to use for the fatty acid oxidation assay. Concentrations of 25, 50, 100, and 200μM with the goal being to use the highest concentration that yielded a discernible difference in COR. We found that concentrations of 100 and 200μM palmitic acid yielded similar increases in OCR and opted for the lower concentration of 100μM to use in proceeding assays. Prior to each assay, fresh palmitic acid/BSA conjugate or BSA only for controls, were made fresh from frozen stocks and aliquoted into the microinjection port of the XF-24 flux plate.

Metabolic Profile

T cells were isolated and cultured as previously described. After 20 hours, T cells were harvested and washed in 5 mL of pre-warmed Seahorse XF assay medium and resuspended to a concentration of 10 X 10^6 cells/mL and 50 μL of this suspension was added to a poly-l lysine coated dish for a final count of 500,000 cells/well and incubated at 37°C for 30 minutes in a non-CO_2 incubator. 450μL of XF assay medium was then added to each well, the top of the plate containing the injection ports and probes added, and the metabolic profile read with the XF-24 Extracellular Flux Analyzer (Seahorse Bioscience).

Fatty Acid Oxidation Profile

T cells were isolated and cultured as previously described. After 20 hours, T cells were harvested and washed in 5 mL of pre-warmed minimal KHB assay medium (111 mM NaCl, 4.7 mM KCl, 2 mM $MgSO_4$, 1.2 mM Na_2HPO_4, 2.5 mM glucose, and 0.5 mM carnitine) and resuspended to a concentration of 10 X 10^6 cells/mL and 50 μL of this suspension was added to a poly-l lysine coated dish for a final count of 500,000 cells/well and incubated at 37°C for 30

minutes in a non-CO_2 incubator. 450µL of KHB assay medium was then added to each well, the top of the plate containing the injection ports and probes added, and the metabolic profile read with the XF-24 Extracellular Flux Analyzer (Seahorse Bioscience).

Statistical Analysis

Statistical significance was determined using one-way analysis of variance due to the non-normal distribution of data. Data are considered significant from control values when $P < 0.05$. Post hoc analysis was conducted using Tukey's multiple-comparison test with GraphPad Prism (San Diego, CA, USA).

3.4 Results

Total mitochondrial content was quantified by mitotracker staining. GPAT-1 KO T cells showed a small but significant decrease in mitochondrial content compared to WT controls (Fig. 3.1). This difference could be the result of either reduced mitochondrial number or smaller mitochondria.

We have previously reported that proliferation in GPAT-1 KO T cells is significantly decreased at 20 hours when assessed by MTT assay. Since GPAT-1 deficiency may result in a loss of mitochondrial redox potential, we sought to assess proliferation by a more direct means such as BrdU incorporation. To control for heterogeneities that can occur with multiple T cell isolations and rounds of stimulation we pooled T cells from 10 WT or 10 GPAT-1 KO age matched mice. Total and CD4+ GPAT-1 KO T cells incorporated approximately 10% and 11% less BrdU than their WT counterparts respectively (Fig. 3.2). Interestingly, there appeared to be

no difference in BrdU incorporation between WT and GPAT-1 KO CD8+ T cells (data not shown). Furthermore, we previously reported that a significant difference in tetrazolium reduction was only observed at 20 hours and not at earlier or later time points which suggests that previous observations are indeed more reflective of a mitochondrial defect and not loss of proliferative capability

Metabolic Profiles of WT and GPAT-1 KO CD4+ T cells

CD4+ T cells were isolated and stimulated with CD3/CD28 antibodies or not for 20 hours in complete RPMI media after which cells were harvested and plated onto poly-l lysine coated 24 well plates and their metabolic profiles read with the Seahorse XF analyzer. Under basal conditions, stimulated GPAT-1 KO T cells showed significantly less respiratory and glycolytic activity as determined by their respective oxygen consumption rate (OCR) and extracellular acidification rates (ECAR) (OCR: Fig. 3.3a ECAR: Fig. 3.5a). However, there appeared to be no significant difference between the metabolic profile of unstimulated GPAT-1 KO and WT T cells (Fig 3.4). Basal readings of the OCR/ECAR ratio revealed no significant difference between stimulated or unstimulated WT and GPAT-1 KO T cells suggesting that glycolysis and OXPHOS are engaged in a proportionally equivalent manner (Fig. 3.4f).

Injection of oligomycin, an ATP synthase inhibitor, followed by intermittent metabolic monitoring over a 24 minute time period revealed a proportional decrease in OCR and ECAR in stimulated GPAT-1 KO compared to WT controls while no significant difference was observed in unstimulated samples, indicating a blunted metabolic profile for the GPAT-1 KO (OCR: Fig. 3.4b, ECAR: Fig. 3.6b) . Likewise, treatment with FCCP, an uncoupler resulted in decreased

maximal respiratory capacity and ECAR in stimulated GPAT-1 KO T cells, however no difference was observed in unstimulated controls (OCR: Fig. 3.4c, ECAR: Fig.3.6c). When maximal respiration was subtracted from basal respiratory levels in stimulated samples we found that GPAT-1 KO T cells have significantly reduced spare respiratory capacity (SRC) strongly implicating an inherent mitochondrial defect that may hinder their ability to upregulate energy production in response to stimulation (Fig. 3.4d). The SRC shows that there is something more than merely a proportional difference in the metabolic profile of GPAT-1 KO T cells and is indicative of a specific defect in the mitochondrial stress coping capability of the GPAT-1 KO. Treatment with rotenone shuts down electron transport and allows for the measurement of non-mitochondrial respiration, of which we found no significant difference between GPAT-1 KO and WT under stimulated or unstimulated conditions, however ECAR did appear decreased in the stimulated GPAT-1 KO compared to the stimulated WT (OCR: Fig. 3.4d, ECAR: 3.6d). This data suggests that decreased oxygen consumption rates observed in the GPAT-1 KO are the result of an inability of these cells to upregulate mitochondrial respiratory metabolism in response to stimulation. It did not appear in any case that glycolytic means of energy production were upregulated in response to these energy deficits as determined by no compensatory increase in ECAR. This indicates that GPAT-1 KO T cells suffer from a severe energy deficit in response to stimulation which may underlie many facets of their previously reported dysfunctional phenotype [7].

We have previously reported elevated CPT-1a protein levels in GPAT-1 KO thymocytes [13] so in the current study we sought to determine whether this correlated with increased lipid oxidative modes of energy production. Basal readings were acquired for 24 minutes followed by

injection of 100 μM palmitate/BSA conjugate intermittent monitoring of OCR and ECAR for 80 minutes in WT and GPAT-1 KO T cells.

Basal OCR and ECAR readings differed significantly between stimulated GPAT-1 KO and WT T cells even more so than basal levels reported for the respiratory assay (Fig. 3.7a and 3.8a). One possible explanation for this is that the KHB formulation used for this assay is very minimal in regard to micronutrients (see materials and methods for composition) compared to the Seahorse XF media used in the respiratory assay exacerbating any underlying defects in metabolism. Much like the respiratory assay, we observed no significant difference in OCR or ECAR in unstimulated samples. Interestingly, no difference in OCR was observed between stimulated GPAT-1 KO and unstimulated WT controls implying that stimulation is unable to overcome the metabolic defect in GPAT-1 KO T cells in minimal KHB media. Following palmitate/BSA injection OCR readings for stimulated GPAT-1 KO T cells were significantly elevated compared to unstimulated samples and significantly reduced compared to WT. However, similar to basal readings, no significant difference was observed between stimulated GPAT-1 KO and unstimulated WT. ECAR readings following palmitate /BSA injection appeared significantly different between all samples tested. Despite increased CPT-1 protein levels as previously reported, there appear to be no significant increases in lipid oxidative metabolism in GPAT-1 KO T cells.

Interestingly, FA/BSA injection significantly increased OCR readings in all unstimulated samples compared to BSA only injected unstimulated samples suggesting that the presence of fatty acid itself is sufficient to upregulate respiratory metabolism in resting T cells. FA/BSA injection also had the unexpected effect of increasing ECAR in stimulated samples while no effect was observed in unstimulated samples. FA/BSA injection significantly increased ECAR

readings in stimulated WT samples compared to BSA injected alone, while no increase was observed in GPAT-1 KO samples. Recently it has been demonstrated that in skeletal muscle cells that palmitate treatment rapidly upregulates GLUT4 expression and stimulates glucose uptake [111]. To the best of our knowledge this is the first time that a similar phenomenon has been observed in primary murine T cells.

3.5 Discussion

Conventional thought is that activated T cells become heavily reliant on glycolysis following stimulation and switch to an "anabolic" program of metabolism that supports clonal expansion even in the presence of adequate oxygen to support mitochondrial oxidative phosphorylation (OXPHOS) [112],[85]. However, the exact role of mitochondrial OXPHOS during T cell stimulation remains unclear. Our data clearly show that stimulated T cells up regulate both OXPHOS and glycolysis in response to stimulation. Furthermore, the OCR/ECAR ratio is significantly increased in response to stimulation demonstrating that OXPHOS is indeed significantly upregulated in $CD4^+$ T cells while unstimulated T cells appear to rely more on glycolysis. Interestingly, offering palmitate as a fuel source, even in the presence of glucose, significantly increased mitochondrial oxygen consumption. This effect was observed in both stimulated and unstimulated cells. We observed no difference in the respiratory profile of unstimulated WT and GPAT-1 KO T cells suggesting that T cell dysfunction in the absence of GPAT-1 only becomes apparent under conditions of increased energy demand. That there is no compensatory increase in glycolysis following any of the treatments suggests that the GPAT-1 KO T cell is not properly responding to stimulation and thus not engaging normal modes of

metabolism. Furthermore, a failure to upregulate glycolysis implies that GPAT-1 $^{-/-}$ T cells suffer from a severe energy sink which they may be unable to overcome a consequence of which may be decreased IL-2 production and decreased proliferative capability. Significant decreases in SRC observed in the GPAT-1 KO are indicative of an inherent defect in the mitochondrial stress response more so than a proportional decrease in the metabolic profile. It is therefore likely that GPAT-1 deficiency is pleitropic in its effects on T cell metabolic function.

Our data demonstrate that GPAT-1 is necessary for regulating the increased energy demand placed on T cells following stimulation. In the absence of stimulation we saw no general difference in the metabolic profile of GPAT-1 KO T cells compared to their WT counterparts suggesting that GPAT-1 dependent lipid biosynthesis plays a critical role in regulating the stimulation induced metabolic switch. One possible explanation is that GPAT-1 KO T cells have reduced mitochondrial mass implying that either mitochondrial size or number is reduced, a consequence of which is decreased respiratory capability. However, it is more likely that GPAT-1 deficiency results in smaller, compromised mitochondria rather than physically fewer mitochondria in light of observations in the liver showing increased susceptibility to Ca^{2+} induced opening of the MPTP [14]. This is intriguing in context of the T cell as substantial increases in intracellular Ca^{2+} are necessary for activation of critical factors responsible for driving cytokine production and pro proliferative signaling. This presents the possibility that GPAT-1 plays a critical role in maintaining mitochondrial integrity, most likely by providing a source of GPL for mitochondrial membranes. Another possibility is that GPAT-1 KO T cells are less responsive to CD3 stimulation similar to old T cells [113]. Changes in membrane phospholipid ratios are thought to interfere with proper activation of membrane based signaling complexes with age[10]. It is interesting then, like old T cells, GPAT-1 KO T cells have significantly reduced

levels of nearly all of the major GPLs and analysis of molar lipid mass per cell makes it unlikely that this reduction in GPL is solely due to altered mitochondrial membrane composition. Instead, it is more probable that GPAT-1 deficiency results in decreased GPL mass in all cellular membranes. Defects in cellular membranes are capable of interfering with membrane based signal transduction such as that required during T cell stimulation through CD3 and the CD28 co receptor. In a related study, we have recently found that phosphorylation of Zap-70, a key proximal effector kinase involved in early T cell signal transduction is significantly reduced in GPAT-1 (GPAM, human homologue) deficient Jurkat T cells, possibly indicating a defect in transmission of the stimulatory signal. GPAT-1 KO T cells have a dysfunctional phenotype similar to old cells characterized by decreased GPL to cholesterol ratios, decreased IL-2 production, and a pro-inflammatory polarization of T cell subsets [7,105]. In addition GPAT-1 KO mice are more susceptible to coxsackievirus B3 infection, suggesting that the *in vivo* adaptive immune response is indeed impacted by *ex vivo* dysfunction [8]. The data presented here show that T cells deficient in GPAT-1 have a blunted metabolic profile and reduced SRC compared to their WT counterparts. In addition, these defects only become apparent when the T cell is stimulated suggesting that GPAT-1 is necessary to meet increased energy demand placed on the cell following stimulation. In light of our previously reported finding that GPAT-1 activity is reduced in old rats [9], it is possible that T cell dysfunction associated with aging can be at least partly attributed to an inherent loss of GPAT-1 activity. Thusly, modulation of GPAT-1 activity may emerge as a principle target in the treatment of T cell dysfunction as we grow old. It will be interesting in the future to see how GPAT-1 KO T cells compare to old T cells in regard to their metabolic profile.

Figure 3.1 Mitochondrial Mass Determination with Mitotracker Staining

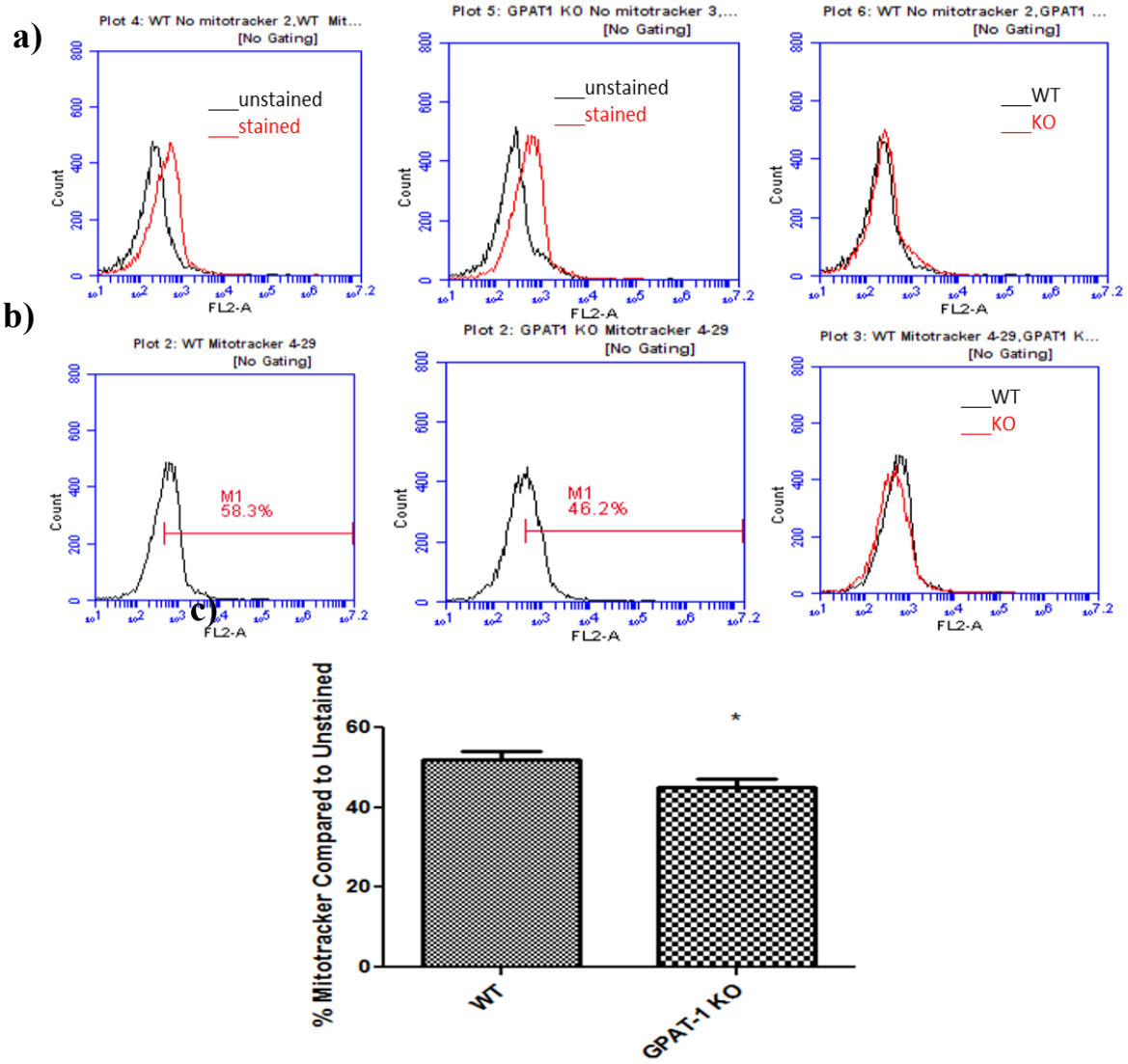

Figure 3.1. Freshly isolated WT and GPAT-1 KO T cells were stained with 100 nM mitotracker red and fluorescence was analyzed by flow cytometry. Values respresent the mean +/- SEM of five individual mice. * Significantly different ($p<0.05$).

Figure 3.2 BrdU Proliferation Assay

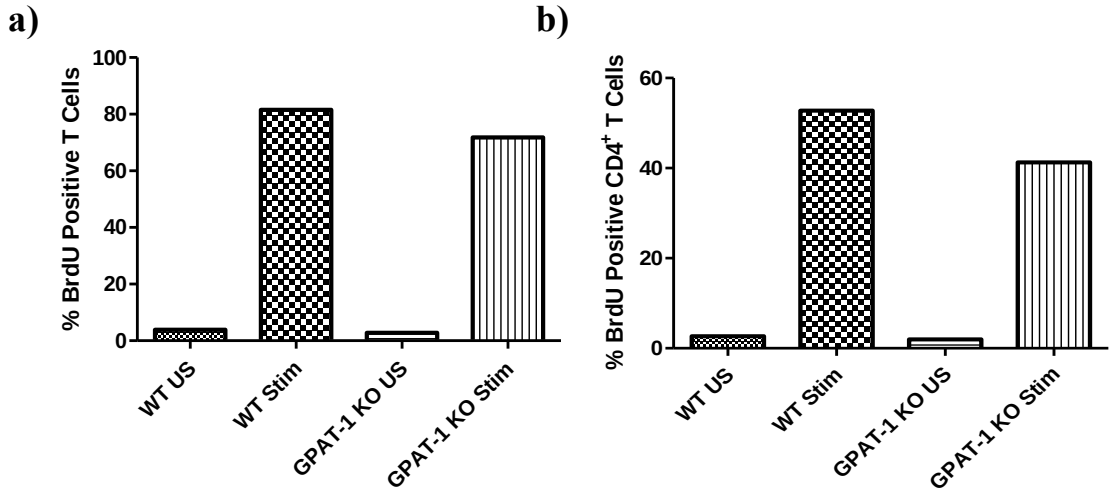

Figure 3.2. T cells were isolated and pooled from 10 WT and 10 GPAT-1 KO mice and cultured in the presence of BrdU for 20 hours. Cells were then stained with antibodies directed against BrdU or CD4. Fluorescence was analyzed by flow cytometry.

Figure 3.3 Respiratory Profile with Inhibitor Treatment

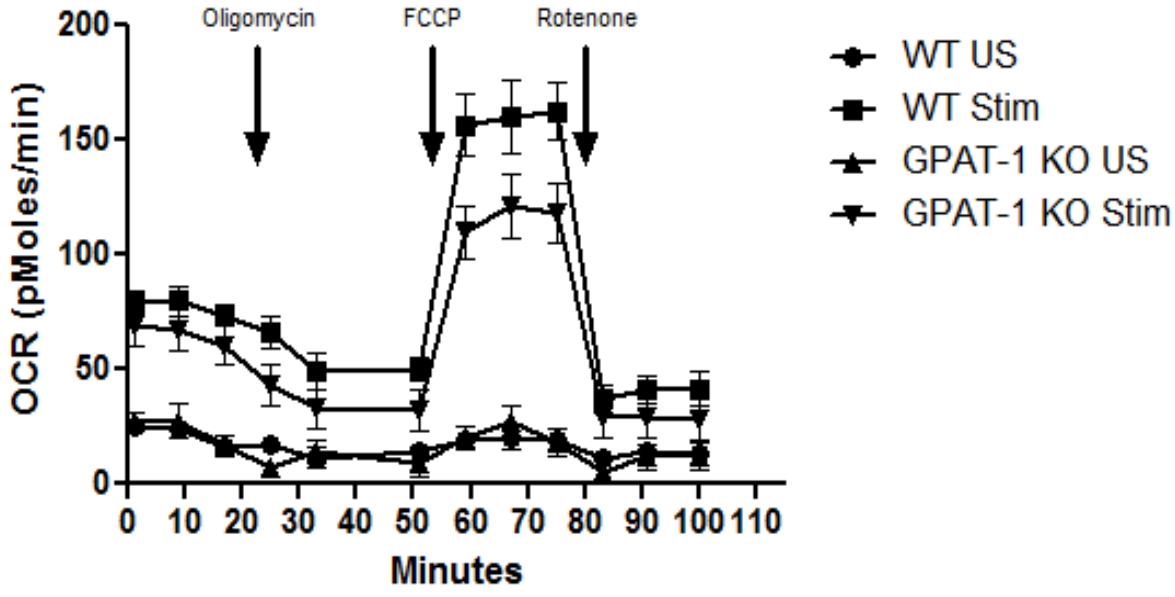

Figure 3.3. $CD4^+$ T cells were isolated from age matched WT or GPAT-1 KO mice and stimulated or not in culture with CD3/CD28 antibody in complete medium. Cells were harvested and plated onto poly-l lysine coated dishes and basal oxygen consumption rate was analyzed followed by time specific injection of oligomycin, FCCP, and rotenone. Each point represents the mean +/- the SEM of five individual mice in triplicate on the plate.

Figure 3.4 Respiratory Response to Inhibitors and Spare Respiratory Capacity

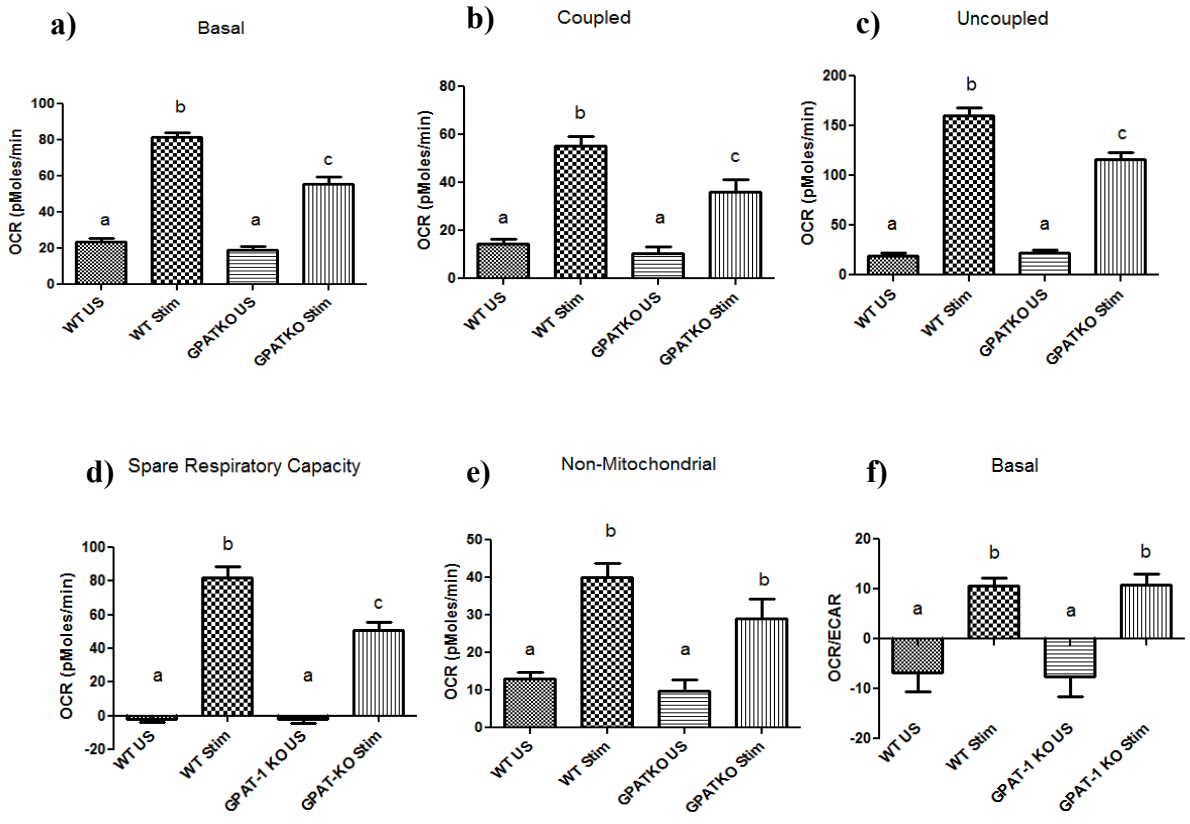

Figure 3.4. a). Basal OCR. b). Coupled, following oligomycin treatment. c). Uncoupled following FCCP Treatment. d). Spare respiratory capacity, calculated by subtracing uncoupled from basal OCR. e). Non-mitochondrial metabolism following rotenone treatment. f). Basal OCR/ECAR. Each bar represents the mean +/- SEM of five individual mice, in triplicate (for inhibitor addittion) or sextuplet (for basal readings),, and averaged across three time points. Significance is indicated by differing letters ($p>0.05$). Bars with same letters are not significantly different from each other. Significance was determined using one-way ANOVA and post-hoc analysis carried out with Tukey's multi-comparison test ($P>0.05$).

Figure 3.5 Glycolytic Profile with Inhibitor Treatment

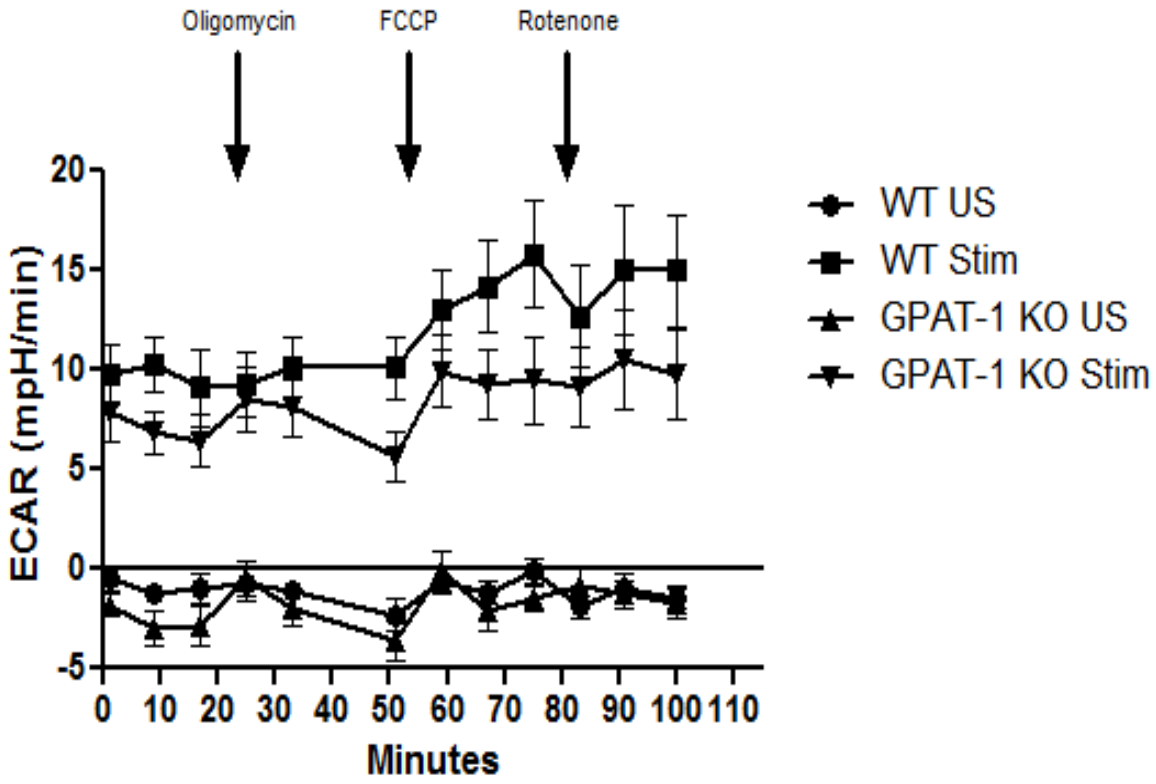

Figure 3.5. Glycolytic metabolic profile (ECAR). a) $CD4^+$ T cells were isolated from age matched WT or GPAT-1 KO mice and stimulated or not in culture with CD3/CD28 antibody in complete medium. Cells were harvested and plated onto poly-l lysine coated dishes and basal oxygen consumption rate was analyzed followed by time specific injection of oligomycin, FCCP, and rotenone. Each point represents the mean +/- the SEM of five individual mice in triplicate on the plate.

Figure 3.6 Glycolytic Response to Inhibitors

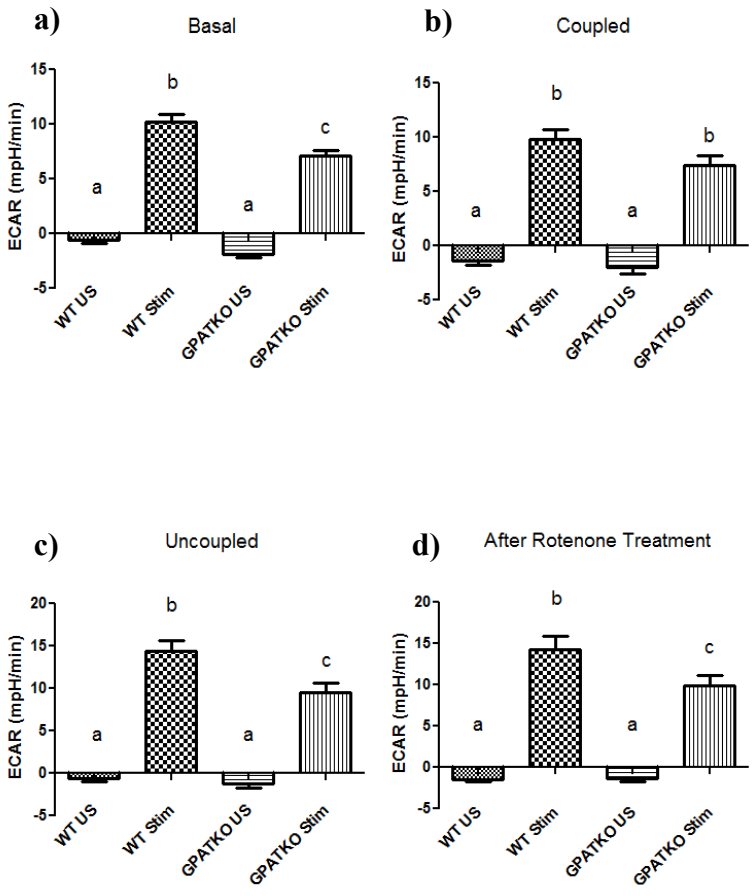

Figure 3.6. a). Basal. b). Coupled, following oligomycin treatment. c). Uncoupled following FCCP Treatment. d). Following rotenone treatment. Each bar represents the mean +/- SEM of five individual mice, in triplicate (for inhibitor addition) or sextuplet (for basal readings), and averaged across three time points. Significance is indicated by differing letters ($p>0.05$). Bars with same letters are not significantly different from each other. Significance was determined using one-way ANOVA and post-hoc analysis carried out with Tukey's multi-comparison test ($P>0.05$).

Figure 3.7 Fatty Acid Oxidation Respiratory Profile I

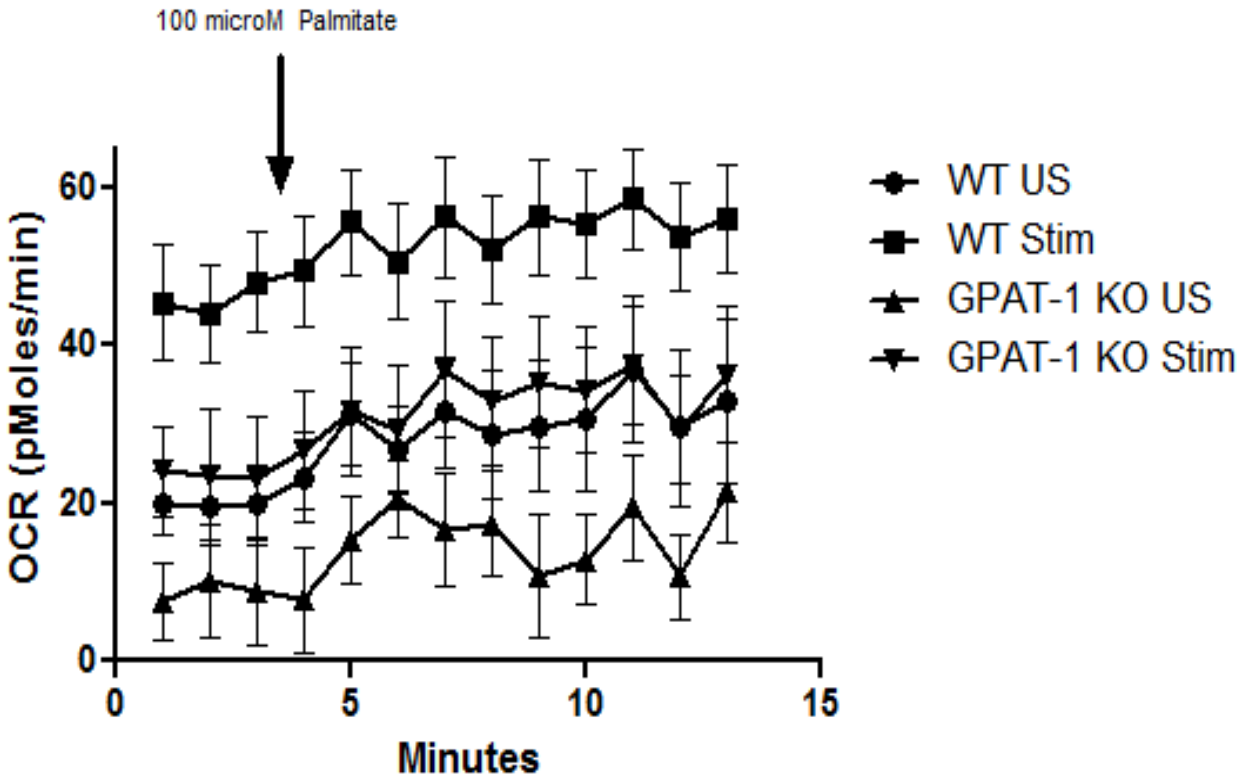

Figure 3.7. $CD4^+$ T cells were isolated from age matched WT or GPAT-1 KO mice and stimulated or not in culture with CD3/CD28 antibody in complete medium. Cells were harvested, transferred to KHB assay specific media and plated onto poly-l lysine coated dishes and basal oxygen consumption rate was analyzed followed by injection of 100 μM FA(palmitic acid)/BSA conjugate. Each point represents the mean +/- the SEM of five individual mice in triplicate on the plate.

Figure 3.8 Fatty Acid Oxidation Metabolic Profile II

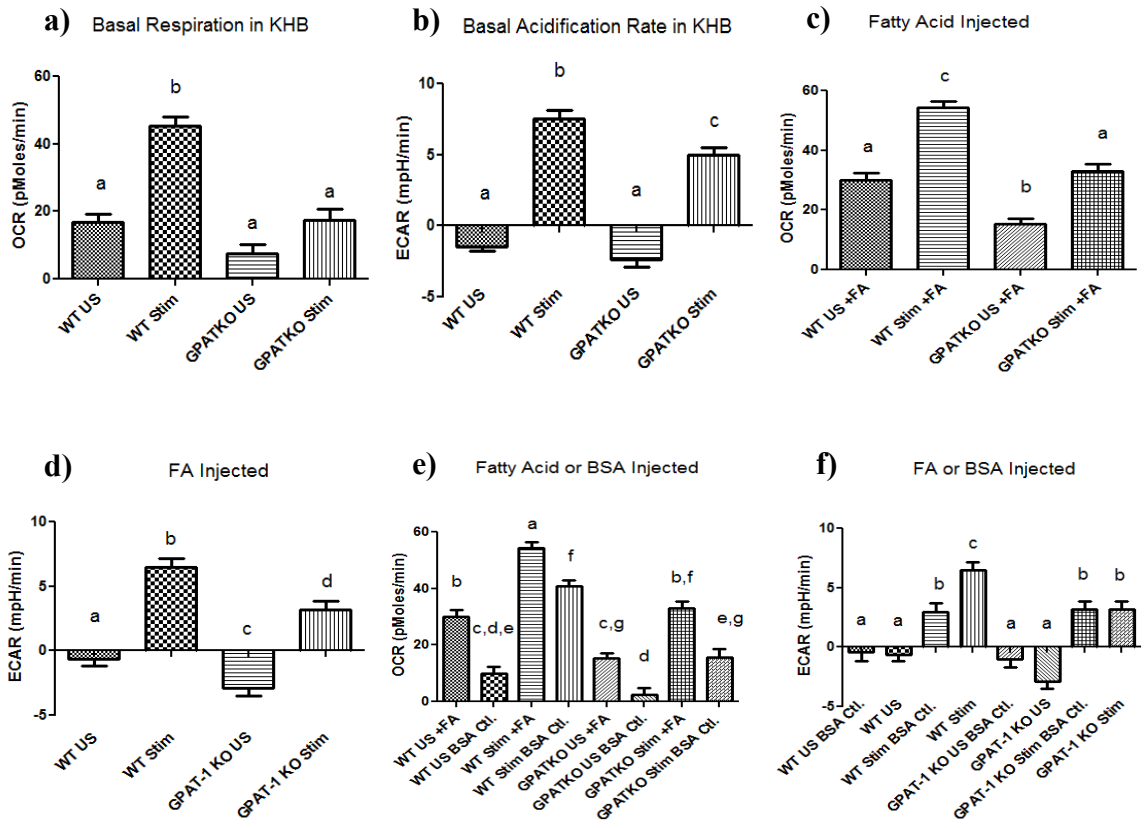

Figure 3.8. a). Basal OCR (average of 3 time points preceding injection). b). Basal ECAR (average of 3 time points preceding injection). c). OCR for FA injected (average of 10 time points following injection). d). ECAR for FA injected (average of 6 time points following injection). e). OCR for FA injected compared to BSA only injected controls (average of 10 time points following injection). f). ECAR for FA injected compared to BSA only injected controls (average of 6 time points following injection) Each bar represents the mean +/- SEM of five individual mice, in quintuplet (for basal readings) or triplicate (for FA injected) or duplicate (for BSA only injected), and averaged across multiple time points as indicated above. Significance is indicated by differing letters ($p>0.05$). Bars with same letters are not significantly different from

each other. Significance was determined using one-way ANOVA and post-hoc analysis carried out with Tukey's multi-comparison test ($P>0.05$).

Chapter 4

Discussion

T cells are principle effectors of the adaptive immune system and as such, are able to determine the type and extent of an immune response. Aging and more recently alterations in cellular metabolism have been demonstrated to significantly alter the subset polarization and functionality of the T cell compartment. Changes in T cell subset proportions or functionality can have profound effects on the adaptive immune response. Alterations in membrane glycerophospholipid levels are thought to be a major underlying cause of T cell dysfunction with aging. It is therefore important to understand how changes in lipid metabolism can alter T cell function.

The goal of this project was to identify mechanisms that contribute to the dysfunctional phenotype of GPAT-1 $^{-/-}$ T cells. The first objective was to determine how and to what extent T cell metabolism is altered in the absence of GPAT-1. Recent studies have suggested that changes in the metabolic profile of T cells are responsible for defining specific effector functions and T cell subsets. So we asked the question as to whether our previous observations regarding T cell dysfunction in GPAT-1 $^{-/-}$ T cells could possibly be explained by changes in the T cell response to stimulation or fatty acid addition. Our experiments revealed several key metabolic defects in GPAT-1 $^{-/-}$ T cells. First, we observed a significant reduction in mitochondrial mass in GPAT-1 $^{-/-}$ T cells compared to their WT counterparts indicating that GPAT-1 deficiency results in either fewer or smaller mitochondria. We have previously reported that proliferation is significantly reduced in GPAT-1 $^{-/-}$ T cells as determined by MTT assay, however only at the specific time point of 20 hours. MTT reduction is actually an indicator of mitochondrial redox capacity, so in

light of our observations concerning mitochondrial mass we hypothesized that our previous results for the MTT assay were more indicative of mitochondrial dysfunction as opposed to proliferation. Therefore, we measured proliferation by a more direct means via BrdU incorporation assay. Consequently, we found decreased BrdU incorporation in GPAT-1 $^{-/-}$ T cells although these differences were much smaller than those reported for MTT assay. Next we sought to characterize the metabolic profile of WT and $CD4^{+}$ T cells. To accomplish this, we monitored the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of WT and GPAT-1 $^{-/-}$ T cells in order to precisely identify metabolic inconsistencies concomitant with GPAT-1 deficiency. We identified striking decreases in both the OCR and ECAR of GPAT-1 $^{-/-}$ T cells indicating an inherent cellular defect in energy production. These differences were observed throughout a series of mitochondrial stress tests using oligomycin, FCCP, and rotenone to detect OCR under coupled, uncoupled and non-mitochondrial respiration respectively. Treatment with FCCP and subsequent subtraction from basal readings allowed us to calculate the spare respiratory capacity (SRC) of GPAT-1 $^{-/-}$ T cells, a key indicator of the cells ability to cope with mitochondrial stress. We found that the proportional decreases in the metabolic profile observed basally and in the presence of inhibitors were underscored by an inherent mitochondrial defect as evidenced by significantly decreased SRC in in GPAT-1 $^{-/-}$ T cells. There also appeared to be no compensatory up regulation of glycolysis as measured by ECAR suggesting that GPAT-1 $^{-/-}$ T cells suffer from an energy sink which they may be unable to overcome. Intriguingly, we observed no significant difference in OCR or ECAR between WT and GPAT-1 $^{-/-}$ T cells in the absence of stimulation strongly implying that GPAT-1 is absolutely critical for the cell to mount a proper metabolic response to the increased work load and stress associated with stimulation. We have previously reported increased CPT-1a protein expression in GPAT-1 $^{-/-}$ thymocytes, so

we hypothesized that lipid oxidative modes of energy production may be skewed in $CD4^{+}$ GPAT-1 $^{-/-}$ T cells. Addition of fatty acid (palmitate/BSA conjugate) and intermittent monitoring of OCR and ECAR in WT and GPAT-1 $^{-/-}$ T cells actually revealed similar results to those observed in the respiratory assay with GPAT-1 $^{-/-}$ T cells having consistently reduced metabolic profiles. However, one interesting difference was that stimulated GPAT-1 $^{-/-}$ T cells responded to fatty acid addition in a similar manner to unstimulated WT cells. We also observed no compensatory increase in glycolytic modes of energy production as evidenced by decreased ECAR readings in GPAT-1 $^{-/-}$ T cells compared to their WT counterparts. Another interesting finding from this study was that addition of fatty acid significantly increased OCR in all samples tested compared to BSA injected controls. That OCR was significantly increased in unstimulated samples suggests that fatty acid itself is capable of upregulating the metabolism in quiescent T cells. This is to the best of our knowledge a completely novel finding in regard to general T cell metabolic responses and may have far reaching implications in regard to the current paradigm. Taken together these results indicate that GPAT-1 is essential for regulation of the stimulation induced metabolic switch in T cells. Furthermore, this data indicates that GPAT-1 $^{-/-}$ T cells have an inherent mitochondrial defect. These results are significant in regards to the field for three main reasons. First, we show here for the first time that deletion of a lipid biosynthetic protein has deleterious effects on total cellular metabolism under conditions of increased energy need. Second, altered metabolism in response to stimulation may be the defining mechanism underlying T cell dysfunction in GPAT-1 $^{-/-}$ T cells and furthermore, in light of the similarities between GPAT-1 $^{-/-}$ T cells and old T cells, loss of GPAT-1 activity with age likely leads to a similar phenotype in old T cells. Thusly, modulation of GPAT-1 activity may emerge as a principle target in the treatment of T cell dysfunction as we grow old. Lastly, we for the first time

link GPAT-1, an integral mitochondrial membrane protein to an inherent mitochondrial defect. This is important as the pleiotropic effects of reduced GPL have made it difficult to identify potential mechanisms related to GPAT-1 deficient T cell dysfunction.

The second objective of this project was to generate and characterize a cell line deficient in GPAT-1. We used an shRNA knock down (KD) strategy to target GPAM (human GPAT-1 homologue) in Jurkat T cells with the goal being to generate a stable cell line deficient in GPAM. We achieved a 7.3 fold and 2.8 fold decrease in GPAM mRNA and protein respectively and were able to maintain this stable KD line (referred to throughout as GPAMKD Jurkat T cells) in culture. To the best of our knowledge, this is the first time that the GPAM has been targeted in a cell line of human lineage. We first asked the question as to whether GPAM KD had any effect on cellular proliferation similar to what we observed in primary murine GPAT-1 $^{-/-}$ T cells. Interestingly, proliferation appeared unaltered in GPAMKD Jurkat T cells compared to scrambled shRNA transduced Jurkat T cells (ScshRNA Jurkat T cells). We next sought to determine whether apoptosis was elevated in GPAM KD Jurkat T cells compared to ScshRNA controls and intriguingly found significantly elevated levels of apoptosis in both stimulated and unstimulated GPAM KD Jurkat T cells. This observation implies that GPAMKD Jurkat T cells may actually proliferate at a higher rate than ScshRNA controls in light of the observed increases in cell death without apparent changes in proliferation. We have previously reported that IL-2, a key cytokine involved in T cell survival and proliferation is significantly reduced in GPAT-1 $^{-/-}$ T cells, so we sought to determine whether the same was true in GPAMKD Jurkat T cells. We found a striking decrease in CD3/CD28 stimulation induced IL-2 production in GPAMKD Jurkat T cells at 24 hours equating to a 9 fold decrease in IL-2 production. However, an important question arises concerning measuring IL-2 production at 24 hours regarding whether the

cytokine is being secreted and subsequently taken back up by the cell. To address this question, we looked at IL-2 secretion at the peak production time point of 6 hours and saw nearly identical reductions (approximately 9 fold) as those observed at 24 hours. We next sought to further characterize the phenotype of GPAMKD Jurkat T cells by determining whether there were alterations in membrane GPL similar to those in the murine GPAT-1 $^{-/-}$ T cells. Interestingly, we found significant decreases in phosphatidic acid (PA) and sphingomyelin (SM). These phospholipids play critical roles in the cell. Specifically, PA serves as the precursor to all GPL and also plays a critical role in mitogenic signaling serving as the docking site for Raf-1. We have previously reported significant decreases in PA in murine GPAT-1 $^{-/-}$ T cells. Interestingly though, many of the other key phospholipids (PC, PE, PS) seemed to narrowly miss significance. It is highly likely that increased sample size and further analysis may reveal significant decreases in these phospholipids as well, similar to the murine GPAT-1 $^{-/-}$ T cells. Another interesting finding was that SM, a key component of lipid rafts was significantly decreased in GPAMKD Jurkat T cells, but was actually the only major phospholipid not significantly decreased in murine GPAT-1 $^{-/-}$ T cells. These findings are rather intriguing, as it presents the possibility that murine GPAT-1 and human GPAM differentially regulate phospholipid metabolism. Another possibility is related to the fact that Jurkat T cells are a human lymphoma cell line which has likely accumulated numerous mutations as immortal cancer cells. It is therefore likely that Jurkat T cells have compensatory mechanisms by which to compensate for GPL sinks not present in murine GPAT-1 $^{-/-}$ T cells, such as up regulation of GPAT-3 or GPAT-4. It is also possible that Jurkat T cells have a second mitochondrial GPAM isoform (GPAT-2) capable of compensating for loss of GPAM. However, it is intriguing none the less that we see such profound effects on IL-2 production and cell death. Since PA is a precursor to all major membrane phospholipids we

hypothesized that supplementing GPAMKD T cells with PA may rescue their dysfunctional phenotype. We generated lipid micelles of two type of PA: dioleoyl (18:1) and dilauroyl (12:0) PA. We then added the resulting micelles back to cultures of GPAMKD T cells and assessed IL-2 secretion under various conditions and time points. We found that in no case was PA supplementation able to rescue the IL-2 deficient phenotype in GPAMKD T cells. One possibility is that the PA delivered was not entering the cell in a useable form or was quickly metabolized through other processes prior to reaching its target for either signaling or as a precursor to other phospholipids. Another possibility is that at the time points looked added PA there was inadequate time for sufficient lipid turnover in membranes to correct the defects associated with GPAM deficiency. It may also be that GPAM serves to regulate other processes such as modulating lipid metabolism by shunting fatty acyl CoA away from β-oxidation that are not corrected through PA addition.

Next, we sought to link reduced IL-2 secretion to a cellular mechanism. Since IL-2 production is directly regulated through signaling originating from the CD3 and CD28 co receptor we hypothesized that GPAM deficiency may be interfering with membrane based signaling. Certain components of the functional T cell signaling complex such as the CD28 co receptor exist on lipid rafts which translocate to the immunological synapse following stimulation. It is interesting then, that we observed decreases in sphingomyelin a principle lipid raft component suggesting that raft associated receptors such as CD28 may not be able to co localize to the TCR/CD3 complex. We therefore hypothesized that IL-2 production may be decreased due to loss of the co stimulatory CD28 signal. To test this hypothesis, we stimulated GPAMKD Jurkat T cells with CD3 or CD28 alone and subsequently measured IL-2 secretion. We subsequently found that IL-2 production was unaltered with CD3 stimulation alone,

suggesting that CD28 activation is dysfunctional in GPAMKD Jurkat T cells. However, the levels of CD3 only induced IL-2 production dwarf those of CD3/CD28 stimulation. We next hypothesized that if a membrane based defect exists in GPAMKD Jurkat T cells that prevents sufficient activation of signaling downstream of CD3 and CD28, then bypassing membrane based signaling should rescue IL-2 secretion. To test this, we stimulated GPAMKD and ScshRNA Jurkat T cells with PMA/Ionomycin, two compounds capable of inducing IL-2 production in T cells through their ability to increase intracellular calcium levels and activate protein kinase C s (PKCs). The Jurkat T cell response to PMA/Ionomycin is well characterized with regards to their stimulatory effect on IL-2 production. We subsequently found that PMA/Ionomycin increased IL-2 secretion in ScshRNA controls approximately 4 fold and approximately 20 fold in GPAMKD Jurkat T cells compared to CD3/CD28 stimulation. This finding suggests that bypassing membrane based signaling partially rescues IL-2 production in GPAMKD Jurkat T cells. This data serves to further support our postulation that GPAM/GPAT-1 deficiency results in perturbance of membrane based signaling as a consequence of altered membrane phospholipid ratios. To further investigate the possibility that GPAM deficiency results in dysfunctional TCR/CD3/CD28 induced signaling we sought to determine whether Zap-70 phosporylation was decreased in GPAMKD Jurkat T cells. Zap-70 is a key effector kinase activated very early (within seconds) following T cell stimulation. Zap-70 plays a critical role in propagating the TCR/CD3/CD28 stimulatory signal. We found that following stimulation, Zap-70 phosphorylation is significantly reduced in GPAMKD Jurkat T cells compared to ScshRNA controls suggesting that the dysfunctional T cell phenotype observed in GPAMKD Jurkat T cells is likely due to inadequate activation of the TCR/CD3/CD28 complex. These findings are highly significant for three main reasons. First, we show here for the first time that Jurkat T cells

deficient in GPAM have many of the same dysfunctional T cell characteristics as primary murine GPAT-1 $^{-/-}$ T cells. That many aspects of the phenotype are recapitulated in a human cell line strongly suggests that GPAM serves a conserved function in mammalian T cells. The importance of GPAM in T cell function is highlighted by the fact that such a profound phenotype was observed in a T cell lymphoma cell line. Second, we show that GPAM/GPAT-1 deficiency is a cell inherent defect. The GPAT-1 $^{-/-}$ mouse is a whole animal GPAT-1 knock-out. As such, certain questions arise regarding the origin of the dysfunctional T cell phenotype and specifically to what extent developmental or peripheral processes influence the phenotype. Since we recapitulated nearly all key aspects of the dysfunctional phenotype in GPAMKD Jurkat T cells, it is highly probable that GPAT-1/GPAM deficiency results in a cell inherent defect in T cell functionality. Lastly, we demonstrate that T cell dysfunction in GPAM deficient Jurkat T cells can at least in part, be attributed to a defect in membrane based signaling. This finding supports our metabolic work in GPAT-1 $^{-/-}$ T cells where we show they are unable to upregulate their metabolism to meet the increased energy needs associated with stimulation.

These data pose a number of important questions that future experiments should be designed to address. First, the results from our studies investigating the metabolic profile of GPAT-1 $^{-/-}$ T cells identified a profound metabolic defect in response to stimulation and decreased mitochondrial mass. However, the precise mitochondrial status and health of GPAT-1 $^{-/-}$ T cells remains unknown. Future experiments should examine indicators of mitochondrial health including redox and mitochondrial outer membrane potential. Work from the lab of Rosalind Coleman revealed that GPAT-1 $^{-/-}$ hepatocytes suffer from a mitochondrial defect related to increased susceptibility to opening of the mitochondrial permeability transition pore (MPTP) [14]. The MPTP is a protein pore formed in the inner mitochondrial membrane in response

to stress that can result in mitochondrial swelling and apoptosis. Elevated intracellular $Ca2^{+}$ is capable of inducing MPTP opening. It is therefore important that one of the principle events following T cell stimulation is a rise in intracellular $Ca2^{+}$, so then, it follows to question whether stimulation induces opening of the MPTP in GPAT-1 $^{-/-}$ T cells. It is possible that altered phospholipid composition in cellular membranes has profound effects on mitochondrial membranes that influence both mitochondrial membrane potential and permeability. MPTP opening and subsequent loss of mitochondrial outer membrane potential (MOMP) could be a key contributing factor in the reduced metabolic profile we observe in GPAT-1 $^{-/-}$ T cells. Specifically, the finding that spare respiratory capacity (SRC) is significantly decreased in GPAT-1 $^{-/-}$ T cells is highly suggestive of an integral mitochondrial defect in the cellular response to stress or increased work load as brought on by stimulation. Interestingly, old T cells have also been shown to undergo enhanced activation of the MPTP [110] . Furthermore, aging has also been associated with decreased SRC in a variety of cell types which may contribute to their inability to deal with increased cellular stress such as ROS, which is also consequently elevated with age [108]. Future experiments should examine stimulation induced opening of the MPTP and monitor loss of MOMP. Furthermore, experiments should be designed to more closely examine the metabolic profile of old T cells. These data in combination with the results of the current studies would solidify a role for GPAT-1 in maintaining mitochondrial health and stability not previously described and further strengthen our hypothesis that loss of GPAT-1 activity with age is a principle contributor to T cell dysfunction and that consequently the GPAT-1 $^{-/-}$ mouse is an ideal model to study the aged T cell phenotype in a young mouse.

Second, GPAMKD Jurkat T cells recapitulate many of the key aspects of the GPAT-1 $^{-/-}$ T cell phenotype including reduced IL-2 production, and altered membrane phospholipid mass.

The GPAMKD Jurkat T cell line is an ideal model to study mechanisms by which changes in phospholipid mass concomitant with loss of GPAM/GPAT-1 cause T cell dysfunction. Our results suggest that GPAMKD T cells suffer from a defect in TCR/CD3/CD28 activation and subsequent downstream signal transduction possibly as a result of membrane perturbances that prevent or interfere with aggregation of lipid raft localized receptors such as CD28. Future experiments should be designed to more closely examine formation of a functional immune synapse in GPAMKD Jurkat T cells. This can be accomplished using the Raji B cell lymphoma line loaded with SEE superantigen. Superantigens are capable of non-specific T cell activation resulting in subsequent stimulation and cytokine production. Raji B cells loaded with SEE superantigen are capable of stimulating Jurkat T cells in culture and forming cell-cell immunological synapses. Labeling of CD3, CD4, and CD28 with fluorescently conjugated antibodies followed by fluorescent confocal microscopy could reveal whether GPAMKD Jurkat T cells are forming a proper immunological synapse. Fluorescence resonance energy transfer (FRET) could also be used to specifically determine whether CD28 is co localizing to the immunological synapse following stimulation. FRET works by two chromophores interacting when they are in close enough proximity which induces a shift in the fluorescence wavelength that can subsequently be quantified. These experiments would provide insight as to whether alterations in phospholipid mass observed in GPAMKD Jurkat T cells prevent adequate co receptor aggregation at the immunological synapse. Failure to form a functional immune synapse could explain nearly all characteristics of the dysfunctional T cell phenotype we have described in both GPAMKD Jurkat T cells and primary murine GPAT-1 $^{-/-}$ T cells. If a defect is indeed present in GPAMKD Jurkat T cells the next step would be to replicate these experiments in primary GPAT-1 $^{-/-}$ T cells. One possible way to accomplish this task would be to cross GPAT-1

$^{-/-}$ mice with commercially available TCR transgenic mice expressing a αβ TCR specific for a defined peptide of a commonly used antigen such as influenza hemaglutinin (HA) or ovalbumin (OVA) capable of presentation by MHC class II. Several rounds of inbreeding should yield transgenic mice GPAT-1 $^{-/-}$ /TCR ^{specific} mice. Murine antigen presenting cells (APC) could then be pre-loaded with TCR specific peptide and experiments carried out as described for GPAMKD Jurkat T cells.

Lastly, GPAT-1 $^{-/-}$ T cells share many dysfunctional characteristics with old T cells. These observations in combination with our previous data showing that GPAT-1 activity is reduced in T cells from old rats presents the intriguing possibility that T cell dysfunction with age can be at least partially attributed to loss of GPAT-1 activity and alterations in membrane phospholipid mass. The first step in exploring this possibility in more detail would be to determine whether GPAT-1 reconstitution is capable of rescuing the dysfunctional phenotype of GPAT-1 $^{-/-}$ T cells. This experiment could be accomplished by first isolating primary murine GPAT-1 $^{-/-}$ T cells and transducing them with a lentivirus capable of delivering a functional *GPAT-1* gene under the control of a strong murine promoter in addition to a reporter gene such as *GFP*. Transduce GPAT-1 $^{-/-}$ T cells could be cultured for a sufficient period of time to allow for expression of GPAT-1 and GFP and then subsequently sorted by FACS on GFP. The experiments to follow would include: phospholipid mass analysis, cytokine analysis, proliferation, and metabolic profile. This proof of principle experiment would form the foundation for further studies aimed at determining whether GPAT-1 is capable of rescuing the dysfunctional phenotype of old T cells. An important point to make concerning these experiments is that T cells from old rats have reduced levels of GPAT-1 activity while protein expression is unaltered [9]. Therefore, it appears that aging reduces activation-dependent

mitochondrial GPAT-1 activity. Further studies by our group in Rat T cells revealed that GPAT-1 activity is positively regulated through phosphorylation by casein kinase 2 (CK2) and, protein kinase C theta (PKC-θ), which plays a critical role during T cell activation [27]. CK2 has also been shown to activate rat michondrial GPAT-1 activity [114]. Studies aimed at determining whether GPAT-1 can rescue T cell dysfunction with aging should therefore be focused on modes of enhancing GPAT-1 activity. CK2 is capable of phosphorylating a wide variety of cellular targets in many tissues, so future experiments in T cells should focus on enhancing PKC-θ mediated phosphorylation of GPAT-1. However, a confounding "which came first" problem surfaces when considering PKC-θ mediated phosphorylation of GPAT-1. If GPAT-1 activity is contingent upon adequate stimulatory signal leading to PKC-θ activation and this stimulatory signal is dependent on GPAT-1 to provide a proper phospholipid ratios in the membrane to provide this signal, how does the T cell reach this point and what should be targeted first? Initial experiments should be designed to determine whether indirect activation of PKC-θ is capable of improving the phenotype in old T cells. It is very important to point out here that the dysfunction in GPAT-1 $^{-/-}$ T cells is much less present or not at all when T cells are stimulated with PMA/Ionomycin which can directly activate PKCs bypassing membrane dependent signals. This is a critically important observation and strongly suggests that T cell dysfunction in the absence of GPAT-1 is modulated through defects in membrane based signaling. Early studies investigating the effects of PMA/Ionomycin in the aged T cell response reported that IL-2 supplementation in addition to PMA/Ionomycin treatment is capable of fully restoring the aged T cell response to that of young T cells [115]. This provides a foundation that allows for more direct probing of the role of GPAT-1, since it has been shown that bypassing membrane based signaling and providing IL-2 is already capable of rescuing the aged T cell dysfunction. Future

experiments should therefore be directly aimed at enhancing GPAT-1 activity in old T cells. One possible way to carry out these experiments would be to transfect old primary T cells with a constitutively active GPAT-1 followed by pheonotypic characterization. These experiments could potentially identify GPAT-1 as a critical effector of immunosenescene and thereby provide a target to improve immune function with aging, or possibly slow or even stop age associated T cell mediated immune dysfunction.

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