A ROLE FOR LYSOPHOSPHATIDYLCHOLINE TRANSPORTER MFSD2A IN CD8+ T CELL MEMORY AND SECONDARY RESPONSE TO INFECTION

by

Ann Renee Piccirillo

BS, Duquesne University, 2010

MS, University of Pittsburgh 2016

Submitted to the Graduate Faculty of

the Department of Human Genetics

the Graduate School of Public Health in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2018

UNIVERSITY OF PITTSBURGH

Graduate School of Public Health

This dissertation was presented

by

Ann Renee Piccirillo

It was defended on

October 30th, 2018

and approved by

F. Yesim Demirci, MD
Associate Professor
Department of Human Genetics
Graduate School of Public Health
University of Pittsburgh

Quasar Padiath, MBBS, PhD
Associate Professor
Department of Human Genetics
Graduate School of Public Health
University of Pittsburgh

William Hawse, PhD
Assistant Professor
Department of Immunology
School of Medicine
University of Pittsburgh

Committee Chair:
Louise D'Cruz, PhD
Assistant Professor
Department of Immunology
School of Medicine
University of Pittsburgh

Louise D'Cruz, PhD

A ROLE FOR LYSOPHOSPHATIDYLCHOLINE TRANSPORTER MFSD2A IN CD8+ T CELL MEMORY AND MAINTENANCE

Ann Renee Piccirillo, PhD

University of Pittsburgh, 2018

ABSTRACT

Immunometabolism is fueling breakthroughs across oncology, infection control, and inflammation research. It is appreciated that CD8 T cells are important players in all of the above-mentioned fields. Access to metabolic nutrients is critical for an effective CD8 T cell immune response to infection. Other groups have successfully identified transporters for exogenous import of sugars and amino acids for CD8 T cells, however, there is still a knowledge gap for how CD8 T cells can actively transport exogenous fatty acids. Here, I propose the lysophosphatidylcholine (LPC) transporter, Major Facilitator Super Family Domain Containing 2a (MFSD2A), is upregulated on activated CD8 T cells and is essential for memory T cell maintenance. MFSD2A deficiency in mice resulted in decreased import of LPC esterified to long chain fatty acids (LCFAs) into activated CD8 T cells, reduced memory T cell formation and maintenance, and reduced response to secondary infection. Import of LPCs was required to maintain T cell homeostatic turnover, that when lost resulted in a decreased memory T cell pool and therefore a reduced secondary response to secondary infection. I hypothesize that de novo fatty acid synthesis (FAS) is upregulated to compensate for loss of MFSD2A. These data were determined by using a thorough and multidisciplinary approach that combines the fields of immunology and human genetics. Importantly, there are families with known mutations in MFSD2A. These families have not been immunophenotyped for CD8 T cell dysregulation due to

loss of MFSD2A, but it is reasonable to suggest that they may suffer from a decreased response to infection. This project is relevant to public health because there is currently a knowledge gap in how exogenous lipid species imported by MFSD2A can affect the CD8 T cell immune response to infection, including how MFSD2A and LPC deliverables could be used for future immunotherapy targets.

TABLE OF CONTENTS

LIST OF FIGURES

PREFACE

Looking back at my PhD, there are many people that helped me to reach the finish line and I am eternally grateful for all of your support over the years. Completing this has been one of my most proud accomplishments to date and I would not be here without the help of many.

Thank you to my thesis advisor, Dr. Louise D'Cruz, for taking a chance on a hybrid research technician that happened to be a part time graduate student. Thank you for further encouraging me to continue onto my PhD after completing my master's degree. Thank you to the rest of my thesis committee – Drs. William Hawse, Quasar Padiath, and Yesim Demirci. It has been rewarding having a committee with members from such different research backgrounds and a joy merging immunology with human genetics.

Many thanks to my PIs and advisors in the old department of immunogenetics. You are responsible for truly getting me started on this path and encouraging me to start graduate school while knowing that I was most likely not going to be with you when I finished. Thank you especially to Drs. Tatyana Votyakova, Henry Dong, Yong Fan, and Massimo Trucco for welcoming me into your labs and first getting me interested in immunology (and human genetics!).

Thank you to all of my professors in the department of human genetics for teaching me a multi-disciplined genetics and public health curriculum while also allowing me to take relevant electives in immunology and to be fairly liberal with my dissertation topic. Thank you to my

academic advisor, Dr. Candace Kammerer, for always pointing me in the right direction and sending me Candy-esque weekly emails forcing me to network. It has helped tremendously. Thank you to me my fellow students for your sense of comradery.

Thank you to my labmates, past and present. Thank you Dr. Heather Buechel, Adolfo Frias, and Lisa Beppu for your advice, comic relief, and listening to me rant after a bad day. I've enjoyed all of our happy hours and mutual love of food over the years. A special thanks to all of my collaborators in the department of immunology and University of Pittsburgh because without you, this work would not be possible. Aarika MacIntyer, Dewayne Falkner, and Uzo Uche – thank you for dealing with my numerous technical questions. And of course, a shoutout to all of the students who have helped out on this project over the years – your genotyping and assistance with routine lab work helped to expedite this process and for that I thank you.

None of this would be possible without my family and friends outside of lab. Thank you for dealing with nights where I have been stuck in lab and nerdy scientific discussion. Thank you to my parents for never telling me not to try something, including getting this PhD. Tyler, you have put up with me being a student almost our entire relationship, I am excited to finally lose that descriptor! To Sidney and Sadie: thanks for being the best puppies and always cheering me up.

The last few years have been an excellent journey and I am looking forward to the future and saving the world with this PhD!

1.0 BACKGROUND AND SIGNIFICANCE

1.1 INTRODUCTION TO THE IMMUNE SYSTEM

The immune system is a mix of both quick acting innate cells and slower but more specific adaptive immune cells that work together to keep the host clean of invading pathogen. Without one or the other, the body is not capable of fighting off foreign invaders such as bacterial or viral infections. Upon pathogen infection, the bacteria or virus will rapidly replicate and elicit an immune response. After pathogen entry at the barrier tissues, the innate immune response will be initiated by pattern recognition receptors (PRRs)[1]. Innate cell types like macrophages will engulf and destroy the pathogen as well as signal for other cells to join the immune system cascade to prevent the pathogen from further infecting the host[2]. Pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPS) along with the immune complement system will further strengthen immune signaling, recruitment, and inflammation to continue to ward off the invading microbe[3-6].

An important component of this immune surveillance system is the dendritic cell (DC). Naïve DCs are constantly surveying the host in patrol of pathogen through PRRs. DCs are the only cell type that is capable of taking up self and non-self-antigen. Once internalized, these antigens are processed into proteolytic peptides and loaded onto major histocompatibility complex class I or class II (MHC I and MHC II) molecules[7]. DCs unique ability to take in

antigen, degrade it, and load it onto MHC complexes is called antigen presentation[8, 9]. Potent, mature DCs are known as antigen presenting cells (APCs) and are capable of activating adaptive immune cells by presenting peptide MHC[7]. In order to accomplish this, mature DCs need to travel through circulation, enter the peripheral lymph tissue, release various chemokines and signaling molecules, and eventually come in contact with naïve CD8 and CD4 T cells[6, 10, 11]. A summary of this is found in Figure 1, below. The remainder of this text will focus on the CD8 T cell immune response to infection.

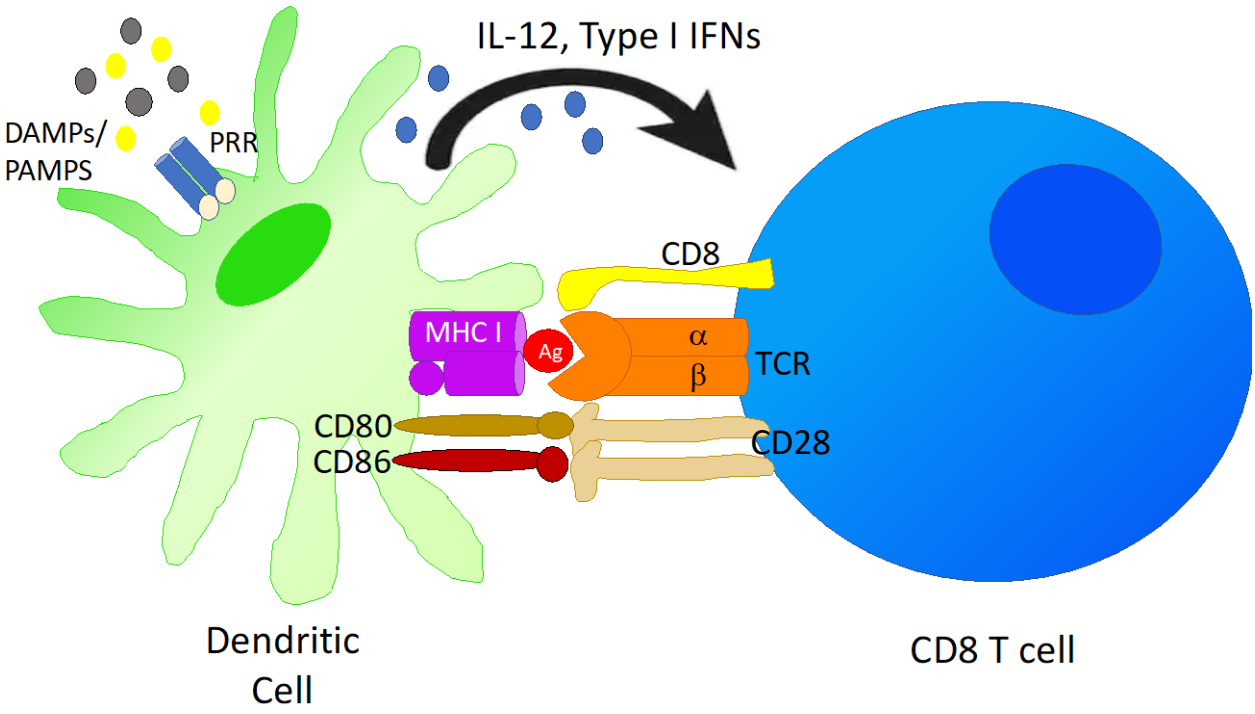


Figure 1. Antigen presentation by DC and CD8 T cell activation

An immune response to pathogen is initiated by DAMPs/PAMPs on PRRs on a dendritic cell (DC). The DC will take in antigen from the pathogen, degrade it, and load it onto MHC I complex in a process known as antigen presentation. The DC will migrate to the peripheral lymphoid tissue where it will encounter a naïve CD8 T cell. T cell activation is initiated by the T cell receptor (TCR) binding to MHC I on the antigen presenting cell (APC). The second required T cell activation stimulus is the co-stimulatory receptor CD28 binding to CD80 and CD86 on the APC. Cytokine signals like IL-12 and Type I interferon (IFN) released from the APC enhance CD8 T cell stimulation.

1.2 INTRODUCTION TO THE CD8 T CELL IMMUNE RESPONSE

CD8 T cells undergo conventional αβ T cell development in the thymus where they then migrate out into the periphery for constant immune surveillance[9, 12]. They will remain in a naïve, inactivated state until making contact with a unique glycoprotein peptide bound to MHC I. In order to become active, they require two stimuli[9, 13-15]. The first being the cell's T cell receptor (TCR) binding to MHC I. The second required stimulus is the co-stimulus surface receptor CD28 on the T cell binding to CD80 CD86 on an APC, usually a dendritic cell. This co-stimulus can be enhanced or replaced by certain cytokines, including IL-12 and Type I interferon (IFNα/β) [13, 14, 16]. T cell activation is illustrated in Figure 1. An example of CD8 T cell activation is OVA-albumin peptide binding to the MHC I receptor on an APC and being presented to the TCR of the CD8 T cell, initiating an adaptive immune response and cytotoxic activities of the CD8 T cell. After T cell activation, the newly activated effector CD8 T will undergo rapid clonal expansion and release both inflammatory and cytotoxic molecules to assist in pathogen killing such as IFNγ[14, 15, 17]. Importantly, IL-2 release will be critical for CD8 T effector cell formation and function and may play a role in determination of memory cell fate[18, 19]. Once pathogen is cleared from the system, the majority of these clonal specific CD8 T cells will undergo a contraction phase of programmed cell death in the form of apoptosis. A small portion of these cells will maintain for years as memory CD8 T cells. A summary of this CD8 T cell response to pathogen can be found in Figure 2.

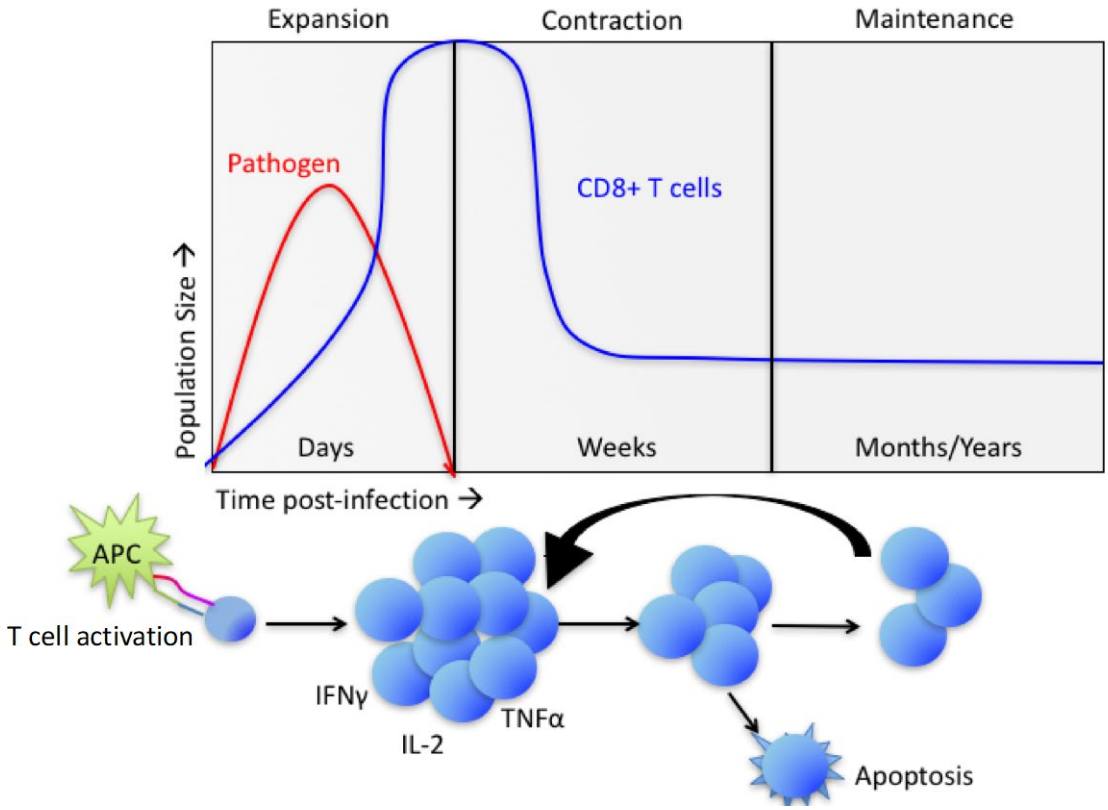


Figure 2 The CD8 T cell immune response to pathogen

The adaptive immune response to infection kicks in days after infection whenever a naïve CD8 T cell encounters peptide MHC on an antigen presenting cell (APC). Activated T cells will undergo a rapid clonal specific proliferative burst accompanied by inflammatory cytokine production, peaking at day 7 post-infection (pi). Most cells will contract and die via apoptosis however some will go on to be maintained as memory T cells, enable to quickly respond to recurrent antigen exposure.

1.2.1 MEMORY CD8 T CELL PHENOTYPING: A CLOSER LOOK

Memory CD8 T cells are a unique and important variety of CD8 T cells as they are able to provide a quicker and more robust clonal expansion response relative to naïve CD8 T cells if the body comes into contact with a secondary infection with the same pathogen[20, 21]. This idea of immunological memory is a critical component to vaccine development[22-27]. Traditional vaccines make use of generating protective antibodies, however newer approaches

have focused on using CD8 T memory cells – HIV and tumor vaccines included[28-30]. For the most part, memory CD8 T cells can be classified by a classical phenotypic signature based on cell surface marker expression, which will be discussed in this work under the next subtopic. In addition to surface marker classification, murine memory CD8 T cells can be more broadly classified as central memory, effector memory, and tissue resident memory based on location within the body[31]. The predominant subtype is known as central memory CD8 T cells (T_{cm}). T_{cm} are long-lived memory CD8 T cells that home to the secondary lymphoid tissue and provide a rapid, frontline defense upon secondary infection. Once restimulated, these T_{cm} are capable of producing a robust recall response to at the site of infection. These cells are phenotyped as $CD44^{hi}CD62L^{hi}KLRG1^{lo}CD127^{hi}$[13, 32-34]. The second subtype of memory CD8 T cells are effector memory CD8 T cells (T_{em}). T_{ems} are found in the circulation between lymphoid tissues and are known to be superior producers of effector cytokines upon restimulation[33, 34]. They are usually phenotyped as being $CD44^{hi}CD62L^{lo}KLRG1^{hi}CD127^{lo}$ cells. The third type of memory CD8 T cells in mouse is the tissue-resident (T_{rm}) memory CD8 T cells. T_{rms} are noteworthy for being a top-line immune defender in non-lymphoid, common barrier tissues such as the skin, lung, gut, and adipose[35]. T_{rms} have a characteristic surface marker phenotype of being $CD69^{hi}$ and (usually) $CD103^{hi}$ in addition to being $CD44^{hi}KLRG1^{lo}ESL^{mod}$[35, 36].

My thesis will focus primarily on central memory CD8 T cells, or those that are found within lymphoid tissue. There is currently a large gap in the knowledge of what determinants make a T_{eff} become a $T_{mem.}$ It has been accepted in the field that memory CD8 T cells typically are those cells that are IL-2 producing effector CD8 T cells and that they are regulated by mTOR transcription[37], however, there is still much to be elucidated about memory CD8 T cell

development and what signaling, metabolic, genetic, and epigenetic changes may lead to this cell fate[9, 15, 31, 38].

1.2.2 LISTERIA MONOCYTOGENES AS A MODEL TO MEASURE THE CD8 T CELL IMMUNE RESPONSE TO INFECTION

Listeria monocytogenes (Lm) is a gram positive, facultative intracellular bacteria most commonly associated with food-borne illness[39, 40]. It can be of most concern to those who are immunocompromised, pregnant women, and infants[9, 39, 40]. Lm has been used as a model pathogen as early as the 1960's whenever researchers infected mice intravenously (IV) with Lm and noticed T cell mediated bacterial clearance (Fig. 2)[41]. A nice complement to studying Lm T cell dynamics is that there are numerous antigen specific epitopes available to track the clonal specific effector response and memory cell formation in these systems[40, 42]. Additionally, like most T cell immune response models, Lm responding effector and memory CD8 T cells can be phenotypically characterized by up or down regulation of surface marker expression of CD44, CD62L, KLRG1, and CD127 (IL-7R), where $CD44^{hi}CD62L^{lo}KLRG1^{hi}CD127^{lo}$ cells are deemed effector CD8 T cells and $CD44^{hi}CD62L^{hi}KLRG1^{lo}CD127^{hi}$ cells are deemed (central) memory CD8 T cells [20, 43-48].

1.2.3 TRACKING CLONAL-SPECIFIC CD8 T CELLS TO MEASURE THE CD8 T CELL IMMUNE RESPONSE

The use of transgenic, clonal specific T cells to track an immune response has been well documented[40, 49-52]. Briefly, transgenic T cell receptor (TCR) inserts that recognize a specific MHC peptide are inserted into the mouse genome. One prominent example of this is a mouse model with TCR engineering to recognize ovalbumin residues 257-264 in the context of $H2K^b$ (MHC I). Roughly 20% of all T lymphocytes will recognize this specific OVA residue. These mice can then be used to track recombinant OVA peptide specific pathogens over the course of *listeria* infection[40, 53, 54] when Lm has OVA antigen attached and are deemed Vα2 Vβ5 OT-I transgenic mice [55-57].

It is also important to note that the OT-I model can be used for mixed bone marrow chimera generation and adoptive transfer studies, where cells from up to three different animals can be distinguished in the host mouse. This is made possible due to genetic manipulation of the leukocyte common antigen CD45. WT (wildtype) B6 (C57BL/6J) mice commonly have the CD45.2 allele. However, allelic variants exist as seen in the "pep boy" ($pepc^b$/BoyJ) CD45.1 mouse or heterozygous CD45.1.2 mice[58, 59]. All variants are functionally identical and enable an easy way to track competitively adoptive transferred donor CD45.2 and CD45.1.2 cells into a CD45.1 congenically labeled recipient mouse. All leukocytes from a given donor will express their allelic CD45 counterpart which makes this model ideal for transplantation studies, including the above-mentioned adoptive transfer approach. Use of this system has been well documented [58, 60-62]. Further discussion of adoptive transfers can be found in the materials and methods as well as diagramed in Appendix D.

1.3 CD8 T CELL METABOLISM AND METABOLIC REPROGRAMMING

1.3.1 INTRODUCTION TO EUKARYOTIC CELLULAR METABOLISM

All living organisms require carbohydrates, lipids, and proteins for fuel. Carbohydrates are broken down into glucose prior to entering glycolysis, the process outside the mitochondria where one molecule of glucose is broken down into two molecules of pyruvate and a net yield of 2 ATP molecules[63, 64]. Pyruvate will then go into the mitochondria where it will enter the tricarboxylic acid (TCA) cycle and be converted into acetyl-CoA. The majority of this acetyl-CoA will become further fuel for cellular respiration, however some will also exit and go on to be banked as sterols and fatty acids, ketone bodies, or for protein acetylation. Importantly, the TCA cycle is also where fatty acids and amino acids join the central metabolism pathway[65, 66]. Here, a series of oxidation reactions yields NADH, FADH, and GTP that go on to further fuel subsequent steps of cellular respiration to produce even more ATP. These metabolites enter the electron transport chain (ETC) where they undergo a series of reductions, where electrons are "transported" across various protein complexes with O_2 being the final acceptor (oxidative phosphorylation)[67]. During this process, a proton gradient of released H+ is formed which eventually leads to the phosphorylation of ADP to ATP, establishing a net gain of 32 ATP molecules[68].

Where do proteins and lipids enter these processes? Most general biology textbooks do not discuss where macromolecules besides glucose enter the metabolic pathway. As mentioned above, the site of convergence is at the TCA cycle. Proteins undergo proteolysis and are broken down into amino acids prior to deamination and oxidation to acetyl Co-A[68]. A cartoon

combination of all of these processes with emphasis on β-oxidation can be visualized in Figure 3. For the purposes of this dissertation, lipid metabolism well be analyzed in greater detail below.

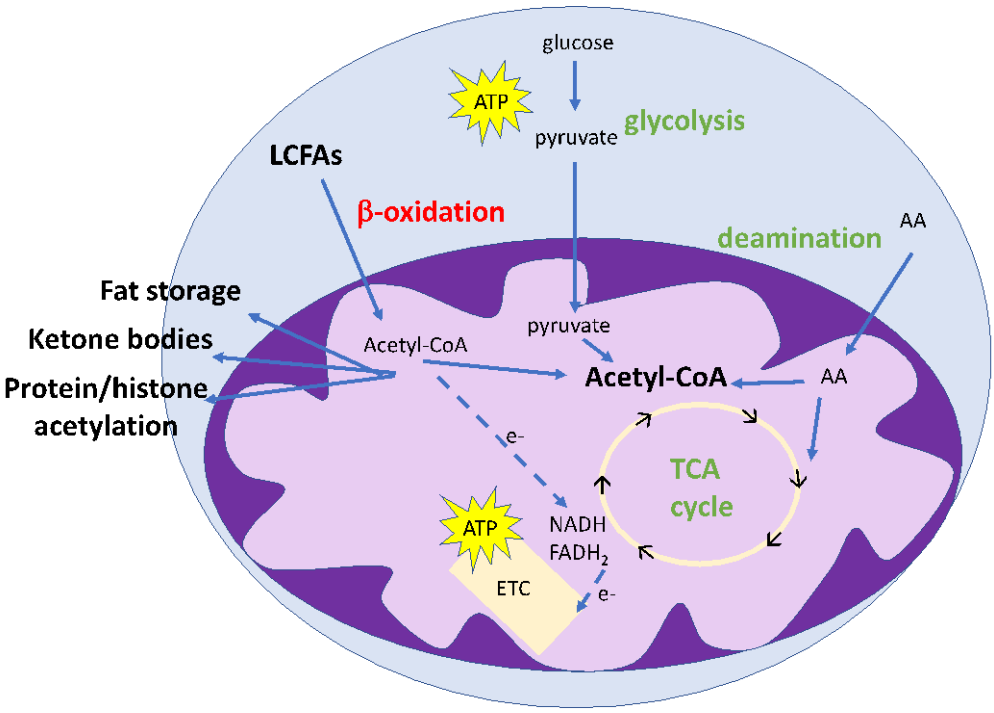


Figure 3. Overview of cellular metabolism with focus on β-oxidation

Eukaryotic cells use metabolites from sugars, proteins, and fats for energy. Sugars (glucose) typically undergo glycolysis prior to entering the TCA cycle and electron transport chain/oxidative phosphorylation in the inner mitochondria. Amino acids (proteins) undergo deamination prior to entering the TCA cycle as various intermediates. Fats (LCFAs) are broken down via b-oxidation, where some will enter the TCA cycle as acetyl-CoA while others will be used for fat storage, ketone body formation, and protein/histone acetylation. An in-depth lipid analysis follows bellow.

1.3.2 A CLOSER LOOK AT LIPID METABOLISM

Dietary fats are degraded intro triacylglycerols (TAGs) in the intestine. They are then taken up by the intestinal mucosa where they join cholesterol and apolipropoteins and are incorporated into chylomicrons. Chylomicrons migrate through the bloodstream where they are

broken down into fatty acids and glycerol and enter extra hepatic target cells via various fatty acid transporters. Here, they can either be oxidized as fuel or re-esterified for storage. Free fatty acids will be specifically broken down by a process known as β-oxidation[68-70]. Prior to this, LCFA must cross the mitochondrial membrane, however, they are not able to do so without using the carnitine shuttle. The most critical and rate-limiting step in this shuttle process relies on the enzyme carnitine palmitoyltransferase 1 (CPT1), with the isoform of most interest to this dissertation being CPT1α[71, 72]. This enzyme enables LCFAs to cross the inner mitochondrial membrane by catalyzing the transfer of the acyl group of a long chain fatty acetyl-CoA from coenzyme A to l-carnitine, a product capable of mitochondrial membrane transport. Once in the mitochondria, a series of enzymatic processes is initiated by the oxidative removal of successive 2-carbon units in the form of acetyl-CoA. In the case of 16 carbon palmitic acid, this means in 8 cycles there will be a net of 8 two-carbon acetyl groups of acetyl-CoA. After the removal of 4 hydrogen atoms, this will mean a net gain of 8 acetyl-CoA molecules that can enter the TCA cycle or fat storage. The delicate balance between fatty acid oxidation (FAO) and fatty acid synthesis (FAS) is regulated by the PPAR transcription factor family.

FAS is regulated by a family of proteins known as sterol regulatory element-binding proteins (SREBPs)[73-76]. These proteins control *de novo* FAS using a canonical insulin-AKT-mTOR-SREBP pathway that involves trafficking between the rough endoplasmic reticulum (ER), golgi, and nucleus[77]. SREBPs come in 3 flavors: SREBP1a is necessary for global lipid synthesis and growth, SREBP1c is involved in FAS and energy storage, and SREBP2 regulates cholesterol production[77].

1.3.3 INTRODUCTION TO CD8 T CELL METBOLISM

Given that CD8 T cells undergo such drastic phenotypic changes upon antigen encounter, it is not surprising that this is accompanied by changes in metabolism. Naïve CD8 T cells are in a relatively quiescent state and predominately use oxidative phosphorylation (OXPHOS) as their metabolic means[78-80]. However, when activated CD8 T cells undergo clonal expansion, they require a much higher energy yield in order to grow, proliferate, and maintain effector cytokine production[64, 78-82]. Activated T cells use an unconventional approach to accomplish this. Similar to cancer cells, activated T cells will rely primarily on aerobic glycolysis, or the Warburg Effect, in order to meet their increased energy demands. This means that even in the presence of oxygen, activated CD8 T cells prefer to quickly use aerobic glycolysis rather than the more energetically productive OXPHOS[83]. The most likely explanation for this phenomenon is that aerobic glycolysis enables proliferating cells to free up energy for important metabolites that growing cells need to produce more biomass[82-84]. Only a small portion of these effector CD8 T cells differentiate into memory CD8 T cells that will again metabolically reprogram to use both OXPHOS and perhaps lipolysis to fulfill energy requirements [38, 85, 86]. A general overview of this can be found in Figure 4.

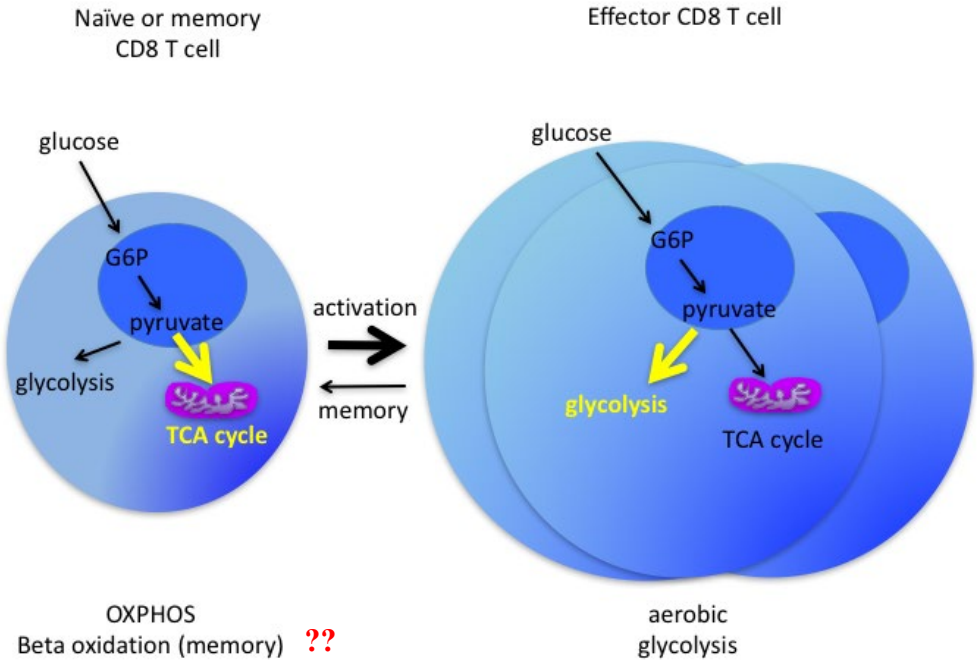


Figure 4. CD8 T cell metabolic switching

As they switch from phenotypic states, CD8 T cells also perform metabolic switching. Naïve T cells are low energy and rely primarily on cellular respiration via the TCA cycle and oxidative phosphorylation (OXPHOS). Upon activation, T cells switch to primarily using aerobic glycolysis. Memory cells again switch to OXPHOS but, there are also reports that they may utilize lipid metabolism (beta oxidation), as indicated by red question marks.

Is there a possible link between CD8 T cell metabolism and memory CD8 T cell fate? Numerous reports suggest differential gene transcription and surface marker signaling are critical for memory CD8 T cell survival[33, 37, 43, 87] but there is also a strong belief in the field that the metabolic switching from glycolysis to OXPHOS and FAO is another important determinant[79, 88, 89]. Reports show that T cells use FA signaling as key cell fate determinants, including whether or not for effector cells to differentiate into memory cells [90]. When FAS is inhibited early in T cell priming to chronic viral infection, it was shown to limit memory precursor effector cell (MPEC) frequency[91], suggesting an important role for LCFAs

early in T cell activation and differentiation. It was previously discovered that when chemical inhibitors of glycolysis are administered during the effector phase that there is an enhancement of memory cell formation[92]. This is supported by seminal studies, performed by Pearce et al., that show that memory CD8 T cells prefer to use FAO metabolism. Early reports illustrated this by inhibiting TRAF6, an adaptor protein in the TNF-receptor, in all T cells of infected mice and saw an enhanced effector response but very poor memory CD8 T cell formation that was found to be a result of decreased transcription of genes related to FAO[38]. The group went on the show that when etomoxir is used to inhibit the rate limiting enzyme of FAO (CPT1α), that memory CD8 T cell formation is also inhibited[93].

Recent reports have sparked controversy by suggesting that the effects of etomoxir were off target and a result of oxidative stress in the system[94, 95]. These studies suggest that much lower levels of etomoxir are all that are necessary to inhibit CPT1α and FAO and that when the physiological relevant level of inhibitor is used, that there is no effect on memory CD8 T cell formation[96] or macrophage polarization, another function thought to be determined by FAO[97]. In light of these recent studies, it is suggested that memory CD8 T cells do not rely directly on FAO, but rather DAG storage during the effector response in combination with *de novo* lipid synthesis.

Lipid metabolism in T cells is a critical topic in the field of immunometabolism. How effector and memory T cells acquire external metabolites such as glucose[98, 99], glycerol[52], and neutral amino acids[100] has already been investigated. Even with the recent contradictory reports listed above, there is still evidence suggesting that CD8 T cells are importing exogenous lipids, as found when looking at another important group of memory CD8 T cells -- tissue resident memory (T_{RM}) CD8 T cells[101] – and their preferential use of lipid transporters FABP4

and FABP5, critical for their formation [36]. Besides this information, very little is understood overall about how CD8 T cells use fatty acids and how, if at all, they use the import of exogenous free fatty acids (FFAs) during effector and memory responses. This knowledge gap represents an area of CD8 T cell immunometabolism worthy of further pursuit.

1.4 INTRODUCTION TO LYSOPHOSPHOTIDYLCHOLINE

Lysophosphotidylcholine (LPC) is a product of lysophospholipid (LPL) metabolism that can be generated from its precursors phospotidylcholine (PC) by the enzyme phospholipase $A_{1/2}$ [102-104]. It is either directly formed in the liver or by altering low-density lipoprotein (LDL) cholesterol *in vitro*. LPC consists of one hydrophobic fatty acyl chain and one hydrophilic polar choline group attached to a glycerol backbone. LPCs are present in roughly 3% of phospholipid membranes and make up roughly 8-12% of blood plasma[105]. In addition to this, many LCFAs are found in the cell plasma in their esterified form with LPC, the most common of these being LPC-palmitate and LPC-oleate[106]. LPC-docosahexanoic (LPC-DHA) also exists at lower levels. LPC species are critical for maintaining cellular homeostasis as well as added benefits such as wound healing and potential therapeutic treatments for both autoimmunity and neurodegenerative conditions [107]. There are hints that LPC may be important to the immune system – others have found a role for LPC as a negative regulator of innate and adaptive immune cell proliferation, under G2A signaling motifs[108] and that LPC is a significant component to viral transmission in Chagas disease through LDLs in infected insect saliva left at the point of infection on the skin[109]. The general form of LPC is shown in Figure 5 and is considered to be $C_{24}H_{50}NO_7P$ with the addition of various R group esterified LCFAs.

Once transported into the target cell's cytoplasm, LPCs are processed into multiple products such as PC, phosphatidic acid (PA), diacylglycerol (DAG) and triglycerides (TAGs) to be used metabolically by the cell[102]. Since LPC acts as the chemical transporter of many species of LCFAs between cells, it is considered an important component to cellular metabolism. It is appreciated that DHA is the preferred dietary fatty acid for brain and eye development, particularly in infants as well as known other positive anti-inflammatory qualities of supplementation[110-113], however, until recently, it was not known how DHA was able to cross the blood brain barrier (BBB) and blood retinal barrier (BRB) and be delivered into the brain[114]. It was discovered in 2014 that Major Facilitator Superfamily Domain Containing 2a (MFSD2A) is the lipid transporter responsible for transporting LPCs across the BBB, BRB, and blood placental barrier[106, 115-117].

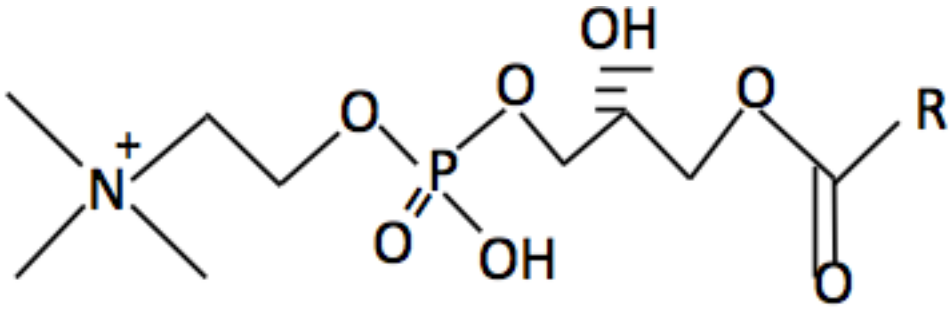


Figure 5. Chemical structure of LPC

The chemical structure of LPC where R is the variable fatty acid chain.

1.4.1 LPC IN T CELLS

Very little is known about LPCs and T cells, including how and if CD8 T cells may be importing and using LPC species. Preliminary studies show a role for LPC as a chemoattractant for T cells, suggesting it may be important for T cell recruitment to atherosclerotic plaques[118]. There is also evidence that LPCs produce reactive oxygen species (ROS) and have (possible activation) signaling properties in Jurkat cells, an *in vitro* model for human CD8 T cells[119, 120]. In $CD4^+$ $FOXP3^+$ $CD25^+$ T regulatory (Treg) cells, LPC was found to increase their suppressive abilities through altering TGFβ signaling, a hallmark of Treg cells[121].

A unique subset of T cells, deemed invariant natural killer T cell's (iNKT) known to have a invariant T cell receptor restricted to responding to glycolipids presented by the CD1d molecule[122, 123] show that they can be activated by CD1d-LPC complexes[124, 125]. This dissertation will not discuss iNKT-LPC interactions but will acknowledge my own preliminary data showing a potential relevancy of iNKT cells and LPC.

1.5 INTRODUCTION TO MFSD2A

MFSD2A is a member of the major facility superfamily (MFS) protein transporter family which consists of 74 diverse subfamilies and nearly 25% of all membrane transporters in prokaryotes[126]. MFSD2A is a 12 domain transmembrane protein. It was previously believed to be an orphan transporter but now has a well-established role as a lipid transporter that bears a close resemblance to the bacterial Na^+/melibiose supporter [106, 116, 127]. Spanning 14 exons and 7.7 kb, it is conserved through vertebrate evolution and has a wide tissue distribution in mouse with highest expression levels in kidney with cellular localization at the rough endoplasmic reticulum (ER) [116, 128, 129]. MFSD2A acts in a sodium-dependent manner to transport LPC species by a unique mechanism where LPCs are "flipped" by turning about Lys436 which carries the LPC from the outer to the inner cell plasma membrane[130]. The complete mechanism of active MFSD2A-LPC species transport is currently unknown, however recent studies have determined three critical steps to transport function: The LPC headgroup binding site, a hydrophobic cleft occupied by the fatty acyl tail of LPC, and a combination of three sets of ionic locks[130].

MFSD2A was first discovered as a fasting-induced gene that regulated PPARα and glucagon in the liver and brown adipose tissue (BAT), suggesting its role in lipid metabolism, growth, and thermogenesis [127, 128]. MFSD2A was subsequently found to be an important sodium-dependent tucinamycin antibiotics transporter in humans and when knocked out in human cell lines, tunicamycin uptake was significantly decreased [129]. This experiment supports MFSD2A's importance at the ER and its role in the cellular stress response as well as the potential for transport roles for MFSD2A[131]. MFSD2A has also been found to be protective against intracerebral hemorrhage by inhibiting vesicular transcytosis post-

hemorrhage[132]. More recently, MFSD2A has been implicated in BBB development and its important role as lipid transporter of DHA in the form of LPC across the blood brain barrier (BBB) [106, 133, 134]. DHA has been well documented for its critical role in brain development [110, 114, 135]. It is essential for normal brain growth and cognition. However, since the brain cannot synthesis DHA *de novo*, the mechanism of how it crossed the BBB was unknown until recently. In 2014, MFSD2A was found to be the major transporter of DHA across the BBB into brain endothelium, and it does so by transporting LPC-DHA in the form of LPC in a sodium-dependent manner [106]. A cartoon illustrating how MFSD2A imports LPC-DHA across the endothelia of the BBB is illustrated in Figure 6. Using conditional KO models, it was shown that the brain tries to compensate for loss of exogenous lipid import by using FAS to synthesize its own, suggesting that MFSD2A is a negative regulator of SREBP1 and SREBP2[74].

In the years since this seminal finding, MFSD2A has been found responsible for transporting DHA across the BRB [115] and placental barrier[117, 136, 137], noting, however, that maternal DHA supplementation in pigs does not increase fetal brain accretion[136]. MFSD2A is also an important marker of central nervous system injury, as it was found to be a biomarker for spinal cord injury where the amount of endothelial cell damage correlated with the amount of MFSD2A loss in mice[138]. Interestingly, this study was the first to show a link between epigenetics and MFSD2A, showing that when miR-155 is expressed at lower levels after spinal cord injury and when this is combined with increased levels of MFSD2A, there is a reduced rate of paralysis.

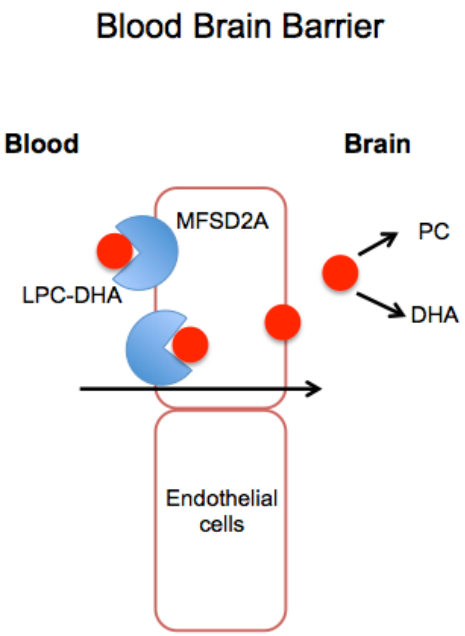


Figure 6. MFSD2A and LPC-DHA interaction at the BBB

MFSD2A actively transports LPC-DHA across the BBB in a sodium-dependent manner. It acts as a transmembrane pore enabling exogenous LPC-DHA to cross the endothelium of the BBB into the brain. Once there, LPC-DHA dissociates into DHA and phosphatidylcholine (PC). The complete mechanism of MFSD2A is currently unknown.

Could there be potential therapeutic benefits to using MFSD2A as a transporter that carries LPC species across the BBB? One suggestion is that MFSD2A could be used as a target for drug delivery, potentially by coupling drug delivery with LPC[139]. MFSD2A co-localizes with GLUT1 in the brain, the transporter for exogenous glucose[106]. Studies have shown in mice that when dietary fish oil is supplemented in the diet, there is an increase in MFSD2A and GLUT1 levels in the BBB, which could increase overall brain health and decrease the risk of disease[140]. Contradictory to this, others have shown that fish oil makes its way across the BBB (and liver) independent of MFSD2A[141], so further research into using MFSD2A as an area of therapeutic benefit must be performed.

Although rare, there are a few families with autosomal recessive MFSD2A mutations on chromosome 1p within their pedigree[116, 142, 143]. The main phenotype is microcephaly, developmental delay, and intellectual disability, all of which may be not surprising given MFSD2A's known importance in the brain. All families show point (missense) mutations responsible for protein changes although the exact point mutations vary by family. These data were able to create a link between loss of MFSD2A and brain disease due to lack of LPC-DHA. Immunophenotyping was not performed on the above individuals, so no known link between MFSD2A mutated individuals and CD8 T cells in humans is currently known.

1.4.1 MFSD2A AND A LINK TO THE IMMUNE SYSTEM

Immunological phenotypes due to alterations in MFSD2A have been reported. The earliest reports link back to MFSD2A and its importance in brain health. These include a role for MFSD2A and brain cancer metastasis prevention, showing a striking correlation between the ability of brain cancer to metastasize out of the brain with loss of MFSD2A. When the BBB loses MFSD2A expression and upstream TGFβ and bFGF signaling, tumors are no longer inhibited from progressing across the BBB[144]. The authors also note that these brain metastases show decreased lipid metabolism and decreased amount of DHA import. These data taken together suggests there may be a novel role for MFSD2A and DHA supplementation in preventing brain tumors from spreading. A role for MFSD2A mutations in gastric cancers has also been reported[145].

MFSD2A may be an important candidate gene for ulcerative colitis (UC). Patients with active UC were found to have decreased amounts of both DHA and MFSD2A, suggesting a role

for MFSD2A transport of DHA across the gut endothelium and that, when lost, causes increased inflammation. Rescue experiments were successfully performed in mice where overexpression of MFSD2A caused a reversal of intestinal inflammation of UC[146]. This is the first study to show that MFSD2A is critical for resolving inflammation in UC and to suggest that lack of MFSD2A expression may indicate a possible autoimmune phenotype as UC is thought to be at least partially caused by autoimmunity[147].

Finally, MFSD2A+ cells are a contributing player to tissue regeneration. It was discovered that hepatocytes that express MFSD2A are capable of expansion following partial hepatectomy or chronic injury while those that are MFSD2A- are not capable of regrowth[148]. Furthermore, MFSD2A was determined to be a marker of liver zones and was the marker that distinguished final zonation following liver repopulation. These data show that MFSD2A is an important marker for tissue regeneration and that it may be possible to take these data to the field of tissue transplantation, specifically to promote cell turnover and fend off necrosis.

1.6 LPC, MFSD2A, AND CD8 T CELLS

I previously showed that MFSD2A and LPC were both increased following innate iNKT (not shown) and adaptive T cell activation[149]. I accomplished and generated these data by using a combination of molecular approaches including RNA, protein, and flow cytometry that nicely show that both MFSD2A and LPC increased *in vitro* and *in vivo* (Fig. 7 and Fig. 8) following CD8 T cell activation. These data in particular showed that MFSD2A and LPC are important very early in T cell activation, as mRNA expression is highest as early as 6 hours post *in vitro* activation and returns down to basal by day 5 post *in vivo* activation. Importantly,

MFSD2A expression levels are activation dependent, as shown by flow cytometry analysis where the CD44^{hi} (a marker for T cell activation) cells are also the ones co-expressing increased levels of MFSD2A (Fig. 7C). Taken together, these preliminary experiments gave the basis of this dissertation project.

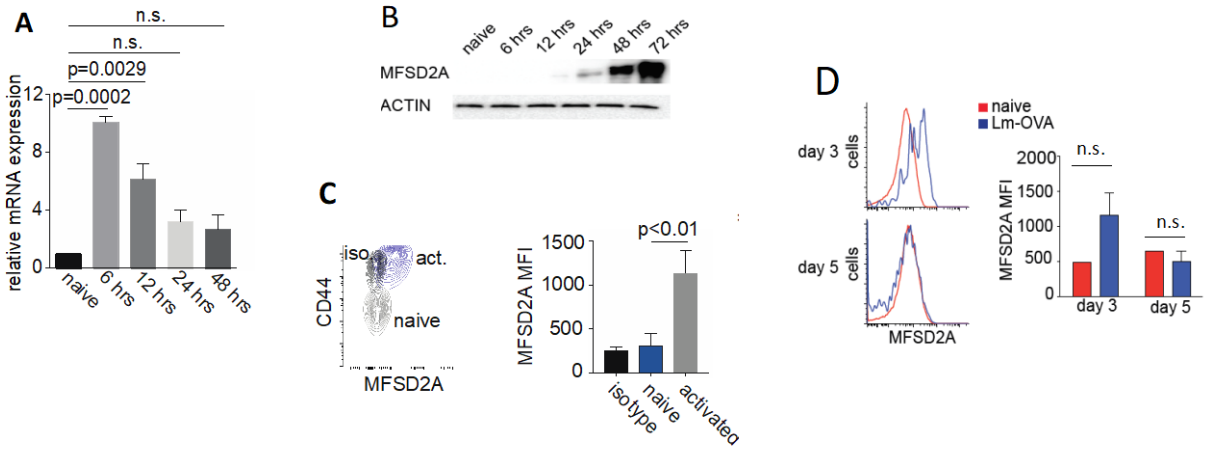


Figure 7. MFSD2A characterization in WT CD8 T cells

MFSD2A mRNA expression levels from CD8 T cells activated until given time point. B. MFSD2A protein levels out to given time point with beta-actin loading control. C. FACS plot of activated versus naïve CD8 T cells measuring MFSD2A relative to CD44. D. Activated or naïve OT-I CD8 T cells from adoptively transferred mice at given time point measuring MFSD2A intensity. All data except D. from *in vitro* activated CD8 T cells stimulated with α-CD3 and α-CD28. Data are representative of at least 3 independent experiments with 3 animals per group with p-values calculated using Student's t test.

I wanted to pursue studies designed to determine what would happen to activated effector CD8 T cells during acute infection with loss of MFSD2A. Would they still be capable of producing an effector response? Memory CD8 T may use FAO, or at least will use lipid metabolism for metabolic fuel to bank biosynthetic precursors for rapid proliferation during secondary antigen encounter. With loss of exogenous lipid import, would memory CD8 T cells form, function phenotypically, be maintained, and be capable of producing an efficient recall response to infection? These were all questions previously left unanswered until now.

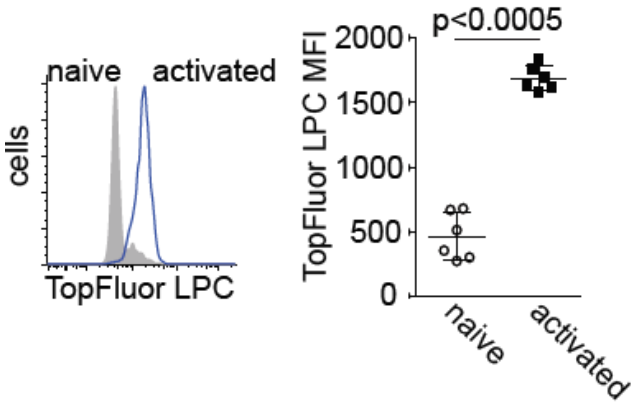


Figure 8. TopFluor LPC levels increase with CD8 T cell activation

CD8 T cells were activated *in vitro* with α-CD3 and α-CD28 for 48 hours. The last 4 hours of incubation, cells were co-cultured with 0.1uM TopFluor LPC prior to being ran on the cytometer. Data are representative of at least 3 independent experiments with 3 animals per group with p-values calculated using student's t test.

1.7 MFSD2A AND PUBLIC HEALTH

T cell metabolomics and metabolic reprogramming are currently an exciting field in immunology because of how cell metabolism can be used as a regulator for various cell processes including cell fate, epigenetic control, tumor regulation, inflammation control, and infection prevention and vaccine development[93, 150-155]. The mechanism by which effector T cells take up LCFAs and how it is regulated is still unknown. This represents a knowledge gap in the understanding of how T cells use exogenous lipids and if these LCFAs play a pivotal role in effector function and memory cell formation. The potential breakthrough of MFSD2A as the carrier molecule of LCFAs into activated T cells by means of chemical form LPC would fill a gap in the current understanding of T cell lipid metabolism by showing where and how CD8 T cells are using exogenous LPCs. Activated T cells require higher energy demands to properly

initiate an immune response, to clear infection, or tumors. Elucidating this pathway is very important to public health, as these metabolic instruments hold potential therapeutic benefits to patients and could provide future cutting-edge immunotherapies. In addition to this, The World Health Organization (WHO) and American Heart Association both recognize DHA as an important component to a healthy lifestyle[156, 157]. The research herein signifies an urgent need to understand and target how phospholipids, LCFAs, and DHA are imported into CD8 T cells and how they impact the immune response to infections, including recurrent infections that depend on a pool of nutrients for memory T cell functionality

1.8 OBJECTIVES, AIMS, AND OVERALL HYPOTHESIS

The main objective of this project was to expand current knowledge on MFSD2A in CD8 T cells. A role for MFSD2A in any one immune cell type has still not been published. I was the first to show a potential role for MFSD2A in CD8 T cell activation during my master's thesis. This current project looked more closely at what are the cellular consequences of loss of MFSD2A and LPC import into CD8 T cells. Until recently, it was accepted that memory CD8 T cells prefer to use FAO and that central memory CD8 T (T_{cm}) cells in particular tend to use more exogenous lipid import rather then *de novo* synthesis. Newer findings suggested that FAO is not critical for memory CD8 T cell formation directly, but it is still within reason to assume that lipid metabolites are critical for biosynthetic precursor generation and regulation of FAS. Without MFSD2A and exogenous LPC import, I proposed there will be a catastrophic failure within CD8 T cells early post-activation due to relatively high MFSD2A expression early on in the effector response, but also that MFSD2A was important for memory CD8 T cell formation and

maintenance well after initial antigen encounter. This hypothesis was further tested by being broken down into two broad specific aims, with multiple sub aims to be discussed for each:

1.7.1 SPECIFIC AIM 1

Specific Aim 1 explored a role for MFSD2A in the activation and differentiation of effector and memory CD8 T cells. This is the more phenotypically descriptive of the two aims of this project. This aim looked to answer the questions of what happens to CD8 T cells over the course of an infection without MFSD2A. This was tested primarily by using an adoptive transfer and listeria-OVA model to investigate effector and memory CD8 T cells with conditional MSFD2A deletion.

1.7.1.1 SPECIFIC AIM 1.1

Specific Aim 1.1 investigated if MFSD2A is required for the effector CD8 T cell response to infection. Before diving in, MFSD2A$^{-/-}$ mice were tested to confirm there was no developmental defect in all T cell subsets. Then, mice were sacrificed at an early infection time point (d4) to measure bacteria clearance, with the idea being that MFSD2A$^{-/-}$ mice may have a delayed clearance since MFSD2A is expressed early. Next, mice were sacrificed at peak expansion and early contraction phases to examine phenotypic CD8 T cell activation marker surface expression as well as inflammatory cytokine production. Since MFSD2A does seem to be elevated early on in T cell activation, I hypothesized that there will be an effect on cytokine production during these early time points.

1.7.1.2 SPECIFIC AIM 1.2

Specific Aim 1.2 investigated if MFSD2A was required for memory CD8 T cell maintenance. In this aim, mice were taken out to d40 post-infection (pi). Cell frequency and phenotype were both analyzed similarly to Aim 1.1. I anticipated that by the time MFSD2A$^{-/-}$ mice reached memory, that I would see critical defects in their memory CD8 T cells, whether it be the relative amount of cells turning over to memory cells or as seen by having decreased fitness relative to MFSD2A$^{+/+}$ mice. I hypothesized that a significantly reduced import of exogenous LPC would alter normal CD8 T cell metabolism and would be responsible for any phenotype seen here. Biochemical LPC levels were further investigated in Aim 2.

1.7.1.3 SPECIFIC AIM 1.3

Specific Aim 1.3 asked if MFSD2A is required for a successful CD8 T cell recall response to recurrent listeria infection. Since I hypothesized that MFSD2A will be critical for memory CD8 T cell formation, I rationalized that without memory CD8 T cells, I would see a futile to none recall response upon secondary infection with listeria. I planned to measure cytokine production in whatever memory cells were available at this time and assessed functionality of recalled memory cells that lack MFSD2A.

1.7.2 SPECIFIC AIM 2

The overall goal of Specific Aim 2 was to determine the metabolic and genetic requirements for exogenous LPCs during CD8 T cell activation and memory cell maintenance. Aim 2 explored what consequences cells were faced with when MFSD2A was lost. It was the more mechanistic of the two aims. Aim 2 was accomplished by using a multi-disciplinary approach, including lipidomics, proteomics, metabolic flux assays, and genetic approaches. There was evidence that exogenous lipids are important for FAO and also contradictory reports that they were not. Due to these recent reports, I hypothesized that loss of MFSD2A may not directly affect FAO processes directly linked to memory cell metabolism and formation, but rather that decreased LPC import early on may result in memory CD8 T cells not banking enough nutrients to sustain their longevity and that this may be held responsible for some of the downstream phenotypes that were seen. Three sub aims were used to answer the questions in Aim 2.

1.7.2.1 SPECIFIC AIM 2.1

Specific Aim 2.1 looked to see if there was decreased exogenous LPC import with loss of MFSD2A. Since MFSD2A was a known transporter for LPC species, I hypothesized that there was a significant decrease in the amount of LPC being imported into CD8 T cells. I investigated LPC import multiple ways, including flow cytometry, thin layer chromatography (TLC), confocal live-cell imaging, and lipidomic mass spec approaches.

1.7.2.2 SPECIFIC AIM 2.2

The goal of Specific Aim 2.2 was to determine what were the metabolic outcomes of uptake of LPC through MFSD2A by effector and memory CD8 T cells. This was performed using a Seahorse Metabolic Flux analyzer to profile both the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR). I anticipated MFSD2A$^{-/-}$ mice would show altered cellular metabolism both at the effector and memory stages. These data showed how loss of LPC import by LPC altered the overall cellular metabolism and how this may be responsible for differences in effector and memory CD8 T cells in the absence of MFSD2A. Since different CD8 T cell subsets have different metabolic profiles, it was possible that with loss of MFSD2A (i.e., being somewhat metabolically deficient) the OCR and ECAR profiles in MFSD2A$^{-/-}$ may differ from what was expected in an MFSD2A$^{+/+}$ mouse.

1.7.2.3 SPECIFIC AIM 2.3

Specific Aim 2.3 investigated the genetic consequences of loss of MFSD2A. Here, I was looking to prove the role of MFSD2A and LPC in genetic regulation in effector and memory CD8 T cells. I wanted to discover what, if any, genes may be expressed differentially with loss of MFSD2A. This included phenotypic CD8 T cell genes as well as genes related to the cell's metabolome including metabolic enzymes, substrates, and pathways. I tried to answer these questions using a mixture of approaches, including qPCR and RNA-sequencing. Overall, I hypothesized that there were differentially expressed genes in the absence of MFSD2A and that these altered transcripts helped to explain why loss of MFSD2A was detrimental to cell health.

The overall hypothesis of this project was summarized in the figure below (Fig. 9). I anticipated based on work by others at the BBB and my own preliminary data that MFSD2A and LPC played a similar role in effector and memory CD8 T cells to what occured in the BBB. After T cell activation, MFSD2A was most highly expressed. I expected MFSD2A to play a role in normal effector function in CD8 T cells. Because memory CD8 T cells were reliant on a bank of metabolites that may be determined early on in the effector phase post-infection, I also expected that memory cell formation and maintenance would be lost with loss of MFSD2A. Overall, I hoped to prove via accomplishing the above Specific Aims that loss of MFSD2A was severely detrimental to both the effector and the memory CD8 T phenotype.

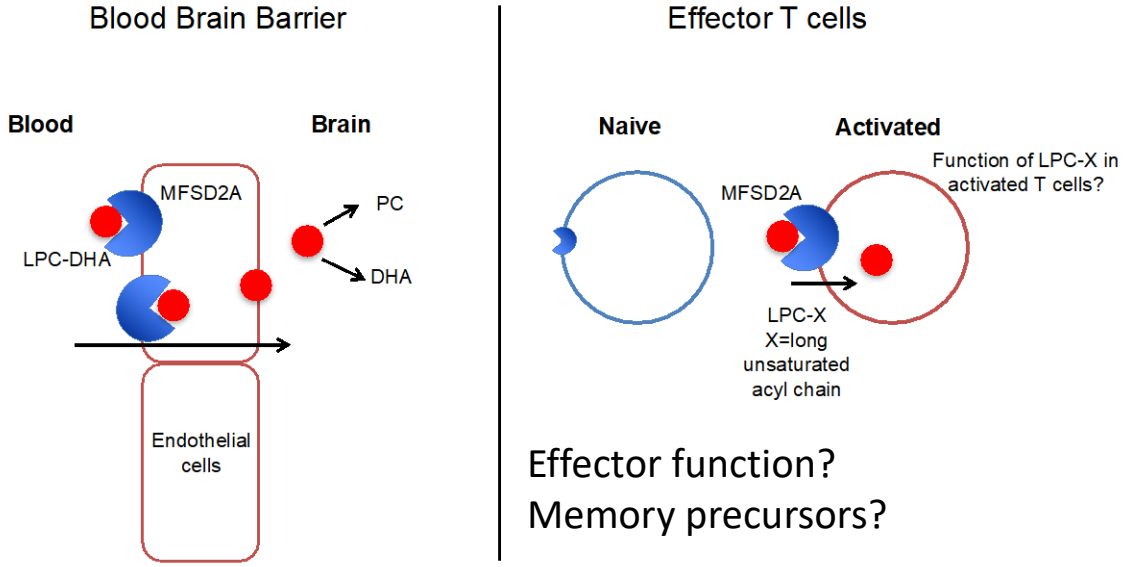


Figure 9. Proposed role of MFSD2A and LPC in activated CD8 T cells

Others have shown a role for MFSD2A and LPC at the BBB (left hand). MFSD2A enables LPC-DHA to cross the BBB endothelium into the brain, where LPC-DHA dissociates into PC and DHA. I hypothesize a similar role for MFSD2A in activated CD8 T cells where MFSD2A transports LPC species across the cell membrane to enter the cell's metabolome. LPC species then play a role in CD8 T cell effector function and memory cell formation and maintenance.

2.0 MATERIALS AND METHODS

2.1 MFSD2A CONDITIONAL KO MOUSE

MFSD2A Flox mice were kindly donated by Dr. David Silver. Loxp sites were inserted above critical exon 3 on chromosome 4. Mice were "conditional ready" (floxed) upon arrival. CD4 cre mice were purchased from the Jackson Laboratory. These mice contain CD4 enhancer, promoter, and silencer sequences driving the expression of cre recombinase. When bred to conditional ready MFSD2A flox mice, cre-mediated recombination will result in deletion of MFSD2A on all T cells during the early double positive stage of thymic development, i.e., MFSD2A will be knocked out of all CD4 and CD8 T cells.

Additionally, most *in vivo* work was performed using conditional MFSD2A knockout mouse that also has a transgenic Vα2 Vβ5 TCR, deemed OT-I mice. The purpose for such mice is listed in the background. Briefly, the use of this transgene serves as a competent model to track antigen-specific CD8 T cell response to infection. Wildtype OT-I mice were bred to MFSD2A conditional knockout mice and all lines were backcrossed to C57BL/6J stain for multiple generations, including a continuation of in-house backcrossing to eliminate any concern for rejection. CD4 cre only littermates were used as controls. $MFSD2A^{-/-}$ mice were phenotyped in the naïve state to show there were no developmental defects (Figs. 10 and 11). Genotyping

primers can be found below in Appendix B. A diagram of mouse models being used can be found in Appendix D.

2.2 LYMPHOCYTE ISOLATION FROM TISSUE

Mice were sacrificed at a given experimental time point according to the University of Pittsburgh's IUCAC protocol. Spleens and thymi were extracted into cold PBS 2% FCS (hereafter referred to as FACS buffer), red cell lysed, and isolated into a single cell suspension prior to counting. Lymphocytes were counted using standard light microscopy/Trypan blue or by using Countess automated cell counter (Life Technologies).

Peripheral blood lymphocytes (PBL) were collected from the submandibular vein via lancet (Goldenrod) into tubes containing 500 μL PBS with 0.5% EDTA. Blood was spun down prior to red cell lysis and staining for surface antigens.

2.3 *IN VITRO* CD8 T CELL ACTIVATION

Lymphocytes were isolated as shown above. CD8 T cells were enriched from total lymphocytes by using negative selection enrichment. Whole lymphocyte suspension was incubated with biotinalyated antibodies to CD4, CD11c, CD11b, B220, and NK1.1 (all eBioscience) and then incubated with streptavidin microbeads (Milteny) followed by negative selection via column prep (Milteny). CD8 T cells were then counted and plated at $2x10^6$ cells/well in complete T cell media (RPMI, 10% FBS, 2% of each l-glutamine and pen-strep, and

0.5% β-mercaptoethanol). Prior to plating, a 24 well plate was coated in anti-CD3 (eBioscience) for a minimum of 2 hours at 37C. Cells were cultured in wells coated with plate bound anti-CD3 and co-cultured with soluble anti-CD28 (eBioscience) to a given time point. Naïve controls were cultured with 1 ng/mL IL-7 to promote viability.

For *ex vivo* OT-I CD8 T cells, whole lymphocyte suspension was plated with soluble OVA peptide. 500x protein transport inhibitor cocktail (eBioscience) was added the last 3 hours of a 6 hour peptide stim.

2.4 ADOPTIVE TRANSFERS AND *IN VIVO* INFECTIONS

$1x10^4$ transgenic CD45.1.2 MFSD2A$^{+/+}$ OT-I and CD45.2 MFSD2A$^{-/-}$ OT-I CD8 T cells were adoptively transferred retrorbitally into congenically labeled CD45.1 C57BL/6J mice. The 50:50 ratio was verified by flow cytometry prior to injection. Mice were sex matched per each experiment to remove any cell rejection due to the Y chromosome. The following day, mice were injected retrorbitally with 5000 CFU *listeria*-OVA. Listeria was grown in tryptic soy broth until reaching an OD_{600} between 0.11-0.09 which is considered to be late-log phase growth. Mice were bled from the submandibular vein at indicated time points into PBS 0.5% EDTA. Spleens were harvested at indicated time points for downstream analysis. Time points correlated to different CD8 T cell phenotypes as seen in Figure 1. A diagram of the overall model is in Appendix D.

2.5 MIXED BONE MARROW CHIMERA GENERATION

CD45.1 mice recipient mice were irradiated with 1000 rads to destroy rapidly dividing (immune) cells one day prior to chimera transplantation. Mice received clean cages, fresh food, and bottled water containing antibiotics sulfamethoxadole and trimethoprim at 2 mg/mL every 2-3 days for the first two weeks following irradiation.

CD45.1.2 MFSD2A$^{+/+}$ and CD45.2 MFSD2A$^{-/-}$ sex matched donor mice were sacrificed the following day and hind limbs collected (femur, tibia, fibula). Muscle and connective tissue was cleaned from bones and bone marrow was isolated in RPMI 20% FCS using 28 gauge needles. Bone marrow was collected, red cell lysed, and counted prior to depletion of all B, T, and NK cells via biotinylated antibodies and streptavidin microbeads (eBioscience and Miltenyi). Bone marrow as recounted following depletion. Bone marrow was injected into irradiated CD45.1 recipient mice retroribitally in sterile PBS in a 50:50 ratio of $5x10^6$ total cells/mouse.

2.6 BACTERIAL CLEARANCE STUDIES

Mice were directly infected with listeria and sacrificed d4 pi. Spleens and livers were harvested for whole tissue homogenate. Whole tissue was diluted 1:10 in sterile PBS and then pipetted onto pre-warmed tryptic soy agar plates in a volume of 200 μl in duplicate. Homogenate was spread evenly across plates using disposable cell spreaders (Fisher). Plates were incubated at 37C 5% CO^2 for 24-36 hours before colonies were counted.

2.7 FLOW CYTOMETRY AND CELL SORTING PROTOCOL

For general flow cytometry, lymphocyte single cell suspensions were aliquoted to 96 well plates, stained with live/dead dye in PBS at RT, washed, then followed by Fc block (BioLegend). Cells were next stained for surface antigens at a 1:200 dilution in FACS buffer at 4C. For intracellular staining, cells were fixed and permeabilized using Cytofix/Cytoperm (BD Biosciences) per manufacturer's instructions. Cells were stained for cytokines at 1:200 dilution in 1x Cytofix/Cytoperm buffer for 30 minutes at 37C prior to being ran on the cytometer. A list of all antibodies used for this project can be found in Appendix C.

For cell sorting, similar protocols to general cell staining were followed, with the exception being that whole spleens were stained in 1 mL PBS or FACS buffer.

2.8 ANNEXIN V AND BRDU STAINING

Annexin V and caspase 3&7 staining was performed according to manufacturers' protocols (BD Biosciences and Thermo Fisher Scientific). Annexin V samples were ran on the cytometer within a half hour of staining.

Mice received BrdU (Millipore Sigma) injections IP at 2 mg/mL on the first day of treatment. They then received bottled BrdU H2O at 200mg/mL plus 1.5% sucralose for 2 weeks with fresh bottles being given every 2-3 days. Water bottles were protected from light. BrdU is considered a chemical hazard and proper labeling and disposal was given in accordance to University of Pittsburgh's Environmental Health and Safety. Mice were given an additional IP injection of BrdU the evening prior to sacrifice. Mice were sacrificed the morning of treatment

d15, surface stained according to previously described flow cytometry protocols, and then stained for BrdU incorporation following the kit's manufacturer's protocol (BD Biosciences).

2.9 REAL TIME PCR

Cells were lysed and RNA was processed using RNeasy micro PLUS kit (Qiagen). cDNA was isolated using a traditional first-strand synthesis kit (Genecopeoia). qPCR was performed on an Applied Biosystems StepOne real-time PCR machine using either SYBR green assays or TaqMan probes. Primer sequences and probe ID can be found in Appendix B.

2.10 WESTERN BLOTTING

For MFSD2A and H3K27ac westerns, enriched or sorted cells were prepped under non-denaturing and non-reducing conditions using RIPA buffer plus protease inhibitors (Roche) with head-over-tail rotation at 4C for 1 hour. Supernatant of MFSD2A micelles was collected following a 10 minute spin at 4C, max speed. Protein concentration of lysed samples was determined using BCA assay (Thermo) and then samples were ran on a 5-20% gradient gel (Bio-Rad) and transferred onto PVDF membrane using a semi-dry Power Blotter (Thermo). Primary antibody for MFSD2A was purchased by Abcam and verified by a homemade antibody kindly donated by Dr. David Silver. Membranes were visualized on Protein Simple FlouroChem machine (Biotechne). Quantification studies were performed using ImageJ software (NIH). A list of all antibodies used can be found in Appendix C.

2.11 GLOBAL LIPIDOMICS

Lipid extraction from blind in vitro activated or naïve MFSD2A$^{+/+}$ and $^{-/-}$ CD8 T homogenates was done according to the Bligh and Dyer method. The organic phases were pooled together and dried in a speed vac. Samples were dissolved in 100 µL of $CHCl_3$/MeOH 1:1 (v/v) prior to MS analysis. Samples were injected into a liquid chromatography-tandem mass spectrometry (LC-MS/MS) instrument (1290 Liquid Chromatography System, and 6460 QqQ, Agilent Technologies). Quality controls and blanks were injected after every 6 sample injections to monitor stability of the instrument response and carryover. Phospholipids were quantified at the sum composition level using multiple reaction monitoring (MRM) with precursor to headgroup transitions. Quantification data were extracted using MassHunter Quantitative Analysis (QQQ) software, and data were manually curated to ensure correct peak integration. T cell samples were generated and then shipped to the lab of Dr. David Silver at Duke-NUS, Singapore where lipidomic profiling was ran and analyzed by Bernice Wong, Juat Chin Foo, Amaury Cazenave-Gassiot, and Markus Wenk.

2.12 CONFOCAL LIVE-CELL IMAGING

CD8 T cells were activated in vitro and cultured with TF-LPC as described above. Prior to imaging, cells were stained with Hoechst at a 1:1000 dilution at 4C. Stain was rinsed 2x with PBS prior to microscopy. Samples were imaged using a Nikon A1 point scanning confocal with a 60x 1.40 N.A. objective and Tokai Hit environmental controller. Complete volumes of cells were acquired at 1 um steps and volumes were reconstructed and analyzed using Nikon's NIS

Elements software. To define perinuclear space a threshold was established using the nuclear signal labeled with Hoechst fluorescent nuclear marker. The nuclear threshold was then dilated and the original nuclear threshold was subtracted from the dilated mask leaving behind a donut shaped region corresponding to the immediate perinuclear space in the cell, under-which intensity measurements were performed. Microscopy experiments were performed in partnership with the University of Pittsburgh Center for Biologic Imaging (CBI) with Callen Wallace under the guidance of Dr. Simon Watkins. Callen Wallace and Eric Hzyny assisted with data analysis.

2.13 SEAHORSE METABOLIC FLUX

CD8 T cells were sorted at a given time point like previously described. Cells were taken over to Hillman Cancer Center where they were plated at a determined density into a 96 well plate. The Seahorse MitoStress test was performed on these samples. Seahorse experiments were performed in partnership with Ashley Menk in the lab of Dr. Greg Delgoffe.

2.14 RNA SEQUENCING

MFSD2A$^{+/+}$ and $^{-/-}$ CD8 T cells were sorted from competitive adoptive transfer mice at d40 p.i. into RLT PLUS lysis buffer (QIAGEN). Samples were delivered to the Sequencing Core at Children's Hospital of UPMC. RNA was isolated and quantified via ScreenTape (Agilent). Library prep was performed prior mid-output single-end read 75bp mRNA-sequencing with 40M

reads/sample. Data analysis was performed using CLC Genomics Workbench 11 (QIAGEN), Partek Genomics Suite (Partek, Inc.), and IPA pathway analysis (QIAGEN).

2.15 HUMAN SAMPLE COLLECTION

Human buffy coats were purchased de-identified from Central Blood Bank. Lymphocytes were isolated from PBL by using a Ficoll gradient (GE Healthcare) and then sorted for whole CD8 T cells or naïve, effector, and memory CD8 T cells prior to *in vitro* stimulation using human αCD3 and αCD28 (eBioscience). Cells were then used for downstream molecular applications.

2.16 CHROMATIN ACCESSIBILITY ASSAY

Chromatin accessibility was assayed by using the EpiQuik^{TM} Chromatin Accessibility Assay Kit (Epigentek). MFSD2A$^{+/+}$ and MFSD2A$^{-/-}$ effector CD8 T cells were sorted at d7 pi as described. Sorted cells were lysed prior to chromatin extraction. Chromatin was either digested or saved as an undigested control prior to DNA clean-up and downstream qPCR analysis using designed qPCR genomic primers for the IFNγ locus. Fold enrichment was calculated by taking the ratio of the Ct values between digested and undigested chromatin samples.

3.0 SPECIFIC AIM I RESULTS

3.1 MFSD2A$^{-/-}$ MOUSE DEVELOPMENTAL PHENOTYPING

MFSD2A conditional knockout mouse was generated as described above. To verify no developmental defects with loss of MFSD2A, I first phenotyped naïve T cells. Naïve phenotyping studies were performed in thymic and peripheral tissue (spleen) in 8 week old mice and analyzed relative to littermate controls. Prior to phenotyping, CD8 enriched splenocytes were activated *in vitro* with anti-CD3 and anti-CD28 for 72 hrs and analyzed by qPCR to confirm conditional deletion of MFSD2A was successful (Figure 10A). These data were further supported by flow cytometry analysis, showing that there is reduced MFSD2A protein expression by FACS (Figure 10B).

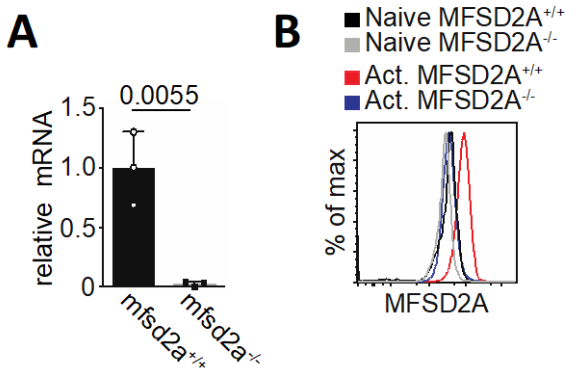


Figure 10. MFSD2A conditional knockout confirmation

Real-time PCR (A) and flow cytometry analysis (B) was performed on 72 hr *in vitro* activated CD8 T cells. CD8 T cells were activated using anti-CD3 and anti-CD28. Data are representative of 2 independent experiments with at least 3 mice per group.

Next, naïve thymocytes were analyzed by flow cytometry. As mentioned above, MFSD2A was conditionally knocked out during the double positive (DP) stage of thymic development, so it was important to verify that cells were undergoing normal thymic development. Mice were analyzed for the ratio of CD4:CD8 positive cells (Figure 11A) as well as total thymocyte cell counts (Figure 11A quantification). No differences were detected in these data. To further confirm, DP and CD4 and CD8 single positive (SP) cells were also quantified to no significant differences (Figure 11B). Thymocytes were critically analyzed for surface markers related to thymocyte development through the double negative (DN), DP, and SP stages and no differences were found (data not shown). Overall, these finding support that there was no significant differences in T cell development due to loss of MFSD2A and that it was okay to move forward with this mouse model.

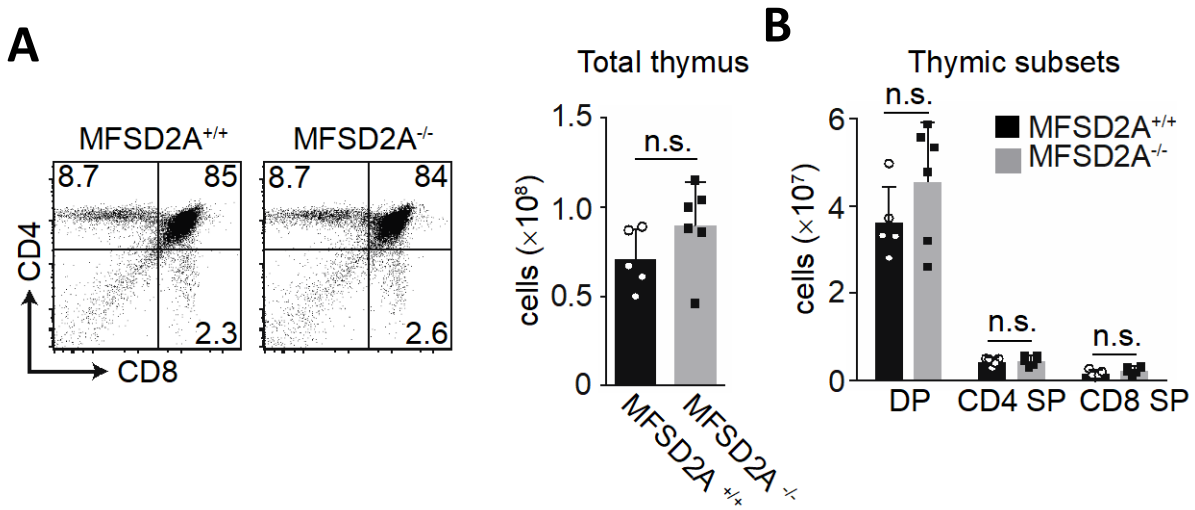


Figure 11. MFSD2A loss does not alter thymic development

Thymocytes were isolated into a single cell suspension and stained for phenotype distinguishing surface markers prior to being ran on a flow cytometer. A. CD4:CD8 staining and quantification of total cells found within the

thymus suspension. B. Further quantification of thymic subsets with and without MFSD2A. Data are representative of 3 independent experiments with at least 3 mice per group.

T cell phenotyping was performed in the periphery to confirm that cells migrated out of the thymus to secondary lymphoid tissue as expected. No noticeable differences were detected in the number of CD4 or CD8 positive splenocytes (Figure 12A) however there was a significant difference in the number of total splenocytes. Spleens from MFSD2A$^{-/-}$ mice appeared larger, however they also appeared to have a normal range of the most common splenic cell subsets including T cells, B cells, NK cells, dendritic cells, and macrophages (data not shown). Why MFSD2A$^{-/-}$ mice had larger spleens with more total cells has yet to be determined and may be further explored in the future. Finally, since T activation studies were planned for future experiments, we wanted to confirm that MFSD2A$^{-/-}$ mice had comparable numbers of CD44^{hi} cells in the naïve state relative to MFSD2A$^{+/+}$ mice (Figure 12C). No significant differences were found between naïve mice. CCR7 levels were also within normal range, indicating no migratory problems with loss of MFSD2A (data not shown).

I originally became interested in MFSD2A because it was found to be one of the most differentially expressed genes in a microarray of NKT cells performed previously by the lab. Because of these findings, I wanted to confirm that splenic NKT cell populations were found within the expected frequency following loss of MFSD2A. Indeed, no differences in NKT cell frequency were determined (Fig. 12B). I was able to conclude that conditional loss of MFSD2A did not affect T cell development in the thymus or migration into secondary lymphoid tissues. Although differences in total splenic cell counts were noted, these data were conclusive enough for us to justify moving on to further phenotype MFSD2A$^{-/-}$ CD8 T cells in the effector and memory states.

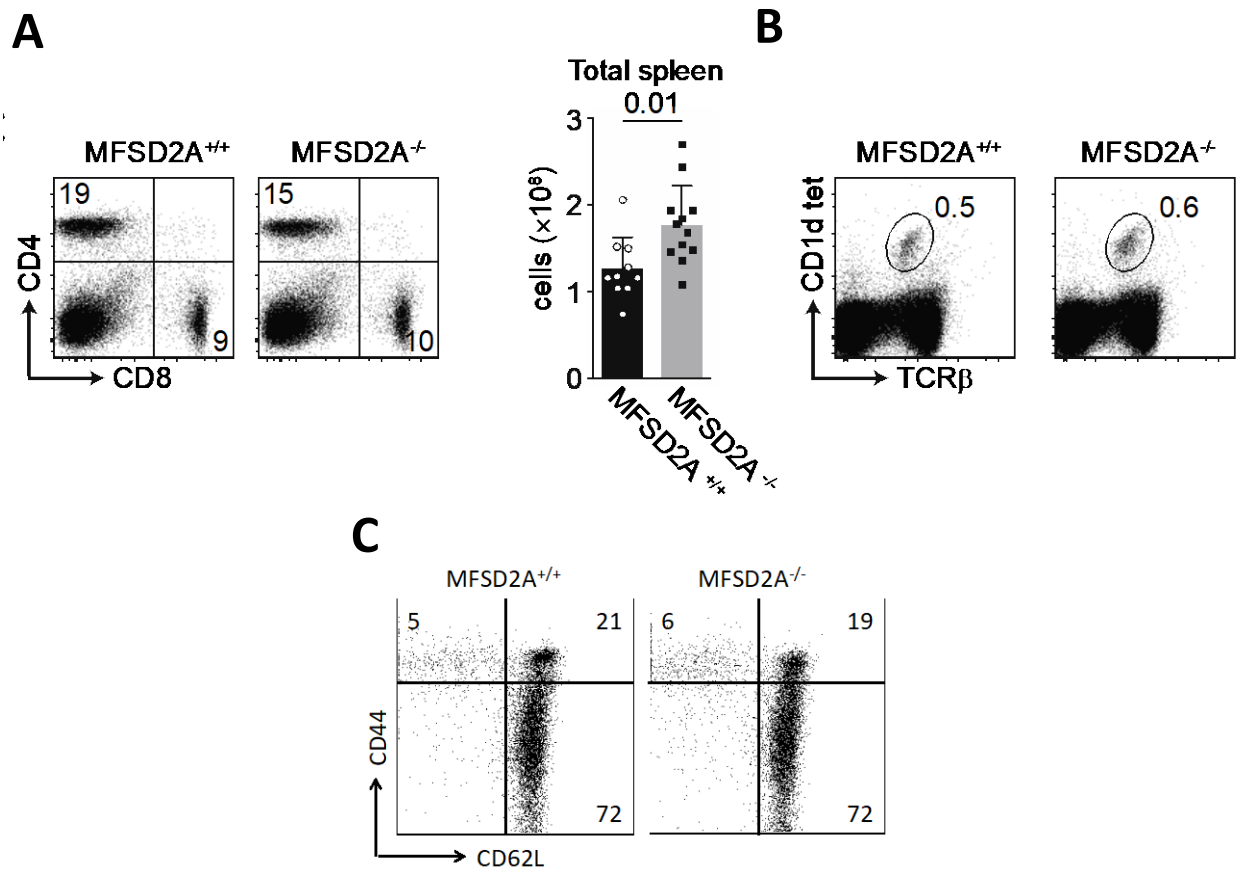


Figure 12. MFSD2A loss does not alter migration to peripheral lymphoid tissue

Naïve spleens were harvested from MFSD2A-/- mice or littermate controls and stained using surface marker antibodies and/or tetramer prior to running samples on a flow cytometer. A. CD4:CD8 T cell frequencies in spleen and quantification of total splenic cells. B. Frequency of NKT cells. C. Measurement of CD44 and CD62L activation markers, backgated on CD8 T cells. Data are representative of 3 independent experiments with at least 3 mice per group with statistical calculations made using student's t-test.

3.2 MFSD2A$^{-/-}$ EFFECTOR CD8 T CELL RESPONSE

After confirming there were no developmental defects accompanied with loss of MFSD2A, I next tested how MFSD2A deficit CD8 T cells would respond to acute infection with *Listeria*-OVA. I accomplished this by directly infecting MFSD2A$^{-/-}$ and littermate control mice retro-orbitally with 5000 CFUs of *Listeria*-OVA. First, I determined if MFSD2A deficient mice were able to clear bacteria as quickly as wild type (WT) mice. Mice were sacrificed d4 pi and whole tissue homogenates were made of spleen and liver prior to plating on tryptic soy agar plates. Colonies formed as early as 24 hrs after incubation, however there was no significant differences in CFUs between MFSD2A$^{+/+}$ and MFSD2A$^{-/-}$ mice in both liver and spleen (Figure 13), which led to the conclusion that MFSD2A deficient mice were not impaired with bacterial clearance. In agreement with this, mice directly infected with *Listeria*-OVA appeared to not have any striking phenotypic differences in their immune responses either(Supplemental Figure 2), so it was decided to try a competitive model to examine of MFSD2A$^{-/-}$ CD8 T cells were less fit in a competitive environment.

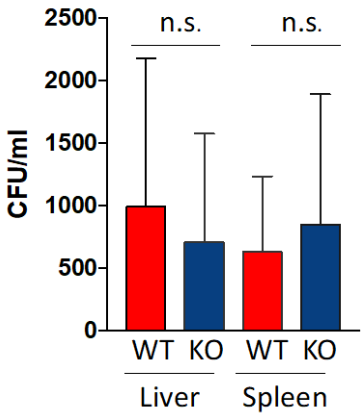


Figure 13. No difference in bacterial clearance with loss of MFSD2A

Quantification of colony forming units (CFU/mL) day 4 post-infection with Lm-OVA in spleen and liver, with and without MFSD2A. Data are representative of 2 independent experiments with at least 3 mice per group. Statistical significance calculated using Student's t-test.

For future experiments, I used a competitive adoptive transfer approach where recipient CD45.1 mice received both sex-matched MFSD2A$^{+/+}$ CD45.1.2 and MFSD2A$^{-/-}$ CD45.2 donor OT-I CD8 T cells. Cells were transferred retro-orbitally at 1:1 ratio of 10,000 cells each (see Appendix D). Mice received 5000 CFUs of lm-OVA retro-orbitally the following day. Mice then undergo a typical acute CD8 T cell immune response following infection which can be tracked by examining the response via the donor OT-I CD8 T cells.

Early post-infection (d5) until d10, there was a slight skewing from the original 1:1 ratio to a 3:2 ratio in favor of the knockout, however by d13 (early contraction) this trend has disappeared (Figure 14A and B). These data were statistically significant at d7 and d10 pi, however, it was hypothesized that these differences are physiologically irrelevant in relation to the overall infection response due to the downstream analysis, to be discussed. When the frequency of CD44^{hi} CD62L^{hi} MPEC cells is examined, there was a trend in reduction of MFSD2A$^{-/-}$ cells as early as d7 pi (data not shown). This discrepancy continued out to d10 and suggested that MFSD2A was important in MPEC and central memory cell formation. These data

were supported by a decrease in the KLRG1^{lo} CD127^{hi} cells by d10, also suggestive of a defect in MPEC generation (Supplemental Figure 1).

To measure effector function, I performed an *ex vivo* stimulation at d7 and d10 pi using OVA peptide. For these experiments, CD8 T cells were unenriched and stimulated with OVA peptide alone for an indicated amount of time. By d10 pi, MFSD2A$^{-/-}$ cells were producing less inflammatory cytokines IFNγ and TNFα (Fig. 14C), suggesting that loss of MFSD2A affects cytokine production as early as d10 post-infection.

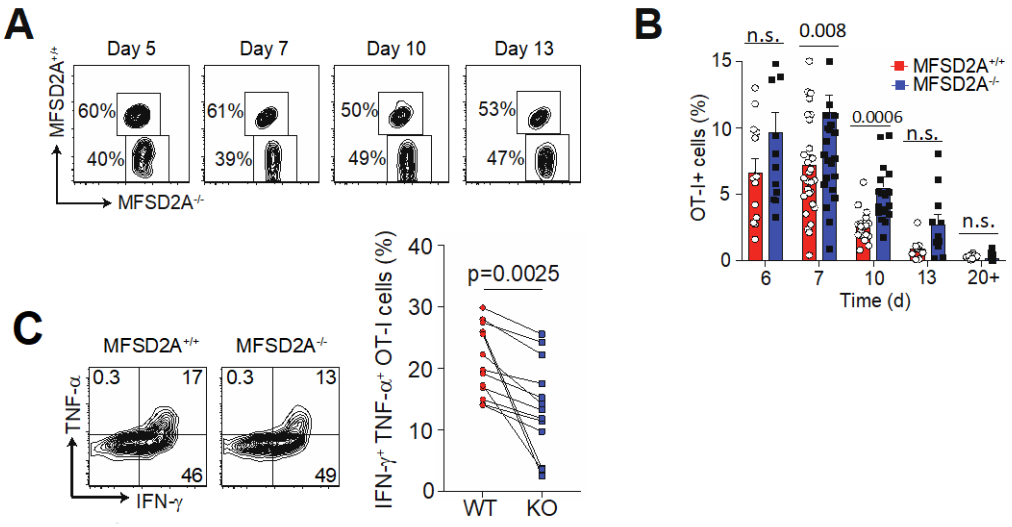


Figure 14. MFSD2A$^{-/-}$ primary effector response following infection

Mice received equal amounts of MFSD2A+/+ and MFSD2A-/- OT-I CD8 T cells prior to infection with 5000 CFUs lm-OVA. A. Time course of effector response showing frequency of MFSD2A$^{+/+}$ and MFSD2A$^{-/-}$ cells at indicated time point. B. Graphical representation of frequency of MFSD2A$^{+/+}$ and MFSD2A$^{-/-}$ cells at indicated effector time point. C. Intracellular staining of inflammatory cytokine production at d10 pi following a 6 hour stim with OVAp with golgi stop the last 3 hours. A and B are from bleeds, C and D are splenic data. Data are representative of 3 independent experiments with at least 3 mice per group with p-values calculated using student's t test or using one way ANOVA adjusting for multiple comparisons.

Next, to confirm if the above findings were cell extrinsic or cell intrinsic, I generated mixed bone marrow chimeras and performed similar effector experiments to those performed using the competitive adoptive transfer approach. Mice showed a similar ratio of cells during the primary expansion phase, including OVA tetramer specific CD8 T cells (Figure 15A and B). However, when stimulated with OVAp, I saw that indeed those cells lacking MFSD2A were poorer at secreting inflammatory cytokines (Figure 15C). These data matched what was found in the mixed adoptive transfer system, suggesting that the MFSD2A$^{-/-}$ CD8 T cells were responsible for the effector phenotype seen here in a cell intrinsic manor.

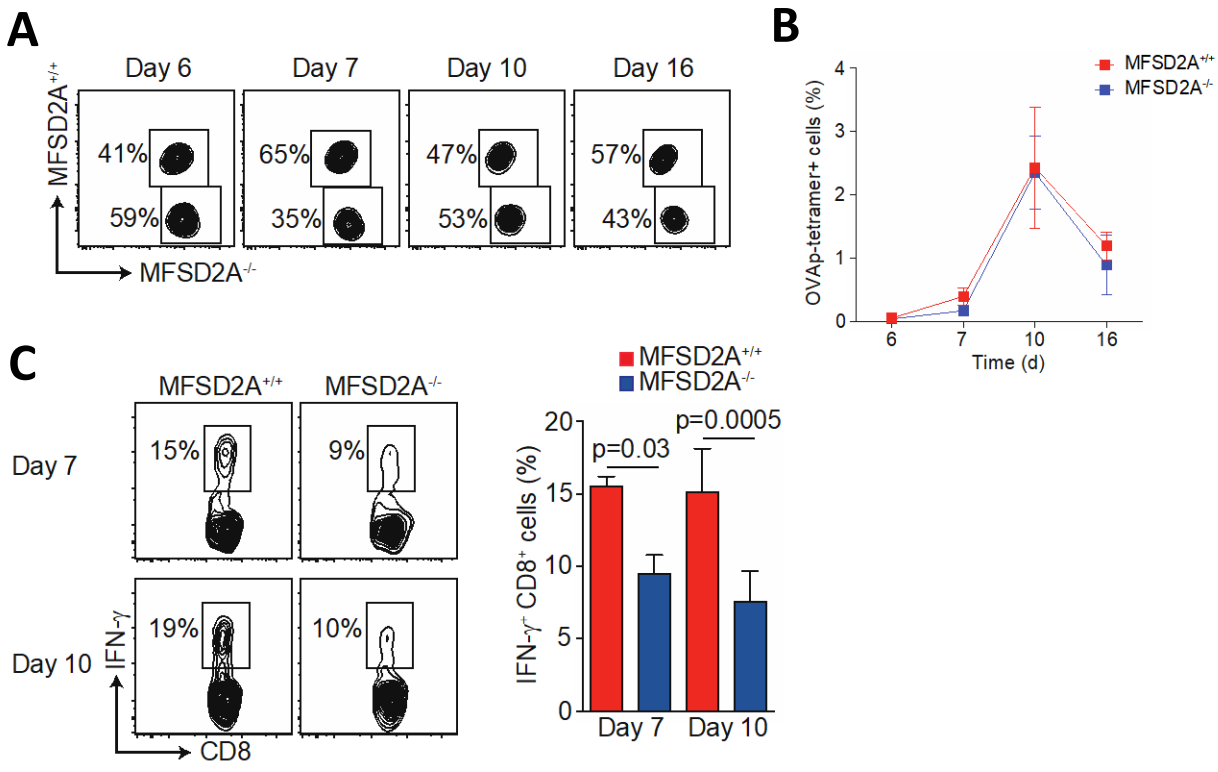


Figure 15. MFSD2A$^{-/-}$ mixed chimera primary effector response following infection

Irradiated CD45.1 host mice received equal amounts of MFSD2A$^{+/+}$ CD45.1.2 and MFSD2A$^{-/-}$ CD45.2 mixed bone marrow cells and were left 8 weeks to recover. Mice were then infected with 5000 CFU lm-OVA. The primary expansion of CD8+ CD44^{hi} cells and OVA-tetramer+ cells was measured at indicated effector and contraction time points (A and B). C. FACS plots and quantification of mice were sacrificed at given time and stimulated ex vivo with OVAp for 6 hrs followed by 3 hrs of golgi stop prior to intracellular IFNγ staining. A and B are from bleeds, C and D are splenic data. Data are representative of 3 independent experiments with at least 3 mice per group with p-values calculated using student's t test or using one way ANOVA adjusting for multiple comparisons.

As a control to confirm that the phenotype was not due to OVA antigen alone, I infected a separate group of mixed-bone marrow chimera mice with *listeria* attached to GP33 antigen. Although a more difficult model to work with due to poor GP33 tetramer-specific FACs staining, cytokine production data as seen in Fig. 15C using listeria-OVA successfully repeated using listeria-GP33 antigen and gating on the $CD44^{hi}$ cells rather than the tetramer positive population (Fig. 16).

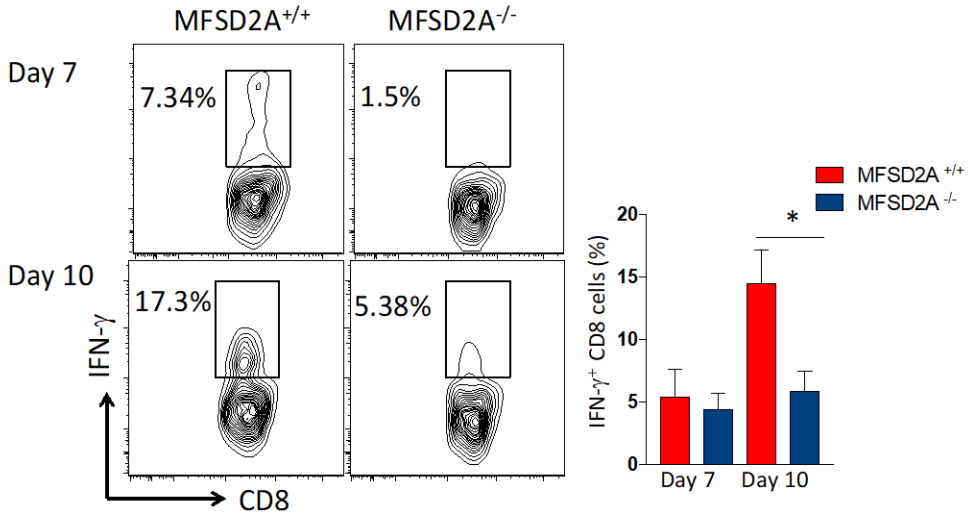


Figure 16. MFSD2A$^{-/-}$ decrease in cytokine production is not restricted to OVA antigen

Irradiated CD45.1 host mice received equal amounts of MFSD2A$^{+/+}$ CD45.1.2 and MFSD2A$^{-/-}$ CD45.2 mixed bone marrow cells and were left 8 weeks to recover. Mice were then infected with 5000 CFU lm-GP33. FACS plots and quantification of splenocytes that were sacrificed at given time pi and stimulated ex vivo with GP33p for 6 hrs followed by 3 hrs of golgi stop prior to intracellular IFNγ staining. Data are representative of 1 independent experiment with at least 3 mice per group with p-values calculated using student's t test.

3.2.1 MFSD2A IN HUMAN EFFECTOR CD8 T CELLS

Because MFSD2A is conserved through evolution and there are known families with MFSD2A mutations, I found it of interest to look at healthy human donor blood for MFSD2A+

CD8 T cells. Buffy coats of human peripheral blood lymphocytes (PBL) were received de-identified from Central Blood Bank and primary lymphocytes were isolated using a Ficoll gradient. Cells were sorted based on distinguishable surface marker expression for naïve and effector CD8 T cells. Sorted cells were processed for qPCR and Western Blot analysis. I found that *MFSD2A* expression was double in human effector CD8 cells compared to naïve (Figure 17A) and that this is also true when looking about protein levels (Figure 17B). Although human studies were not developed for this project past this preliminary work, it may be worth pursuing in the future along with the other future directions that follow.

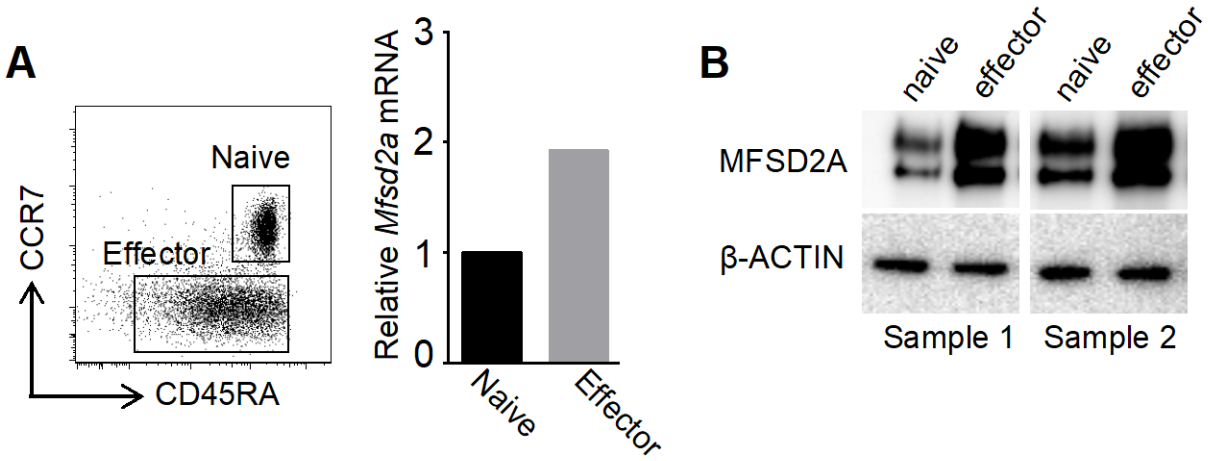


Figure 17. MFSD2A levels in human PBL samples

Human PBL was sorted for naïve and effector CD8 T cells and then analyzed for MFSD2A levels. A. Flow cytometry plot of human PBL sorting schematic and quantification of relative MFSD2A expression following real-time PCR. B. Western Blot image for MFSD2A protein levels in human naïve and effector CD8 T cells with β-actin loading control. Data is representative of 2 unique experiments from 2 sorted human donors (A, left) where 1 donor was used for mRNA (A, right) and 2 were used for western blotting (B).

3.3 MFSD2A$^{-/-}$ MEMORY CD8 T CELL RESPONSE

I wanted to determine if there was a role for MFSD2A in memory CD8 T cell formation. Due to the possibility that memory CD8 T cells use FAO and their reliance on metabolities from the primary response, I hypothesized that those CD8 T cell that were lacking MFSD2A would be critically impaired in generating a robust and functional memory pool compared to wild type. Competitive adoptive transfer mice were taken out to what is considered in the field to be an acceptable memory CD8 T cell time point, d40 pi. Indeed, by d40 pi, there was a highly significant reduction in the memory CD8 T cell pool compared to MFSD2A$^{+/+}$ mice (Figure 18A). These data also repeat when looking at mixed bone marrow chimera mice taken out to memory (Supplemental Figure 3). In addition to their decreased frequency, MFSD2A$^{-/-}$ mice showed an altered surface marker phenotype (Figure 18 B and C). Central memory CD8 T cells are typically CD44^{hi}CD62L^{hi}KLRG1^{lo}CD127^{hi}. In the case of the MFSD2A$^{-/-}$ mice, their surface marker profile at memory more closely resembled effector memory CD8 T cells as they were CD44^{hi}CD62L^{lo}KLRG1^{hi}CD127^{lo}. This effector-like phenotype was not in agreement with the above effector time point data, as I expected there to be a larger memory cell pool if these cells were truly more effector-like. In addition, mice lacking MFSD2A were producing less IFNγ and TNFα under homeostatic memory conditions – this was also the opposite of what I expected of true effector-like memory CD8 T cells (Figure 18B and C).

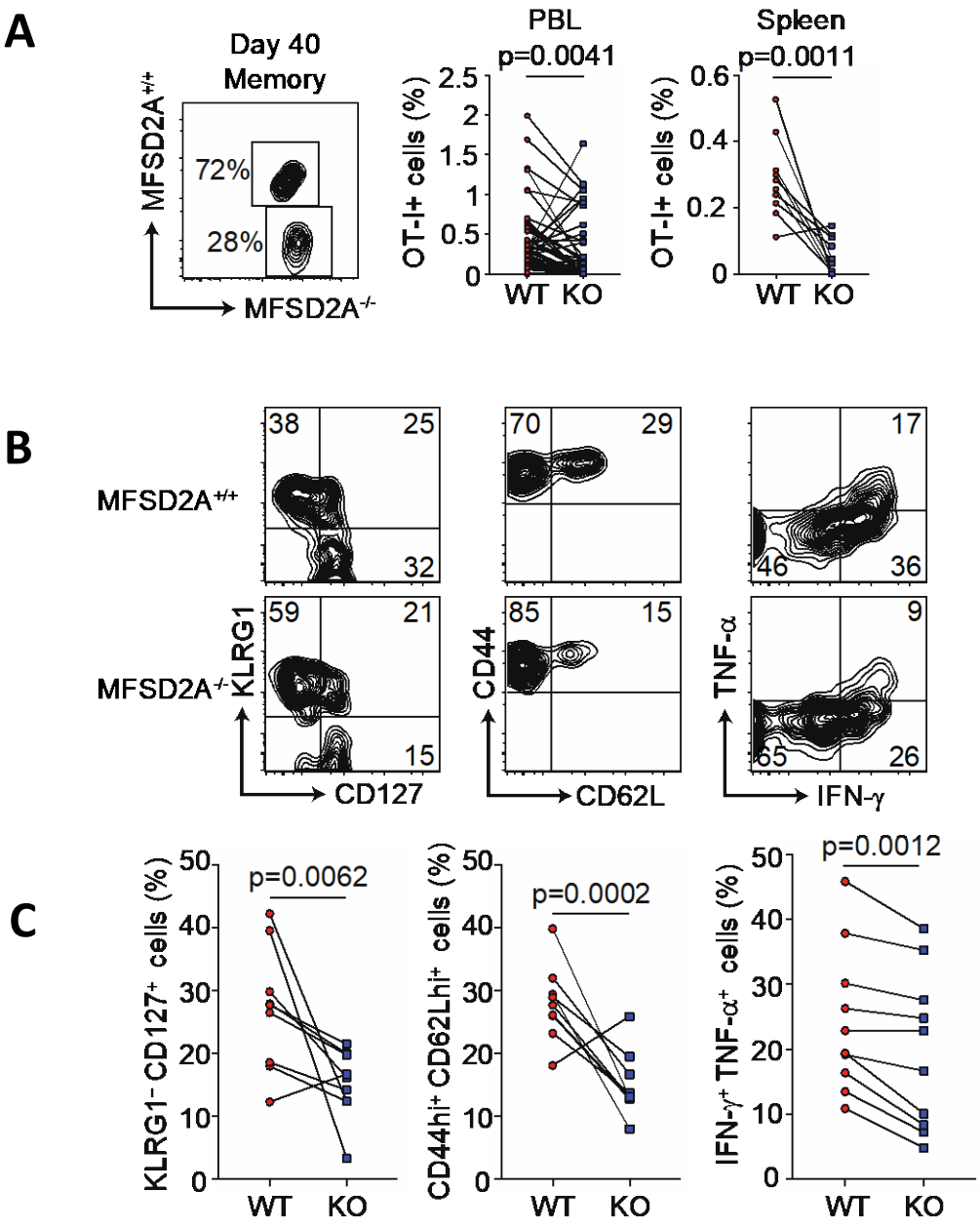


Figure 18. MFSD2A$^{-/-}$ phenotyping at homeostatic memory

Equal numbers of competitively adoptive transferred MFSD2A$^{+/+}$ CD45.1.2 OT-I and MFSD2A$^{-/-}$ CD45.2 OT-I cells were infected with lm-OVA and taken out to memory. A. Frequency of MFSD2A$^{+/+}$ and MFSD2A$^{-/-}$ splenocytes at d40 pi. B. Flow cytometry plots of surface marker expression and intracellular cytokine staining for MFSD2A$^{+/+}$ and MFSD2A$^{-/-}$ cells. For ICS, splenocytes were stimulated *ex vivo* with OVAp for 6 hrs and golgi stop for the last 3 hrs. Data are representative of 3 independent experiments with at least 3 mice per group with p-values calculated using student's t test.

Since fewer MFSD2A$^{-/-}$ CD8 T cells were surviving at memory timepoints, I next wanted to determine if this was due to a defect in homeostatic proliferation or if cells lacking MFSD2A were more apoptotic and dying at a higher rate relative to WT cells. To check if there was a proliferation defect, mice were given BrdU injects IP as well as BrdU supplementation into their drinking water for two weeks prior to sacrificing to measure BrdU incorporation. Interestingly, mice lacking MFSD2A had a highly significant decrease in the amount of actively dividing cells as measured by BrdU (Figure 19A). To confirm these findings, the same cells were stained with antibodies to cell division marker, Ki-67. MFSD2A$^{-/-}$ cells also had a significant decrease in the amount of Ki-67+ cells (Figure 19B). To confirm MFSD2A$^{-/-}$ were not also more apoptotic, splenocytes were stained for Annexin V (Figure 19C) and caspase 3 & 7 (data not shown). There was no significant difference in either apoptotic marker, and I was able to conclude that cells lacking MFSD2A were unable to proliferate normally compared to wild type by memory.

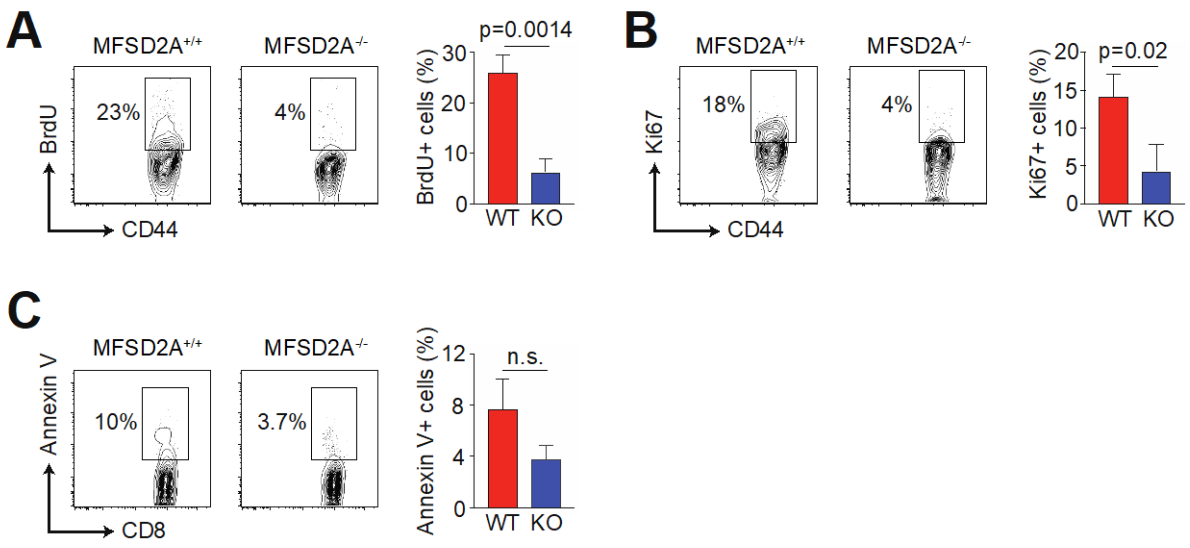


Figure 19. MFSD2A$^{-/-}$ has a proliferation defect at memory

Equal numbers of competitively adoptive transferred MFSD2A$^{+/+}$ CD45.1.2 OT-I and MFSD2A$^{-/-}$ CD45.2 OT-I cells were infected with lm-OVA and taken out to memory. A. Frequency of MFSD2A$^{+/+}$ and MFSD2A$^{-/-}$ memory splenocytes that incorporated BrdU. B. Flow cytometry plots of intracellular Ki-67 staining for MFSD2A$^{+/+}$ and MFSD2A$^{-/-}$ cells. For ICS, splenocytes were stimulated ex vivo with OVAp for 6 hrs and golgi stop for the last 3 hrs. C. Annexin V+ CD8 T cells with and without MFSD2A. Samples were run on the cytometer within one hour of staining according to the manufacturer's protocol. Data are representative of 3 independent experiments with at least 3 mice per group with p-values calculated using student's t test.

Another possible explanation for this phenotype that would not be specific to MFSD2A was that when using loss of function mouse models, there was always a chance that the mouse line is not 100% backcrossed to the background strain. In order to test this, I sent MFSD2A$^{-/-}$ tail samples to Dartmouse (Dartmouth, MS) for a C57BL/6 backcross check screen (data not shown) and mice were found to be >95% backcrossed. To reconfirm these findings, I also set up a competitive adoptive transfer experiment using littermate control mice to test for late graft rejection. Littermate OT-I CD8 T cells did not "disappear" like what was seen with MFSD2A$^{-/-}$ cells (Supplemental Figure 4) so we concluded that the phenotype was real and that the loss of CD8 T cell homoeostatic proliferation was due to loss of MFSD2A.

3.4 MFSD2A$^{-/-}$ RECALL CD8 T CELL RESPONSE

To test if MFSD2A$^{-/-}$ memory CD8 T cells were capable of producing a successful recall response to infection, I again re-challenged these mice with 100,000 CFU of *listeria*-OVA. I had previously performed real-time PCR on wildtype MFSD2A CD8 T cells that were re-challenged with *listeria*-OVA and saw that *MFSD2A* was upregulated early post-reinfection (Figure 20A). This was to be expected since I previously showed that *MFSD2A* is highly expressed early in the primary response (Figure 7A). *MFSD2A* was significantly upregulated 2.5 days post re-infection, in agreement with this hypothesis. MFSD2A$^{-/-}$ memory CD8 T cells were unable to produce a robust (or any) recall response (Figure 20B) compared to MFSD2A$^{+/+}$ cells. By d5 post-reinfection (which would correspond to the peak recall response), only 3% of OT-I cells lacking MFSD2A are still viable, representing a huge reduction in the frequency of MFSD2A$^{-/-}$ cells that remain (Figure 20C). Because there were so few cells at this time point, I re-challenged mixed bone marrow chimeras to stimulate *ex vivo* for cytokine production. MFSD2A$^{-/-}$ cells had a significant reduction in the frequency of IFNγ+ cells (Figure 20D). Overall, it was concluded that without MFSD2A, memory CD8 T cells failed to produce a recall response to infection and that this was due to decreased *MFSD2A* transcripts early post-reinfection.

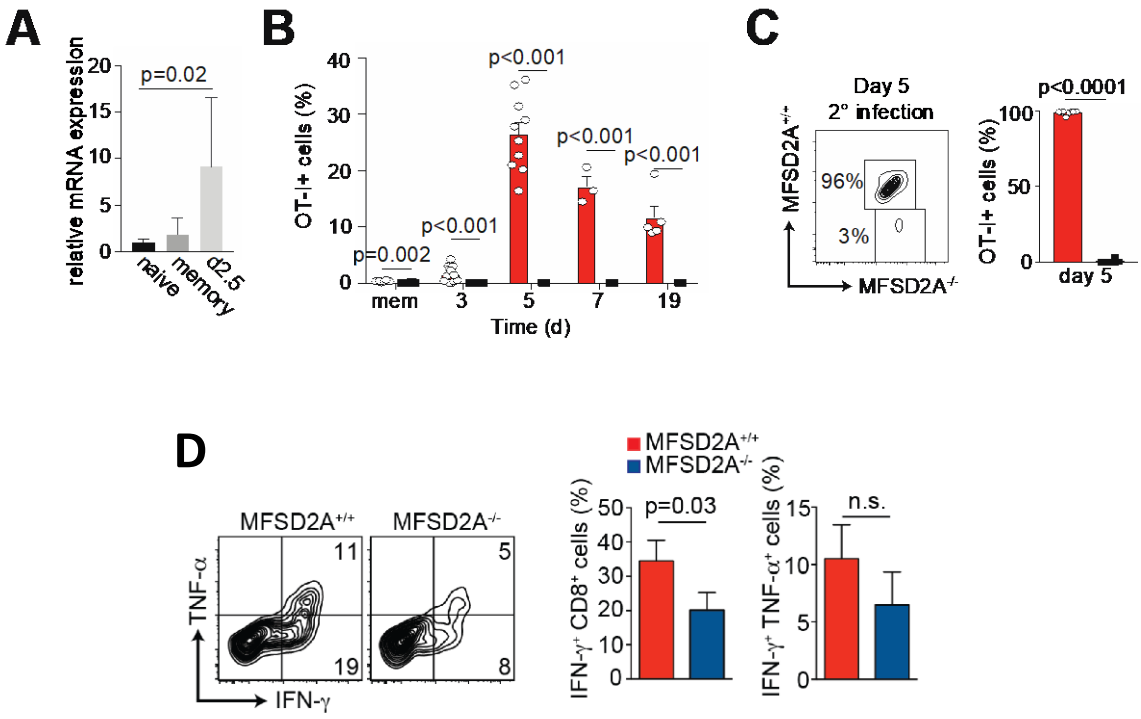


Figure 20. MFSD2A$^{-/-}$ fail to produce a recall response to infection

Competitive adoptive transfer mice from previous experiments were reinfected with 100,000 CFU lm-OVA and analyzed for downstream experiments. A. qPCR analysis for *MFSD2A* mRNA expression levels of sorted naïve, memory, and d2.5 reinfection wildtype OT-I CD8 T cells. B. Frequency of MFSD2A+/+ and MFSD2A-/- cells at memory through d19 reinfection with lm-OVA. C. Flow cytometry plot and quantification of specified OT-I cells at d5 reinfection with lm-OVA. D. Inflammatory cytokine production in mixed bone marrow chimera mice at d5 reinfection with lm-OVA following ex vivo stimulation with OVAp for 6 hrs and golgi stop for the final 3 hrs of culture. All data except for A is representative of at least 3 independent experiments with 3 animals per group. For A, data is representative of 1 experiment with 3 mice per group. P-values were calculated using student's t test.

3.5 AIM 1: FUTURE DIRECTIONS

Based on the above findings, it was clear that there is a negligible role for MFSD2A and LPC in CD8 T cell activation but that both molecules become indispensable in CD8 T cell memory and recall response to infection. There are other avenues to be explored for MFSD2A phenotypical relevance that are worth pursuing. The two with the most potential highlighted below.

3.5.1 MFSD2A AND TISSUE RESIDENT MEMORY CD8 T CELLS

Tissue resident memory CD8 T cells are noteworthy for being a first-line immunological defense in barrier tissues such as the skin and the lung. Studies have shown that T_{rm} cells rely heavily on FAO from FFA metabolism and that when the exogenous LCFA transporters FABP4 and FABP5 are conditionally deleted in T cells that T_{rm} cells (but not T_{cm} cells) were drastically reduced. Since there are numerous lipid transporters that can compensate for each other, it would be worthwhile to investigate a role for MFSD2A in T_{rm} cell maintenance.

Preliminary studies were performed to determine if there may be a link to MFSD2A and T_{rm} development. MFSD2A$^{+/+}$ or $^{-/-}$ OT-I CD8 T cells were adoptively transferred into recipient mice and infected with *listeria*-OVA as described above. On d4, the date considered peak bacterial pathogenicity, mice were shaved on their belly and painted with 2,4-dinitro-1-fluorobenzene (DNFB) to induce contact hypersensitivity in the skin and draw out adoptively transferred cells from circulation into the dermis of the skin, i.e., develop OVA-specific T_{rm} that could be isolated and quantified by FACS and fluorescent imaging of whole skin mounts.

Flow cytometry samples were collected at d7 and d10 pi to gain insight into migration patterns of T_{eff} into the skin to see if any obvious disadvantages were present in the MFSD2A$^{-/-}$ OT-I CD8 T cells. Interestingly, at d7, there was a decrease in the amount of MFSD2A$^{-/-}$ cells within the skin, however, by d10, this relationship had switched and there was actually an increased amount of MFSD2A$^{+/+}$ cells (Fig. 21A). It was worth noting that the size of the skin was not measured at these effector timepoints and that skin size is critical in order to calculate an actual count of CD8 T cells relative to tissue size rather than going off of a relative cell frequency. Noting this disadvantage, skin was quantified and true CD8 T_{rm} cells were measured at d45 pi. As expected, there was a decreased amount of CD8 T cells isolated from the skin at this timepoint (Fig. 21B), indicating that MFSD2A may be an important lipid transporter necessary for T_{rm} development.

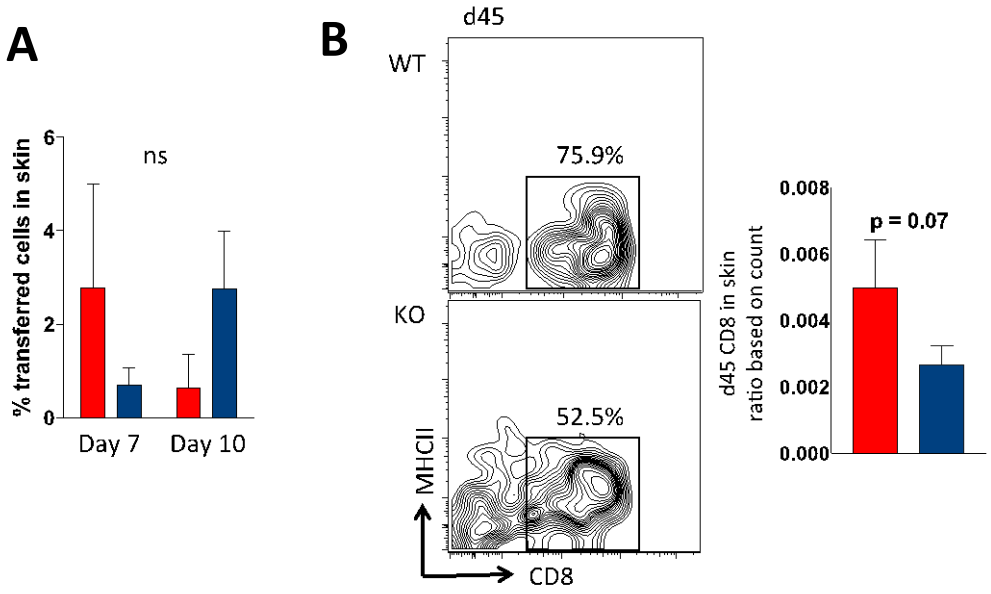


Figure 21. MFSD2A$^{-/-}$ may have a decreased ability to generate T_{rm} as seen by FACS

MFSD2A$^{+/+}$ or MFSD2A$^{-/-}$ OT-I CD8 T cells were adoptively transferred into recipient mice and infected with listeria-OVA. On d4 pi, mice were treated on the belly with DNFB. A. Quantification of the frequency of transferred cells that migrated into the skin at indicated effector day pi. B. FACS plots (left) showing relative amount of donor OT-I CD8 T cells in the skin of recipient mice at d45 pi. CD8 T cells are shown relative to MHCII as a negative control. Quantification data (right) is ratio of the CD8+ CD69+ CD103+ cells in the skin over the total amount of CD45.2+ cells in the skin by area. Data is representative of 1 independent experiment with 2 animals per group. P-values were calculated using student's t test.

Fluorescent imaging was performed on the above skin samples because microscopy is accepted to give a more powerful representation of the cells in the skin compared to flow. Whole epidermal sheet mounts were made of skin sample at d45 pi prior to immunofluorescence. Surprisingly, more CD8^{+}CD45.1^{-} OT-I cells were found in the mice that received MFSD2A$^{-/-}$ OT-I CD8 T cells, indicating that MFSD2A deletion actually creates a larger T_{rm} pool (Fig. 22). This data was in contrast to the FACS data in Fig 21. One possible explanation for this was that images were of various degrees of clarity due to technical errors and inexperience with the immunofluorescent microscope. In any case, flow and imaging data combined show that it may be worth pursuing T_{rm} experiments in the future.

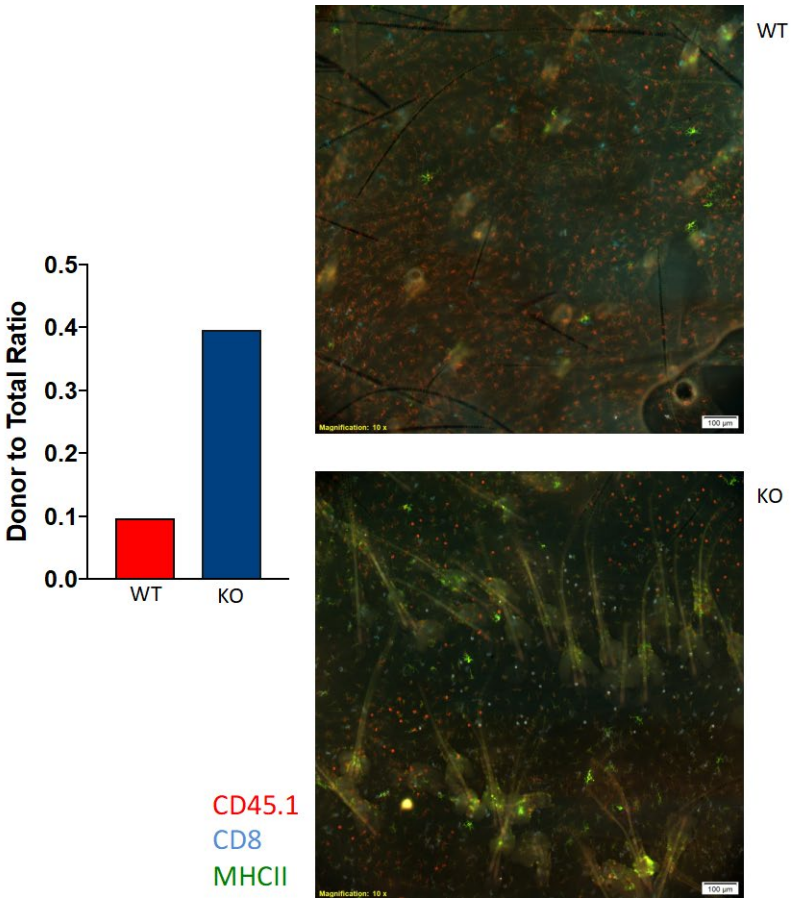


Figure 22. MFSD2A$^{-/-}$ may not have a decreased ability to generate T_{rm} as seen by IF

MFSD2A$^{+/+}$ or MFSD2A$^{-/-}$ CD45.2^{+} CD8 T cells were adoptively transferred into CD45.1^{+} recipient mice and infected with listeria-OVA. On d4 pi, mice were treated on the belly with DNFB. Data is representative of 1 independent experiment with at least 2 animals per group. Representative images of epidermal sheets from WT and KO mice are shown, whereas red indicates CD45.1^{+} cells, cyan indicates CD8^{+} cells, and MHCII indicates dendritic cells as a positive control. Long autofluorscent strands on images are hair follicles. WT and KO cells were considered the MHCII^{-}CD45^{-}CD8^{+} cells. Quantification of donor to total CD8 T cells was calculated taking the sum of amount of MHCII^{-}CD45^{-}CD8^{+} cells over the total amount of CD8+ cells (the MHCII^{-}CD45+or^{-}CD8^{+} population). Because this is a ratio, data cannot be further analyzed for significance without performing a second independent experiment. Data is representative of 1 independent experiment with at least 2 animals per group.

3.5.2 MFSD2A AND AUTOIMMUNITY (AND EXPLORING CD4 T CELLS)

CD4 T cells have not been explored in this dissertation up until now. Their metabolism was outside of the scoop of this work, but it goes without saying that their exogenous LPC import by MFSD2A is worth further exploring. It was noted that as Th0 cells differentiate into their various T helper subtypes, that metabolism is altered in certain subtypes. One subtype in particular, T regulatory (T_{reg}) CD4 T cells, are appreciated for using increased levels of FAO metabolism relative to their other CD4 counterparts. T_{regs} are important for their role as immunosuppressers and preventing autoimmunity related autoreactive T cells.

The mouse model of T_{reg} deletion is known as a "scurfy mouse" due to its scruffy, decrepit appearance. If left untreated, these mice die early in age due to autoimmunity. Like CD8 T cells, it has not been discovered what transporter is importing exogenous lipids in CD4 cells and this (like CD8 T cells) represents a knowledge gap in the field of immunometabolism that, if found, could be targeted for potential immunotherapy treatment.

I was able to work with one scurfy mouse to very preliminarily characterize MFSD2A in an autoimmunity setting. This mouse was found to have decreased relative levels of MFSD2A in its CD4+ T cell population in the lymph nodes (Fig. 23). This phenotype did not repeat when looking at the spleen (data not shown), which indicates a need to repeat this experiment with a larger sample size to better quantify these data. Still, a role for MFSD2A and LPC in all CD4 subtypes is worth further exploring and characterizing in future work.

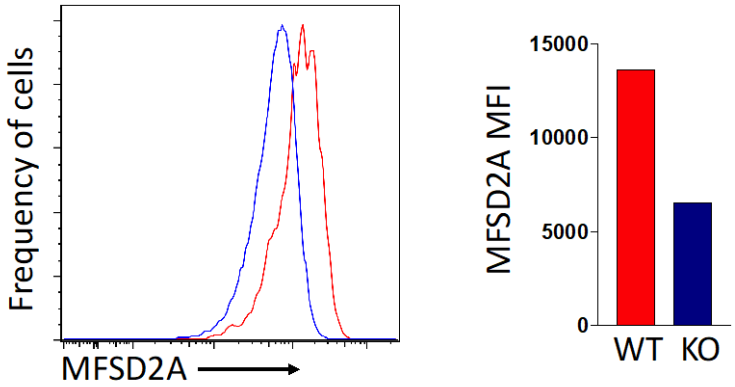


Figure 23. MFSD2A may be decreased in autoimmunity

Age-matched "scurfy" (Foxp3 del) and WT mice were sacrificed and spleen and lymph nodes were harvested. FACS plot (left) shows intracellular staining for MFSD2A gated on the CD4+ cells in the lymph node with quantification (right). Data is representative of 1 independent experiment with 1 animal per group.

4.0 SPECIFIC AIM 2 RESULTS

4.1 MFSD2A$^{-/-}$ AND LPC IMPORT

Even though I have shown the effect of loss of MFSD2A on CD8 T cells, I wanted to relate the lack of memory cell pool and secondary response to infection back to LPC import and FAO/FAS in CD8+ T cells. First, in WT mice, I took *in vitro* activated CD8 T cells and co-cultured them with 0.1μM Top-Fluor LPC (TF-LPC) for the last 4 hours of a 48 hour stimulation. TF-LPC will be taken up by the cells in culture and emit a green fluorescent glow, enabling for a way to visualize and quantify relative amounts of LPC import into CD8 T cells. One way to visualize this is by making use of Thin Layer Chromatography (TLC). TLC separates out extracted lipids as they are drawn up the TLC plate by capillary action. As expected, those lipids extracted from activated CD8 T cells show a very robust migration and TF-LPC brightness intensity (Fig. 24A) however there was zero fluorescence from naïve CD8 T cells, suggesting that naïve cells were not actively taking up TF-LPC *in vitro*.

Another way to visualize this was to use confocal microscopy. This technique was useful to visualize uptake in live-cells during real time and gives a way to view where in the CD8 T cell TF-LPC was migrating to. Interestingly, only in *in vitro* activated MFSD2A$^{+/+}$ CD8 T cells TF-LPC incorporated at the perinuclease (Fig. 24C), suggesting that with loss of MFSD2A I was not able to visualize LPC being taken up by the *in vitro* activated MFSD2A$^{-/-}$ CD8 T cells. This

feature was quite striking since the perinuclear space is continuous with the smooth ER – the site in the cell where phospholipid synthesis, fatty acid elongation, and fatty acid desaturation all take place. These data suggest the LPC species being actively imported into CD8 T cells were being used for these processes.

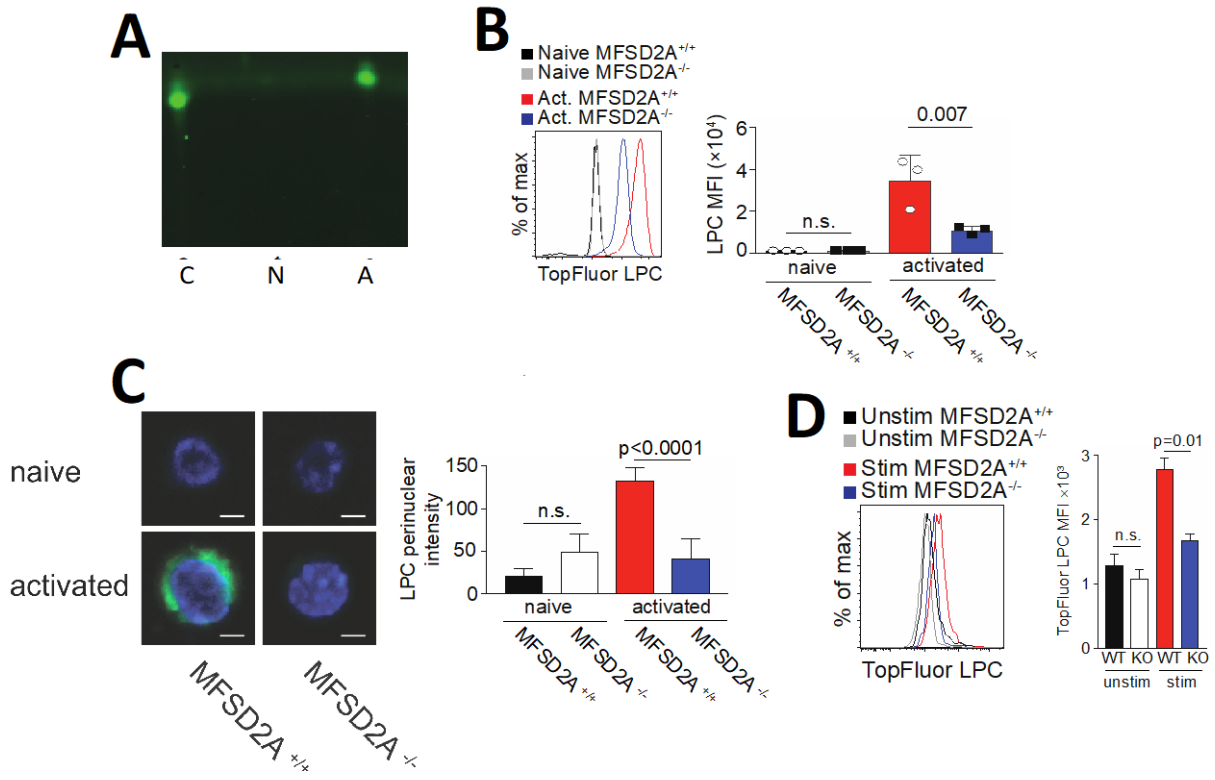


Figure 24. TF-LPC uptake levels in CD8 T cells with and without MFSD2A

Biochemical analysis for LPC performed on *in vitro* (A, B, C) or *in vivo* activated CD8 T cells cultured for 48 hours with anti-CD3 and anti-CD28 (A, B, C) or OVAp (D) and TF-LPC given the last 4 hours of stimulation at 0.1μM. A. Thin layer chromatography (TLC) imaging of loading control LPC (C), naïve CD8 T cells (N) or activated (A) CD8 T cells co-cultured with LPC and lipids isolated. TLC was ran for 4 hours to allow for separation prior to imaging. B. Naïve or Activated CD8 T cells were stained with Hoechst prior to measurement for LPC incorporation at the perinucleus out to 80 min. C. FACS plot and quantification of *in vitro* activated CD8 T cells. D. FACS plot and quantification of adoptively transferred MFSD2A$^{+/+}$ or $^{-/-}$ OT-I CD8 T cells analyzed for TF-LPC uptake via flow cytometry. B and D are representative of at least 3 individual experiments with at least 3 mice per group. C is representative of 3 experiments with MFSD2A$^{+/+}$ CD8 T cells and 1 experiment with MFSD2A$^{-/-}$ CD8 T cells. A is representative of 2 experiments. Statistical analysis was calculated using Student's t test or one-way ANOVA. Part C microscopy data generated by Callen Wallace and Eric Hyzny.

Next, I wanted to characterize LPC uptake yet another way, this time via flow cytometry TF-LPC was visible in the FITC channel. I again took *in vitro* stimulated cells CD8 T cells with and without MFSD2A and co-cultured with LPC as described. As expected, only activated MFSD2A$^{+/+}$ CD8 T cells were taking up TF-LPC relative to naïve cells and unstimulated controls (Fig 24B). This was also true in memory CD8 T cells in MFSD2A$^{+/+}$ and MFSD2A$^{-/-}$ from adoptive co-transfer experiments that were restimulated for 6 hours with OVAp prior to harvest (Fig. 24D), suggesting that LPC import was important for memory CD8 T cells. It was worth noting that in both the *in vitro* activated and restimulated memory MFSD2A$^{-/-}$ CD8 T cells that the level of TF-LPC import was not necessarily at baseline relative to naïve. It was hypothesized that this may be due to compensation by alternative lipid transporters such as CD36 or the FABP family. These studies overall suggest that CD8 T cells were indeed importing LPC species and that with loss of MFSD2A, there was a significant decrease in LPC import.

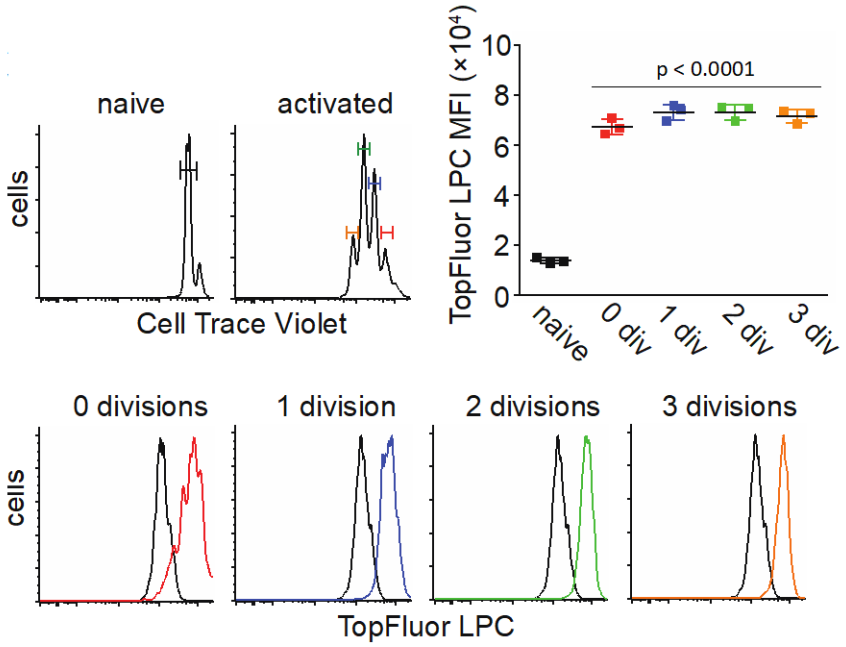


Figure 25. TF-LPC uptake in CD8 T cells is an active process

Wildtype CD8 T cells were pulsed with cell trace violet and then cultured for 72 hours with anti-CD3 and anti-CD28 with TF-LPC given the last 4 hours of stimulation at 0.1μM. Naïve cells received 10 ng/mL IL-7 for maintenance. Quantification histograms detail TF-LPC fluorescence intensity by number of divisions relative to naïve cells. Data is representative of 2 individual experiments with at least 3 mice per group. Statistical significance was calculated using one-way ANOVA with multiple comparisons.

Because stimulated CD8 T cells are much larger in size compared to naïve CD8 T cells and because T cells are capable of passive uptake, I wanted to confirm that TF-LPC uptake was in fact an active process in CD8 T cells and that the increase in TF-LPC was not just due to the activated cells being bigger. To accomplish this, WT CD8 T cells were pulsed with cell trace violet (CTV) prior to being stimulated *in vitro*. CTV labeled and "tracked" cells as they divide as a dilution readout. These cells were then given TF-LPC the last 4 hours of culture like previous followed by flow cytometry analysis. Indeed, activated CD8 T cells were capable of importing TF-LPC regardless of how many divisions the cells have gone through, suggesting that TF-LPC was an active process and not dependent on cell size (Fig. 25).

Finally, in an effort to better quantify the LPC species imported into activated CD8 T cells, I performed a global lipidomic analysis on *in vitro* activated MFSD2A$^{+/+}$ or MFSD2A$^{-/-}$ CD8 T cells (Fig. 26). This experiment was performed in collaboration with Dr. David Silver's group at Duke-NUS in Singapore. Unfortunately, overall cell counts were quite low and biological and technical replicates from each group (naïve samples, activated samples, and naïve B6 samples to calibrate the machine) needed to be pooled and then concentrated together in order to generate these data, so it was not possible to calculate statistics on these data. The heat map, however, showed a trend of decreased PC lipid species in the sample lacking MFSD2A, suggesting that with loss of MFSD2A there was also a loss of LPC import into CD8 T cells were LPC will then dissociate into PC. The most notable down expressed PC variants are PC 32:0, PC 38:4, and PC 36:5. There were also notable differences in PE lipid variant (data not shown). It was also worth noting that overall, the B6 naïve samples, the naïve WT, and naïve KO samples had the largest lipid pool, which was not in agreement with my hypothesis that LPC species were only imported into activated CD8 T cells. One such reason for this was that naïve cells do not have the rapid turnover for new cellular membrane biogenesis as seen in activated CD8 T cells. Another reason may involve use of other lipid transporters used exclusively in the naïve cells. It may be worth doing further testing with labeled C^{13} trace to track lipids in activated and naïve CD8 T cells. It could be, particularly in the case of the activated cells, that the lipids are being broken down which could explain the overall decreased amount of lipids in the activated CD8 T cells.

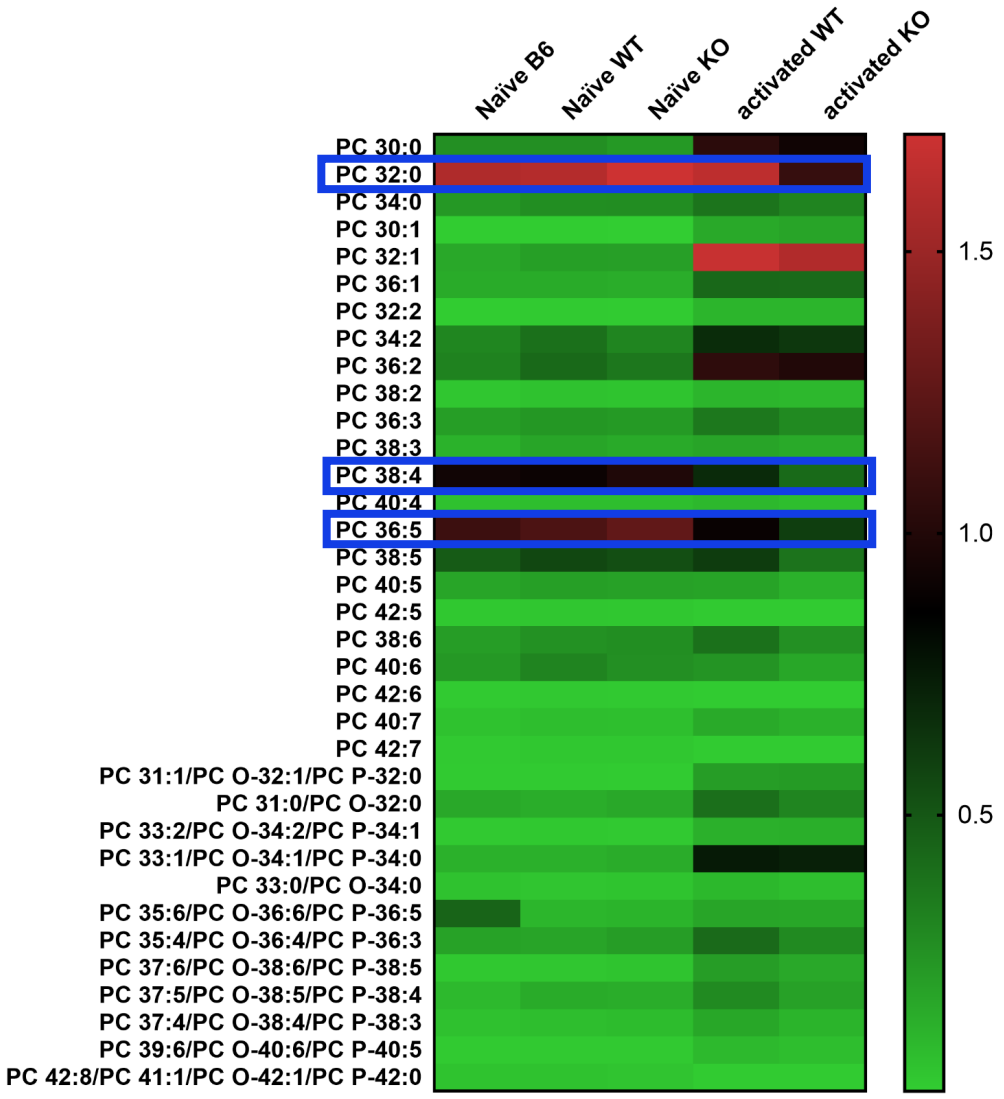


Figure 26. Lipidomic analysis of *in vitro* activated CD8 T cells

MFSD2A$^{+/+}$ and MFSD2A$^{-/-}$ CD8 T cells were activated in vitro as described and then sorted for live cells post-activation prior to lipid extraction and global lipidomics mass spec. Biological triplicates were pooled to enable instrument detection. Naïve B6 are fresh "out of the mouse" CD8 T cells meant to be used for instrument calibration. Blue boxes highlight PC species of interest showing a trend of decreased PC species in activated WT:KO. Data is represented as mean ± S.E. This data was technically challenging to generate and performed only once. Heatmap provided by Dr. David Silver of Duke-NUS, Singapore.

4.2 THE METABOLIC OUTCOMES OF UPTAKE OF LPC BY MFSD2A IN ACTIVATED T CELLS

In an effort to better understand how metabolism may be altered in CD8 T cells without MFSD2A, I performed Seahorse Flux Bioanalyzer analysis on MFSD2A$^{-/-}$ CD8 T cells. Preliminary studies first measured OCR (not shown) and ECAR in 24 hrs *in vitro* activated CD8 T cells as readouts of mitochondrial respiration and glycolysis, respectively. CD8 T cells were cultured in T Cell Media under optimal conditions. I saw a striking difference in both basal and maximal glycolysis (ECAR) rates, with the MFS2A$^{-/-}$ CD8 T cells being more metabolically active in both scenarios (Fig. 27A). This was also true, although to a lesser extent, in *in vivo* activated MFSD2A$^{-/-}$ CD8 T cells at d7 pi (Fig. 27B).

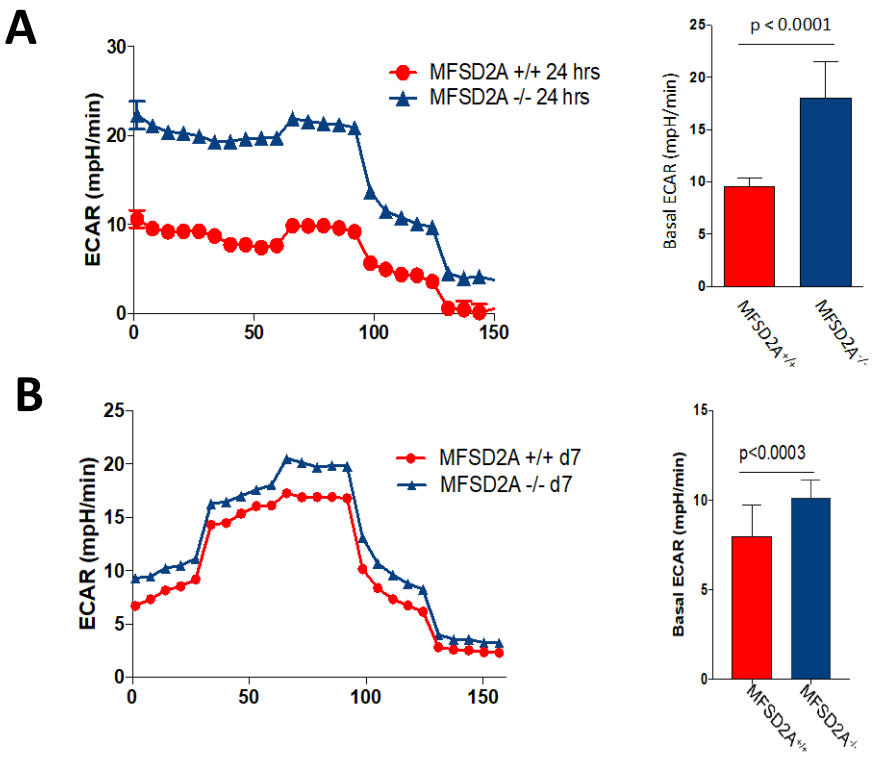


Figure 27. *in vitro* and *in vivo* activated effector CD8 T cell Seahorse analysis

MitoStress Test analysis was performed using a Seahorse Bioanalyzer on either 24 hour *in vitro* activated or 7 day *in* vivo activated CD8 T cells with or without MFSD2A. A. In vitro activated CD8 T cells cultured for 24 hours with anti-CD3 and anti-CD28 prior to ECAR analysis with basal ECAR quantification. B. Adoptively transferred MFSD2A+/+ or -/- OT-I CD8 T cells were sorted at day 7 post *listeria* infection and ECAR rates were recorded on the Seahorse with basal rate quantification. All data is representative of at least 2 individual experiments with at least 3 mice per group where samples were pooled prior to Seahorse. Statistical analysis was calculated using Student's t test.

Because I saw a more drastic phenotype when MFSD2A$^{-/-}$ mice were taken out to a memory time point, I decided to run Seahorse analysis on memory CD8 T cells. Memory CD8 T cells should be respiring primary by cellular respiration (OCR) and at relatively low levels as they are being homeostatically maintained. However, MFSD2A$^{-/-}$ memory CD8 T cells were respiring very robustly compared to MFSD2A$^{+/+}$ cells (Fig. 28A). They were also using more glycolysis compared to WT (Fig. 28A). Both basal (Fig. 28B) and maximal metabolic rates were higher in KO mice, suggesting that they were more metabolically active at memory compared to MFSD2A$^{+/+}$ CD8 T cells. The spare respiratory capacity (SRC) was also higher in MFSD2A$^{-/-}$

(Fig 28B), meaning they should be able to produce more ATP if need be. Overall, these findings suggest that MFSD2A$^{-/-}$ CD8 T cells were more metabolically active compared to MFSD2A$^{+/+}$ CD8 T cells, and that these findings were at higher intensity at memory.

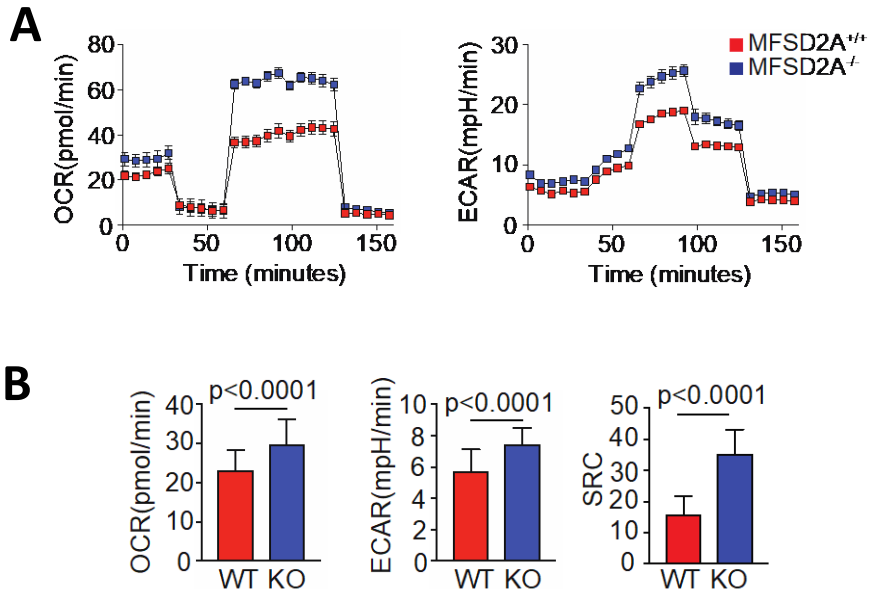


Figure 28. Memory time point CD8 T cell Seahorse analysis

MitoStress Test analysis was performed using a Seahorse Bioanalyzer on adoptively transferred MFSD2A+/+ or -/- OT-I CD8 T cells that were sorted at memory (d45 pi) post *listeria* infection and pooled prior to Seahorse. A Raw run files of OCR and ECAR. B. Quantification of maximum OCR rate, maximum ECAR rate, and SRC between MFSD2A+/+ and MFSD2A-/- memory OT-I CD8 T cells. of All data is representative of 2 individual experiments with at least 3 mice per group where samples were pooled prior to Seahorse. Statistical analysis was calculated using Student's t test.

4.2.1 GLUT1 MAY COLOCALIZE WITH LPC IN CD8 T CELLS

There were reports that GLUT1 co-localized with MFSD2A at the BBB to actively transport glucose into the brain. In addition to this, others found that GLUT1 transported glucose into activated CD4 T cells. Because of these findings, I wanted to investigate if GLUT1 levels were altered with loss of MFDS2A and if this could result in further metabolic disfunction within the CD8 T cell. It was hypothesized that if GLUT1 and MFSD2A co-localized on effector CD8 T cells after activation, that if one was lost at the TCR immunological synapse, that it may impact the other (in this case, loss of MFSD2A impacting GLUT1) and that there will be overall decreased import of exogenous metabolites. MFSD2A$^{+/+}$ and $^{-/-}$ CD8 T cells were stimulated *in vitro* for 48 hours prior to flow cytometry staining for GLUT1. Indeed, with loss of MFSD2A there appeared to be a decrease in the amount of intracellular GLUT1 (Fig. 29A) and net GLUT1, the difference between extra and intracellular GLUT1 (data not shown). In addition to these data, there were no differences in *GLUT1* mRNA (Fig. 29B), suggesting that loss of MFSD2A may only effect GLUT1 on the protein level and not transcriptionally. These findings suggest that, as in the brain, MFSD2A colocalized with GLUT1 and that when MFSD2A was lost, GLUT1 expression was also decreased which could result in further metabolic dysfunction and decreased ability to import exogenous metabolites in activated CD8 T cells.

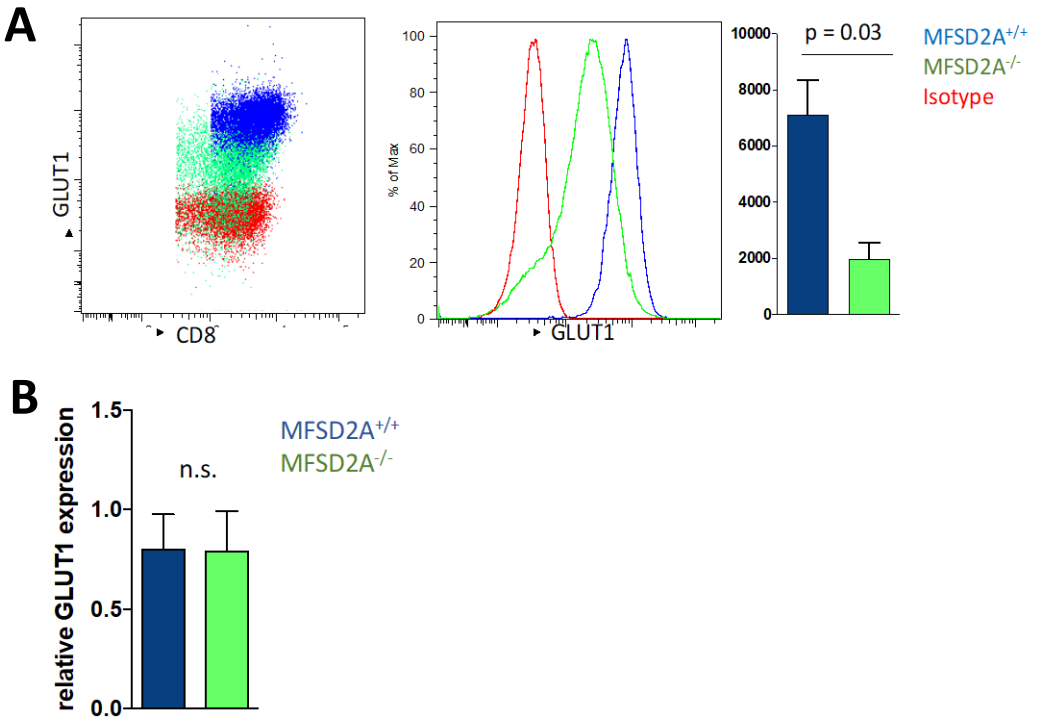


Figure 29. GLUT1 protein and RNA expression data with loss of MFSD2A

MFSD2A$^{+/+}$ and MFSD2A$^{-/-}$ CD8 T cells were stimulated *in vitro* for 48 hrs with αCD3αCD28 prior to analysis for GLUT1 protein and RNA. A. Protein GLUT1 levels are shown relative to live CD8 T cells (left panel) and by histogram (middle panel) with quantification (right panel). B. Real-time PCR expression data for GLUT1. All data is representative of 2 individual experiments with at least 1-3 mice per group. Statistical analysis was calculated using Student's t test.

4.3 THE GENETIC CONTROL OF MFSD2A AND LPC ON CD8 T CELLS

Because exogenous LCFAs imported into the mitochondria are metabolized into acetyl-CoA and the fate of some of this acetyl-CoA will eventually become a key component for histone acetylation, I was interested in the epigenetic control loss of MFSD2A had on histone modification, particularly histone acetylation. After numerous experiments including western blotting for H3K27ac (Fig. 30A) that gave inconsistent results and investigating chromatin accessibility at the IFNγ locus (a gene dysregulated with loss of MFSD2A as illustrated in Aim 1)(Fig. 30B), I concluded that histone acetylation was probably unperturbed in MFSD2A KO.

The next logical step was to determine what genes were altered with loss of MFSD2A: Was there pathway dysregulation? Were there changes in transcription factor expression or in genes required for glycolysis and cellular respiration?

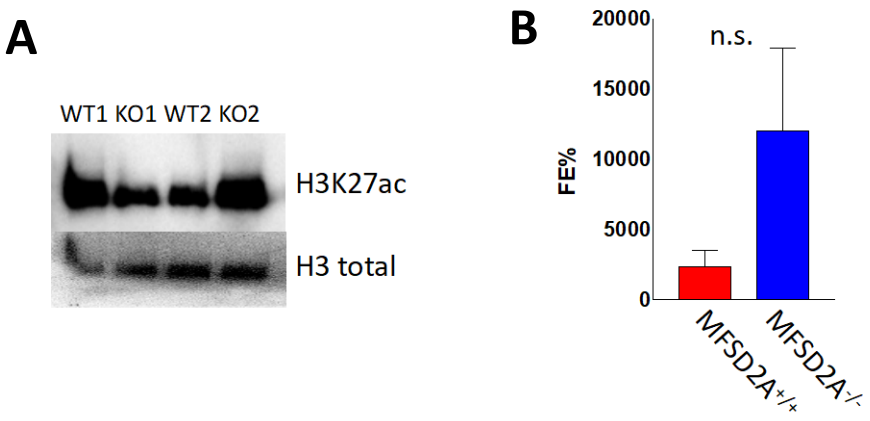
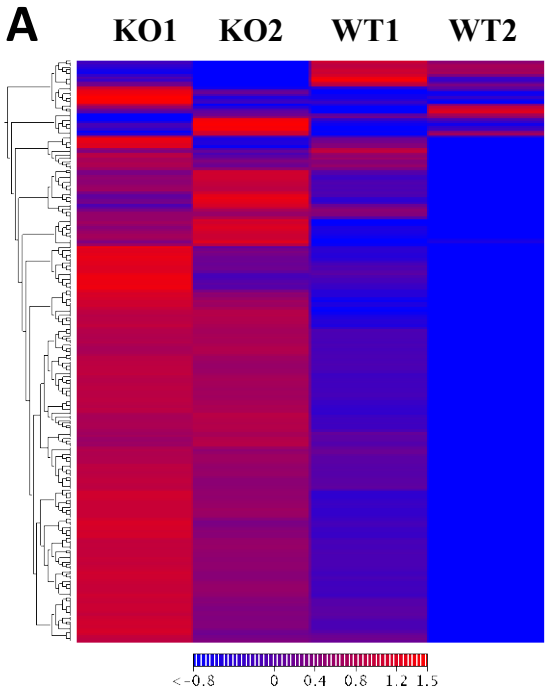


Figure 30. Epigenetic regulation by MFSD2A

A. MFSD2A$^{+/+}$ and MFSD2A$^{-/-}$ CD8 T cells were stimulated *in vitro* for 48 hrs with αCD3αCD28 prior to analysis for H3K27ac protein by Western blot. Acetylated H3K27 was normalized by examining total H3 input B. MFSD2A$^{+/+}$ and MFSD2A$^{-/-}$ effector CD8 T cells were harvested from competitive adoptive transferred recipient mice at d7 pi and sorted for purity prior to lysis, chromatin digestion, and genomic qPCR analysis of samples at the IFNγ locus. Fold enrichment (FE) was calculated by taking the difference of Ct values between digested chromatin samples and non-digested controls. All data is representative of 1-2 individual experiments with at least 2 mice per group. Statistical analysis was calculated using Student's t test.

To answer these questions, I performed RNA-sequencing on adoptively transferred MFSD2A$^{+/+}$ and $^{-/-}$ OT-I CD8 T cells from mice infected with *listeria* and taken out to d40 pi. There were numerous changes in transcription (Fig. 31A) however I did not find one master gene to connect the dots between phenotype and function in the MFSD2A$^{-/-}$. Still, some genes of interest were differentially expressed (Fig. 31B), including downregulation of interferon inducible proteins *GM4955* and *PYDC4* as well as increased expression of genes related to co-stimulatory inhibition and inhibitory receptors, *CD244* and *KLRA9*, respectively. Interestingly, growth hormone was also slightly increased in MFSD2A$^{-/-}$, suggestive of a novel way to increase

glucose and FFA concentration. Overall, the majority of transcripts appeared to be upregulated genes in MFSD2A$^{-/-}$. I hypothesized this was due to the greater metabolic demand that was seen by Seahorse in the KO cells, inducing overall higher levels of gene transcription at homeostatic memory compared to MFSD2A$^{+/+}$ CD8 T cells.



B

Gene	Fold Change	Function
GM4955	-189.95	IFNg inducble protein
KLRA9	119.25	Inhibitory receptor
PYDC4	-53.77	IFNg inducble protein
CD244	14.5	co-inhibitory receptor
Gh	4.24	growth hormone

Figure 31. RNA-sequencing results summary from memory CD8 T cells

Memory MFSD2A$^{+/+}$ and MFSD2A$^{-/-}$ OT-I CD8 T cells were sorted prior to RNA sequencing. A. Heat Map was generated in CLC Genomics 11. B. Genes of notable function with high fold change were picked out and further examined for function as it may relate to MFSD2A. Data are representative of one experiment with 2 mice per group.

To make sense of the sequencing data generated, I decided to investigate pathway analysis and try to piece together where MFSD2A and lipid metabolism fit into my phenotype in Aim 1. I used Ingenuity Pathway Analysis (IPA) software to accomplish this. IPA has a specific 'metabolomics for IPA' pathway program that generated a genetic trail based on known

metabolic pathways and regulators. I performed the metabolomics analysis using the table of differentially expressed genes with $p<0.05$. Interesting, the sequencing data, including one of the top gene hits *CD244*, linked back to the ERK1/2 signaling pathway (Fig. 32). Although this pathway does not directly link to LCFA metabolism, it does have potential regulator control over cell survival, cell cycle progression, and CD8 T cell proliferation – all hallmarks of the memory CD8 T cell phenotype without MFSD2A as shown in Aim 1. ERK activation was not investigated further, but may be an avenue for future experimental work linking changes in T cell signaling pathways to loss of MFSD2A.

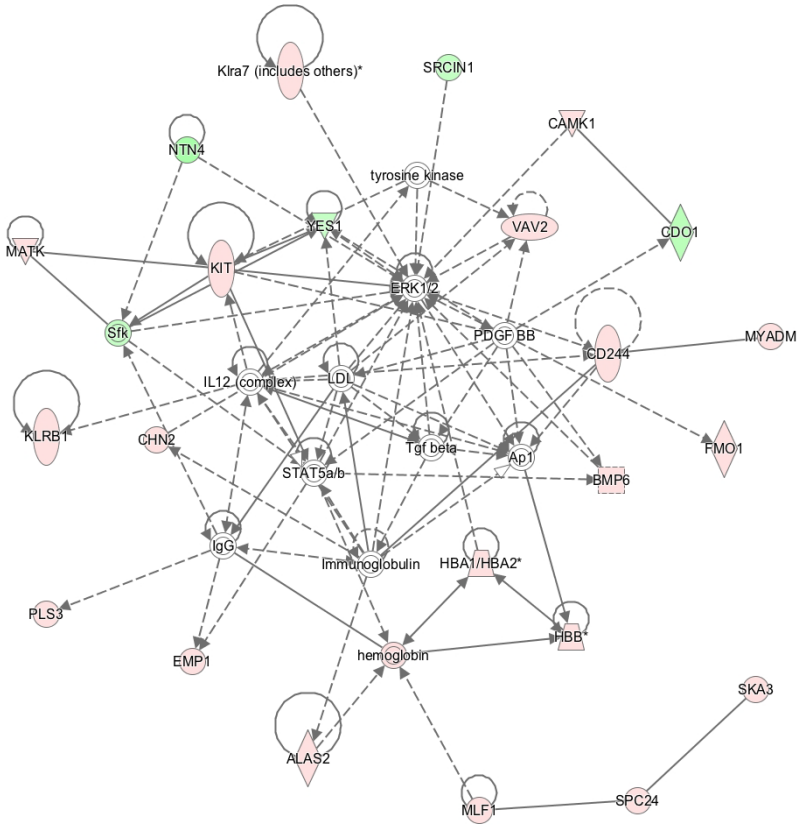


Figure 32. Metabolomics pathway analysis for memory CD8 T cells

Memory MFSD2A$^{+/+}$ and MFSD2A$^{-/-}$ OT-I CD8 T cells were sorted prior to RNA sequencing. A. Heat Map was generated in CLC Genomics 11. B. Genes of notable function with high fold change were picked out and further examined for function as it may relate to MFSD2A. Data are representative of one experiment with 2 mice per group.

Finally, recent reports illustrated that MFSD2A negatively regulates fatty acid synthesis (FAS) in the brain. Fatty acid and cholesterol synthesis in itself is regulated by two proteins, SREBP1 and SREBP2. With loss of MFSD2A in the brain, there was an increase in SREBP1 and SREBP2 protein in the tissue, with the idea that FAS and cholesterol synthesis (SREBP1 and SREBP2, respectively) are being increased to compensate for loss of exogenous LPC import. When MFSD2A$^{+/+}$ and $^{-/-}$ CD8 T cells were activated *in vitro* and analyzed for mRNA expression of *SREBP1* by real-time PCR, there was elevated levels of *SREBP1* after 48 hours *in vitro* activation (Fig. 33A). In addition to this, when competitive adoptive transfer mice were taken out

to a memory timepoint and restimulated *ex vivo* with OVAp, there was again increased levels of *SREBP1* expression in MFSD2A$^{-/-}$ cells (Fig. 33B), suggesting that this increased level of FAS is also seen in MFSD2A$^{-/-}$ memory CD8 T cells re-challenged with antigen. These data suggested that like in the brain, MFSD2A deficient CD8 T cells were able to compensate for lack of LPC import by upregulating genes related to FAS and generate lipids *de novo*.

Overall, MFSD2A deficient CD8 T cells showed an altered gene transcription, including possible alterations in signaling pathways and compensatory genes related to FAS, but future studies would need to be performed in order to confirm these findings.

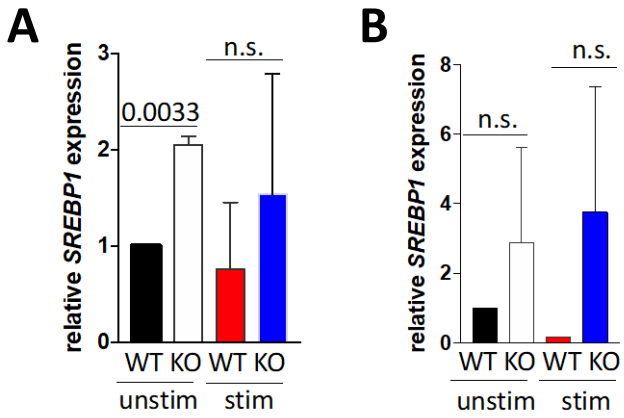


Figure 33. *SREBP1* is elevated in MFSD2A deficient CD8 T cells

A. MFSD2A$^{+/+}$ and MFSD2A$^{-/-}$ CD8 T cells were enriched and stimulated *in vitro* for 48 hrs with αCD3αCD28 or 10 ng/mL IL-7 (unstimulated) prior to sorting for live cells and downstream RNA processing. B. MFSD2A$^{+/+}$ and MFSD2A$^{-/-}$ CD8 T cells were sorted from competitive adoptive transferred mice that were taken out to a memory time point (d40 pi) and either restimulated *ex vivo* with OVAp or media alone for 48 hours prior to sorting for live cells and downstream RNA processing. For A, data are representative of two pooled experiments with 1 mouse per group per experiment and p-value calculation using Student's t-test. For B, data is representative of one experiment with pooled memory cells from 3-5 mice per group.

4.4 AIM 2: FUTURE DIRECTIONS

Aim 2 has explored how MFSD2A is importing LPC in CD8 T cells, where the LPC migrates to once within the cell, the metabolic consequences of loss of MFSD2A on CD8 T cells, and dysregulation of gene expression. Out of all of the above, the gene expression data – especially the RNA-sequencing data – is the most perplexing. One idea as to why may be found above in Fig. 18A. MFSD2A expression was close to basal at memory and goes back up early (d2.5) post-reinfection with *listeria*. Sequencing samples were not stimulated *ex vivo* or reinfected, so it is within reason to think that more genes would be induced upon reinfection and restimulation based on the mentioned MFSD2A qPCR expression data. With that in mind, RNA-sequencing restimulated memory CD8 T cells is an excellent direction to pursue. The other potential pitfall with Aim 2 study design may be found within the mice themselves.

4.4.1 MFSD2A IN THE FASTED STATE

It has previously been published by collaborators Silver et al that MFSD2A was a fasting-induced gene that was induced by PPARα and glucagon signaling. For all experiments in this document, mice were not fasted nor were they on a special low fat/high carbohydrate diet. Is it possible that, if MSFD2A$^{-/-}$ mice were on a special diet or fasted the night before harvest, would the phenotype in these mice be the same or would it be more extreme? Similarly, was there error in these current data based on whether a mouse ate within hours of harvest compared to another mouse that was voluntarily fasting in its cage? These are real variables that need to be taken into

consideration with the work in Aim 2, especially all data related to metabolism of these mice as well as gene expression data.

Another element to this fasting paradox is the *in vitro* data. CD8 T cells were incubated for the indicated amount of time under ideal culture conditions in TCM. What if rather than TCM the system had been challenged? If CD8 T cells were cultured in a nutrient poor media for 48 hours, they would not have exogenous metabolites already in place in their media to readily uptake. It would be worth repeating any and all introductory *in vitro* experiments using a low glucose or serum free media and observe how MFSD2A deficient CD8 T cells compare to their wildtype counterparts under non-ideal culturing conditions.

5.0 DISCUSSION

By investigating phenotype and functional differences in CD8 T cells, this dissertation has given critical evidence in support of a role of MFSD2A in CD8 T cells. MFSD2A expression was significantly increased early post *in vitro* CD8 T cell activation. These data formulated the initial hypothesis of this work suggesting that MFSD2A and import of exogenous LCFA by LPC was necessary during the initial CD8 T effector response following infection with *listeria*, and that MFSD2A remained critical for memory CD8 T cell formation and maintenance due to memory cells' indispensable need for a bank of biosynthetic precursors and TAG storage generated during the primary effector response to help memory cells quickly respond to secondary antigen challenge. Surprisingly, MFSD2A and LPC loss created very little change in the primary effector response, with only subtle differences in KLRG1 and CD127 surface marker expression on those cells apparently pre-determined to become memory CD8 T cells. Once experimental mice reached a true memory time point, the effects of loss of MFSD2A and LPC became more striking. MFSD2A deficient CD8 T cells had a homeostatic proliferation defect by the time they reached memory, and when presented with antigen rechallenge, these cells were unable to produce a secondary response to infection compared to their WT counterparts.

In what was perhaps counterintuitive, MFSD2A$^{-/-}$ memory CD8 T cells were more metabolically active: they were more glycolytic, had increased cellular respiration, and a significantly higher spare respiratory capacity. These data were indicative of the higher basal

rates necessary by MFSD2A deficient CD8 T cells in order to perform an increased amount of *de novo* fatty acid synthesis. This potential increase in FAS is one way that KO cells have developed compensatory effects to counteract the loss of exogenous lipid import. Additional data supporting this increase in FAS was higher relative expression levels of the FAS regulating gene *SREBP1* in restimulated memory MFSD2A$^{-/-}$ CD8 T cells. These data were supported by recent reports showing that when FAS was inhibited early in the CD4 T cell effector response there was a decreased frequency of cells in the CD4 memory cell pool[91]. These higher metabolic rates could also be a reason that I see decreased overall amounts of MFSD2A deficit memory CD8 T cells as it was within reason to hypothesize that eventually these cells 'run out of gas', are unable to sustain homeostatic proliferation, and die.

It is fair to say that other compensatory measures may be at play. Numerous exogenous lipid transporters have been identified in various immune cell types. FABP4 and FABP5 were already mentioned in reference to tissue resident memory CD8 T cell development. There were no significant differences in these genes in my RNA sequencing data but this may be due to not performing sequencing on more energetically demanding restimulated memory CD8 T cells (see future directions for Aim 2). Another important lipid transporter is CD36. CD36 was identified as a critical lipid transporter and signaling molecule in macrophages, where it was shown to be a mandatory signal for macrophages to bind to oxidized low density lipoprotein (OxLDL) and contribute to the formation of foam cells in atherosclerosis[158]. Data on CD36 in CD8 T cells is inconclusive, including pilot experiments conducted in relation to this work, but it is fair to suggest that CD36 and/or members of the FABP family may have increased expression levels in MFSD2A$^{-/-}$ CD8 T cells to pick up the slack for lack of MFSD2A and that this form of compensation may be way MFSD2A loss was detrimental to the CD8 T cell but not 100% lethal.

Looking into any potential compensatory lipid transporter mechanisms in CD8 T cells with loss of MFSD2A is a critical component to this work that will need to be investigated in the future.

As noted in the above text, the phenotype associated with loss of MFSD2A was most striking when using the competitive adoptive transfer approach rather than directly infecting WT and KO mice or when performing signal adoptive transfer experiments where mice received either WT or KO OT-I CD8 T cells rather than both. These data suggested that loss of MFSD2A in CD8 T cells may not be overly terminal to CD8 T cells alone, but rather that the phenotype that was seen at least in due part to the MSFD2A deficient CD8 T cells being "less fit" compared to their WT counterparts. Why would loss of a lipid transporter make CD8 T cells less fit in a competitive environment? The answer may be found within the animals themselves. During competition, cells are under pressure to survive (i.e., survival of the fittest) and to capture all available nutrients within their environment. Those CD8 T cells that contain MFSD2A were able to import exogenous LPC species with ease to bank their metabolites needed in times of memory. However, MFSD2A deficient CD8 T cells need to expend more energy and synthesize their own nutrients, wasting time and energy to do so while their WT counterparts focused on cell survival.

What does all of this mean for humans? The few families with known mutations in MFSD2A suffer from microcephaly-like syndromes but have very little known about their immunopathology. Due to these patients being so rare and so sick, their immune system remains to be investigated for a potential phenotype. As shown in the results section, MFSD2A is known to be conserved through evolution and in my hands there appears to be upregulation of MFSD2A in human CD8 T effector cells in the PBL, similar to what has now been shown in mouse. Although blood has not been analyzed in these patients, it is hypothesized that they would have

increased blood plasma LPC levels compared to healthy, age matched controls and that these patients would have a reduced memory CD8 T cell pool with an inability to use CD8 T cell memory to prevent recurrent infections.

Could MFSD2A ever have clinical relevance? Public health organizations such as the AHA and WHO recognize DHA as an important component to a healthy lifestyle. Along with that, a walk to the local grocery or health store is full of supplements advertising the importance of LCFAs in a healthy diet for everything from improved cognition to their anti-inflammatory properties. I have experimentally shown that MFSD2A is expressed early in infection and does not become of critical importance until immunological memory has been reached. Thus, how fatty acids are imported into CD8 T cells has a clinical relevancy for determining how vaccines can be better designed and how diet can be controlled to better fight infection. If, for example it is possible to better control infection by obtaining more dietary LCFAs? Vaccines may also be able to benefit from a smart design targeting LPC. For instance, by creating small molecule therapeutics to target LPC and boost the immune response from vaccination, leading to a more robust treatment. Similarly, vaccines of engineered memory CD8 T cells have proven promising for treating tumors. MFSD2A is critical to memory CD8 T cell maintenance. If tumor-specific memory CD8 T cells with bioengineered MFSD2A overexpression were injected into the tumor site, they may be more capable of destroying tumors than tumor-specific memory CD8 T cells alone.

Taken together, the data within this dissertation used a combination of molecular and biochemical approaches to prove that there is a role for MFSD2A and LPC CD8 T cells in response to infection. This document is the first of its kind to show a purpose for LCFA import into CD8 T cells and how, when lost, it can have disastrous consequences on the secondary

immune response to infection. It is only a start for what MFSD2A and LPC may be critical for in the immune response to infection, and further investigation needs to be examined. Future directions below each Aim critically analyze possible directions to take this project, including where and how MFSD2A can be relevant in the clinic and in overall public health.

APPENDIX A: LIST OF ABBREVIATIONS

AHA: American Heart Association
APC: antigen presenting cell
BAT: brown adipose tissue
BrdU: bromodeoxyuridine
CD: cluster of differentiation
CPT1: carnitine palmitoyltransferase I
CTV: cell trace violet
DAMPS: danger-associated molecular patterns
DAG: diacylglyerol
DC: dendritic cell
DHA: docosahexaenoic acid
DNFB: 2,4-dinitro-1-fluorobenzene
EDTA: ethlenediaminetetraacetic acid
ER: endoplasmic reticulum
FABP: fatty acid binding protein
FACS: fluorescence-activated cell sorting
FAO: fatty acid oxidation
FAS: fatty acid synthesis
FBS: fetal bovine serum
FCS: fetal calf serum
FE: fold enrichment
FFA: free fatty acids
IF: immunofluorescence
IP: intraperitoneal
IV: intraveneously
KO: knockout
LM: listeria monocytogenes
LPC: lysophosphatidylcholine
MFSD2A: major facilitator superfamily domin containing 2a
MHCI: major histocompatibility complex class I
MHCII: major histocompatibility complex class II
MPEC: memory precursor effector cell
OxLDL: oxidized low density lipoprotein
PA: phosphatidic acid
PAMPS: pattern-associated molecular patterns
PBS: phosphate buffered saline

PBL: peripheral blood lymphocytes
PC: phophatidylcholine
PCR: polymerase chain reaction
qPCR: real-time polymerase chain reaction
Pi: post infection
PRR: pattern recognition receptors
RO: retrooribitally
SRC: spare respiratory capacity
SREBP: sterol regulatory element-binding protein
T_{eff}: T effector cell
T_{rm}: tissue resident memory T cells
T_{mem}: T memory cell
TAG: triacylglycerol
TCA cycle: tricarboxylic acid cycle
TCM: T cell media
TCR: T cell receptor
TNF: tumor necrosis factor
TF-LPC: TopFluor-lysophosphatidylcholine
UC: ulcerative colitis
WB: western blot
WHO: World Health Organization
WT: wildtype

APPENDIX B: LIST OF PCR PRIMER SEQUENCES AND REAL-TIME PCR TAQMAN ASSAY PROBES

Genotyping PCR

MFSD2A:
F: ACG TTG TCG CCA CCA TCA TCA CC
R: CTT AAT AGT GAC ACT ACG AGG TTC CGG G

CD4 Cre:
F: GCG GTC TGG CAG TAA AAA CTA TC
R: GTG AAA CAG CATTGC TGT CAC TT

OT-I
F: AAG GTG GAG AGA GAV AAA GGA
R: CCA GTG CAT GCA TAC CTC AG

Real-Time PCR

GLUT1 (Slc2a1)
F: ATG GAT CCC AGC AGC AAG
R: CCA GTG TTA TAG CCG AAC TGC

IFNγ genomic primers
IFNγ Exon1 F: TAT AGC TGC CAT CGG CTG AC
IFNγ Exon 1 R: GAA AGT CTG AAT AAC TGT TTT

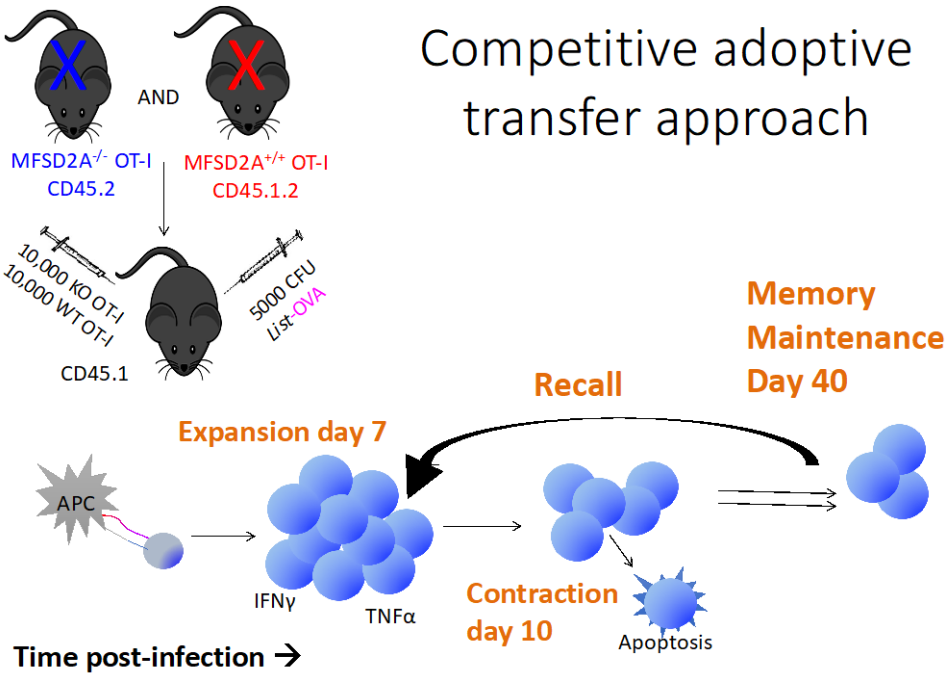
Taqman Assay Probe IDs

MFSD2A: Mm01192208_m1
SREBP1: Mm00550338_m1

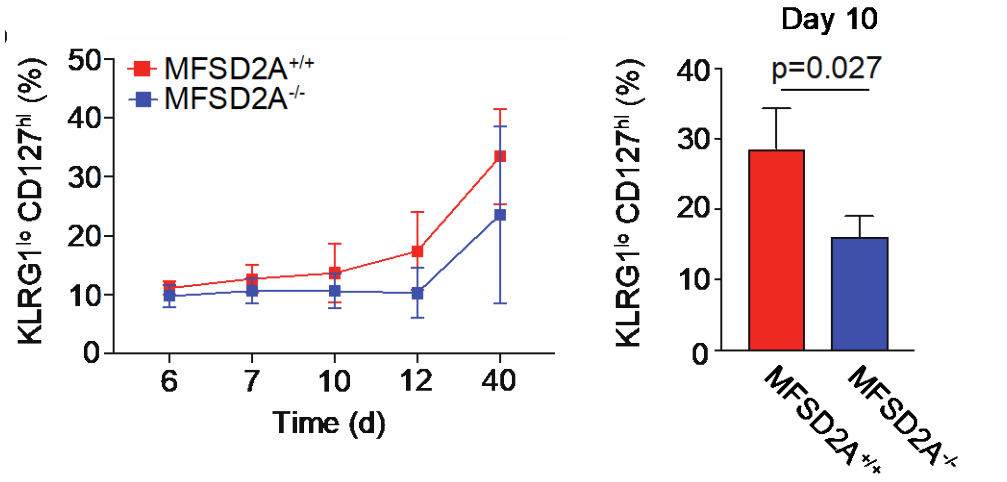
APPENDIX C: LIST OF ANTIBODIES

Antibody/Tetramer	Conjugate	Clone	Manufacturer
CD45.2	APC	104	eBioscience
CD45.1	e450	A20	eBioscience
CD45.1	BUV395	A20	BD Biosciences
CD8	PeCy7	53-6.7	eBioscience
CD8	e506	53-6.7	eBioscience
Va2	PE	B20.1	eBioscience
OVA (H2-kB)	PE	SIINFEKL	MBL Int'l.
CD44	PerCPCy-5.5	IM7	eBioscience
CD62L	e780	MEL-14	eBioscience
KLRG1	e610	2F1	eBioscience
CD127	FITC	A7R34	eBioscience
IFNg	e610	XMG1.2	eBioscience
TNFa	e450	MP6-XT22	eBioscience
BrdU	APC	n/a	BD Biosciences
Ki-67	PeCy7	SolA15	eBioscience
Annexin V	PE	n/a	BD Biosciences
Viability	BV510	n/a	Biolegend
MFSD2A	arab	183-232	Silver Lab/Abcam
GLUT1	arab	KLH	Abcam
anti-rabbit IgG	PE	H + L	eBioscience
anti-rabbit IgG	HRP	H + L	Jackson

APPENDIX D: DIAGRAM OF IN VIVO MODEL

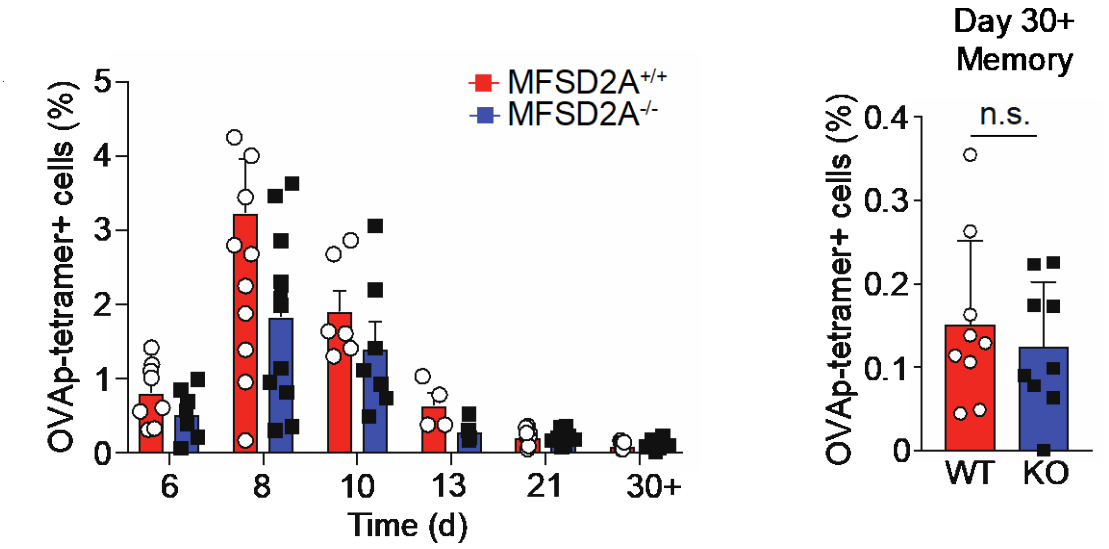


APPENDIX E: SUPPLEMENTAL FIGURES



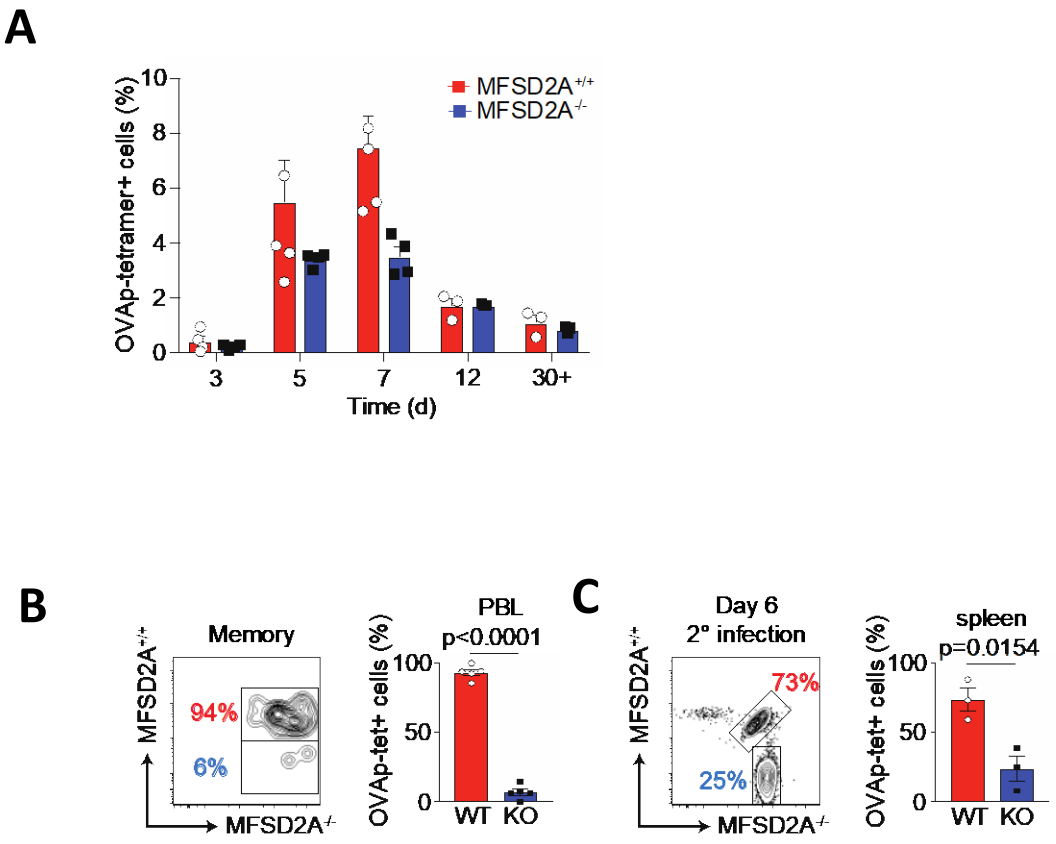
Supplemental Figure 1. MFSD2A$^{-/-}$ has a decreased CD8 memory precursor cell pool

Mice received equal amounts of MFSD2A$^{+/+}$ and MFSD2A$^{-/-}$ OT-I CD8 T cells prior to infection with 5000 CFUs lm-OVA. Time course of effector response showing frequency of KLRG1^{lo}CD127^{hi} MFSD2A$^{+/+}$ and MFSD2A$^{-/-}$ memory precursor cells at indicated time point (left). Quantification of frequency of KLRG1^{lo}CD127^{hi} MFSD2A$^{+/+}$ and MFSD2A$^{-/-}$ cells at d10 pi (right). All data is from blood. Data are representative of at least 3 independent experiments with at least 3 mice per group with p-values calculated using student's t.



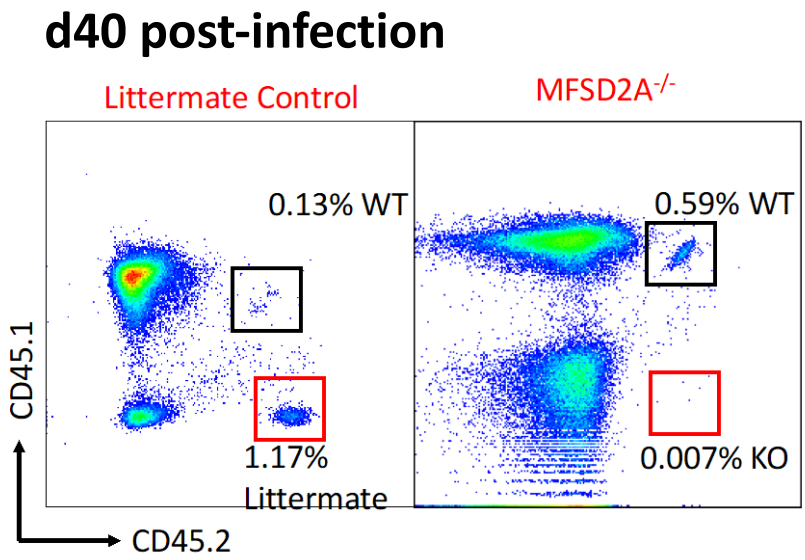
Supplemental Figure 2. MFSD2A$^{-/-}$ has similar effector response in mice directly infected with listeria

MFSD2A$^{+/+}$ and MFSD2A$^{-/-}$ mice were directly infected with 5000 CFUs lm-OVA to measure the endogenous response. OVA specific CD8 effector CD8 T cells were measured using OVA tetramer. Time course of effector response showing frequency of OVA tetramer positive cells at indicated effector time point (left). Quantification of frequency of OVA tetramer positive MFSD2A$^{+/+}$ and MFSD2A$^{-/-}$ CD8 T cells at memory (right). All data is from blood. Data are representative of at least 3 independent experiments with at least 3 mice per group with p-values calculated using student's t. No data was significant.



Supplemental Figure 3. Endogenous response at memory and secondary infection in MFSD2A$^{-/-}$ mice

A. Time course of effector response showing frequency of OVA tetramer positive cells in MFSD2A$^{+/+}$ and MFSD2A$^{-/-}$ mice previously infected with *listeria* that received a high dose (100,000 CFU) rechallenge with *listeria*. at indicated effector time point. B and C: MFSD2A$^{+/+}$ and MFSD2A$^{-/-}$ bone marrow was depleted of lymphocytes and then injected in a 50:50 ratio to irradiated CD45.1 recipient mice. Mice were given 8 weeks to recover. Mice were then directly infected with 5000 CFUs lm-OVA to measure the endogenous response. OVA specific CD8 effector CD8 T cells were measured using OVA tetramer. B. FACS plot and quantification of frequency of OVA tetramer positive MFSD2A$^{+/+}$ and MFSD2A$^{-/-}$ CD8 T cells at memory. C. Mixed bone marrow chimera mice were reinfected with 100,000 CFU lm-OVA. FACS and quantification are from d6 peak post reinfection. All data is from blood. Data are representative of at least 3 independent experiments with at least 3 mice per group with p-values calculated using student's t. For A, no data was statistically significant.



Supplemental Figure 4. MFSD2A littermate OT-I CD8 T cells do not reject at memory

MFSD2A$^{+/+}$ CD45.1.2 and MFSD2A$^{+/+}$ CD45.2 OT-I CD8 T or MFSD2A$^{+/+}$ CD45.1.2 and MFSD2A$^{-/-}$ CD45.2 OT-I CD8 T were competitively adoptively transferred in a 50:50 ratio to CD45.1 recipient mice. Mice were then infected with 5000 CFUs lm-OVA. FACS plot showing littermate OT-I (left) and MFSD2A-/- OT-I (right) as the CD45.2 single positive population d40 pi. All data is from blood. Data are representative of 1 independent experiment with at least 3 mice per group.

BIBLIOGRAPHY

1. Medzhitov, R., *Recognition of microorganisms and activation of the immune response.* Nature, 2007. **449**: p. 819.

2. Varol, C., A. Mildner, and S. Jung, *Macrophages: Development and Tissue Specialization.* Annual Review of Immunology, 2015. **33**(1): p. 643-675.

3. Turvey, S.E. and D.H. Broide, *Chapter 2: Innate Immunity.* The Journal of allergy and clinical immunology, 2010. **125**(2 Suppl 2): p. S24-S32.

4. Iwasaki, A. and R. Medzhitov, *Control of adaptive immunity by the innate immune system.* Nature Immunology, 2015. **16**: p. 343.

5. Mogensen, T.H., *Pathogen Recognition and Inflammatory Signaling in Innate Immune Defenses.* Clinical Microbiology Reviews, 2009. **22**(2): p. 240.

6. Warrington, R., et al., *An introduction to immunology and immunopathology.* Allergy, Asthma & Clinical Immunology, 2011. **7**(1): p. S1.

7. Guermonprez, P., et al., *Antigen Presentation and T Cell Stimulation by Dendritic Cells.* Annual Review of Immunology, 2002. **20**(1): p. 621-667.

8. Purcell, A.W., N.P. Croft, and D.C. Tscharke, *Immunology by numbers: quantitation of antigen presentation completes the quantitative milieu of systems immunology!* Current Opinion in Immunology, 2016. **40**: p. 88-95.

9. Kenneth Murphy, P.T., Mark Walport, *Janeway's Immunobiology*. Vol. 7th edition. 2008, United States: Garland Science.

10. Dempsey, P.W., S.A. Vaidya, and G. Cheng, *The Art of War: Innate and adaptive immune responses.* Cellular and Molecular Life Sciences CMLS, 2003. **60**(12): p. 2604-2621.

11. Banchereau, J., et al., *Immunobiology of Dendritic Cells.* Annual Review of Immunology, 2000. **18**(1): p. 767-811.

12. Zhang, N. and Michael J. Bevan, *CD8⁺ T Cells: Foot Soldiers of the Immune System.* Immunity. **35**(2): p. 161-168.

13. Best, J.A., et al., *Transcriptional insights into the CD8+ T cell response to infection and memory T cell formation.* Nat Immunol, 2013. **14**(4): p. 404-412.

14. Smith-Garvin, J.E., G.A. Koretzky, and M.S. Jordan, *T Cell Activation.* Annual Review of Immunology, 2009. **27**(1): p. 591-619.

15. Russ, B., et al., *T cell immunity as a tool for studying epigenetic regulation of cellular differentiation.* Frontiers in Genetics, 2013. **4**(218).

16. Curtsinger, J.M. and M.F. Mescher, *Inflammatory cytokines as a third signal for T cell activation.* Current opinion in immunology, 2010. **22**(3): p. 333-340.

17. Zhang, N. and Michael J. Bevan, *CD8⁺ T Cells: Foot Soldiers of the Immune System.* Immunity, 2011. **35**(2): p. 161-168.

18. Cui, W. and S.M. Kaech, *Generation of effector CD8(+) T cells and their conversion to memory T cells.* Immunological reviews, 2010. **236**: p. 151-166.

19. Cheng, L.E., et al., *Enhanced signaling through the IL-2 receptor in CD8(+) T cells regulated by antigen recognition results in preferential proliferation and expansion of responding CD8(+) T cells rather than promotion of cell death.* Proceedings of the National Academy of Sciences of the United States of America, 2002. **99**(5): p. 3001-3006.

20. Kaech, S.M., et al., *Molecular and Functional Profiling of Memory CD8 T Cell Differentiation.* Cell, 2002. **111**(6): p. 837-851.

21. Wherry, E.J. and R. Ahmed, *Memory CD8 T-Cell Differentiation during Viral Infection.* Journal of Virology, 2004. **78**(11): p. 5535.

22. Rappuoli, R., et al., *Vaccines, new opportunities for a new society.* Proceedings of the National Academy of Sciences, 2014. **111**(34): p. 12288.

23. Clem, A.S., *Fundamentals of Vaccine Immunology.* Journal of Global Infectious Diseases, 2011. **3**(1): p. 73-78.

24. Vetter, V., et al., *Understanding modern-day vaccines: what you need to know.* Annals of Medicine, 2018. **50**(2): p. 110-120.

25. Farber, D.L., et al., *Immunological memory: lessons from the past and a look to the future.* Nature Reviews Immunology, 2016. **16**: p. 124.

26. Crotty, S. and R. Ahmed, *Immunological memory in humans.* Seminars in Immunology, 2004. **16**(3): p. 197-203.

27. Pichichero, M.E., *Booster Vaccinations: Can Immunologic Memory Outpace Disease Pathogenesis?* Pediatrics, 2009. **124**(6): p. 1633.

28. Butler, N.S., J.C. Nolz, and J.T. Harty, *Immunologic Considerations for Generating Memory CD8 T Cells through Vaccination.* Cellular microbiology, 2011. **13**(7): p. 925-933.

29. Ahlers, J.D. and I.M. Belyakov, *Memories that last forever: strategies for optimizing vaccine T-cell memory.* Blood, 2010. **115**(9): p. 1678.

30. van Duikeren, S., et al., *Vaccine-Induced Effector-Memory CD8⁺ T Cell Responses Predict Therapeutic Efficacy against Tumors.* The Journal of Immunology, 2012. **189**(7): p. 3397.

31. Samji, T. and K.M. Khanna, *Understanding memory CD8+ T cells.* Immunology Letters, 2017. **185**: p. 32-39.

32. Roberts, A.D., K.H. Ely, and D.L. Woodland, *Differential contributions of central and effector memory T cells to recall responses.* The Journal of experimental medicine, 2005. **202**(1): p. 123-133.

33. Obar, J.J. and L. Lefrançois, *Memory CD8(+) T cell differentiation.* Annals of the New York Academy of Sciences, 2010. **1183**: p. 251-266.

34. Mueller, S.N., et al., *Memory T Cell Subsets, Migration Patterns, and Tissue Residence.* Annual Review of Immunology, 2013. **31**(1): p. 137-161.

35. Schenkel, J.M. and D. Masopust, *Tissue-resident memory T cells.* Immunity, 2014. **41**(6): p. 886-897.

36. Pan, Y., et al., *Survival of tissue-resident memory T cells requires exogenous lipid uptake and metabolism.* Nature, 2017. **543**: p. 252.

37. Araki, K., et al., *mTOR regulates memory CD8 T cell differentiation.* Nature, 2009. **460**(7251): p. 108-112.

38. Pearce, E.L., et al., *Enhancing CD8 T-cell memory by modulating fatty acid metabolism.* Nature, 2009. **460**(7251): p. 103-107.

39. Hamon, M., H. Bierne, and P. Cossart, *Listeria monocytogenes: a multifaceted model.* Nature Reviews Microbiology, 2006. **4**: p. 423.

40. Khan, S.H. and V.P. Badovinac, *Listeria monocytogenes: a model pathogen to study antigen-specific memory CD8 T cell responses.* Seminars in Immunopathology, 2015. **37**(3): p. 301-310.

41. Mackaness, G.B., *Cellular Resistance to Infection.* The Journal of Experimental Medicine, 1962. **116**(3): p. 381-406.

42. Stavru, F., C. Archambaud, and P. Cossart, *Cell biology and immunology of Listeria monocytogenes infections: novel insights.* Immunological Reviews, 2011. **240**(1): p. 160-184.

43. Kaech, S.M., et al., *Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells.* Nature Immunology, 2003. **4**: p. 1191.

44. Zhou, X., et al., *Differentiation and persistence of memory CD8(+) T cells depend on T cell factor 1.* Immunity, 2010. **33**(2): p. 229-240.

45. Yuzefpolskiy, Y., et al., *Early CD8 T-cell memory precursors and terminal effectors exhibit equipotent in vivo degranulation.* Cellular And Molecular Immunology, 2014. **12**: p. 400.

46. Joshi, N.S. and S.M. Kaech, *Effector CD8 T Cell Development: A Balancing Act between Memory Cell Potential and Terminal Differentiation.* The Journal of Immunology, 2008. **180**(3): p. 1309.

47. Hand, T.W., M. Morre, and S.M. Kaech, *Expression of IL-7 receptor α is necessary but not sufficient for the formation of memory CD8 T cells during viral infection.* Proceedings of the National Academy of Sciences, 2007. **104**(28): p. 11730.

48. Miller, J.D., et al., *Human Effector and Memory CD8⁺ T Cell Responses to Smallpox and Yellow Fever Vaccines.* Immunity, 2008. **28**(5): p. 710-722.

49. Hogquist, K.A., et al., *T cell receptor antagonist peptides induce positive selection.* Cell, 1994. **76**(1): p. 17-27.

50. Pape, K.A., et al., *Use of adoptive transfer of T-cell antigen-receptor-transgenic T cells for the study of T-cell activation in vivo.* Immunological Reviews, 1997. **156**(1): p. 67-78.

51. Bettelli, E., et al., *Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells.* Nature, 2006. **441**: p. 235.

52. Cui, G., et al., *IL-7-Induced Glycerol Transport and TAG Synthesis Promotes Memory CD8+ T Cell Longevity.* Cell, 2015. **161**(4): p. 750-761.

53. Pope, C., et al., *Organ-Specific Regulation of the CD8 T Cell Response to Listeria monocytogenes Infection.* The Journal of Immunology, 2001. **166**(5): p. 3402.

54. Klonowski, K.D., et al., *CD8 T Cell Recall Responses Are Regulated by the Tissue Tropism of the Memory Cell and Pathogen.* Journal of immunology (Baltimore, Md. : 1950), 2006. **177**(10): p. 6738-6746.

55. Clarke Sally, R., et al., *Characterization of the ovalbumin-specific TCR transgenic line OT-I: MHC elements for positive and negative selection.* Immunology and Cell Biology, 2000. **78**(2): p. 110-117.

56. McKenzie, M.D., et al., *Perforin and Fas induced by IFNγ and TNFα mediate beta cell death by OT-I CTL.* International Immunology, 2006. **18**(6): p. 837-846.

57. Mehlhop-Williams, E.R. and M.J. Bevan, *Memory CD8⁺ T cells exhibit increased antigen threshold requirements for recall proliferation.* The Journal of Experimental Medicine, 2014. **211**(2): p. 345-356.

58. Charbonneau, H., et al., *The leukocyte common antigen (CD45): a putative receptor-linked protein tyrosine phosphatase.* Proceedings of the National Academy of Sciences, 1988. **85**(19): p. 7182.

59. Shen, F.W., et al., *Cloning of Ly-5 cDNA.* Proceedings of the National Academy of Sciences, 1985. **82**(21): p. 7360.

60. Moon, J.J., et al., *Tracking epitope-specific T cells.* Nature protocols, 2009. **4**(4): p. 565-581.

61. Stemberger, C., et al., *A Single Naive CD8+ T Cell Precursor Can Develop into Diverse Effector and Memory Subsets.* Immunity, 2007. **27**(6): p. 985-997.

62. Schluns, K.S., et al., *Cutting Edge: Requirement for IL-15 in the Generation of Primary and Memory Antigen-Specific CD8 T Cells.* The Journal of Immunology, 2002. **168**(10): p. 4827.

63. Li, X.-b., J.-d. Gu, and Q.-h. Zhou, *Review of aerobic glycolysis and its key enzymes – new targets for lung cancer therapy.* Thoracic Cancer, 2015. **6**(1): p. 17-24.

64. Lunt, S.Y. and M.G. Vander Heiden, *Aerobic Glycolysis: Meeting the Metabolic Requirements of Cell Proliferation.* Annual Review of Cell and Developmental Biology, 2011. **27**(1): p. 441-464.

65. Pietrocola, F., et al., *Acetyl Coenzyme A: A Central Metabolite and Second Messenger.* Cell Metabolism, 2015. **21**(6): p. 805-821.

66. Shi, L. and B.P. Tu, *Acetyl-CoA and the regulation of metabolism: mechanisms and consequences.* Current Opinion in Cell Biology, 2015. **33**: p. 125-131.

67. Da Poian, A.T., El-Bacha, T. & Luz, M. R.M.P. *Nutrient Utilization in Humans: Metabolism Pathways*. 2010. **3**, 11.

68. David L. Nelson, M.M.C., *Lehninger Principles of Biochemistry*. Vol. 5. 2008: Freeman.

69. Houten, S.M. and R.J.A. Wanders, *A general introduction to the biochemistry of mitochondrial fatty acid β-oxidation.* Journal of Inherited Metabolic Disease, 2010. **33**(5): p. 469-477.

70. Bartlett, K. and S. Eaton, *Mitochondrial β-oxidation.* European Journal of Biochemistry, 2004. **271**(3): p. 462-469.

71. Sharma, S. and S.M. Black, *Carnitine Homeostasis, Mitochondrial Function, and Cardiovascular Disease.* Drug discovery today. Disease mechanisms, 2009. **6**(1-4): p. e31-e39.

72. Qu, Q., et al., *Fatty acid oxidation and carnitine palmitoyltransferase I: emerging therapeutic targets in cancer.* Cell Death &Amp; Disease, 2016. **7**: p. e2226.

73. Brown, M.S. and J.L. Goldstein, *The SREBP Pathway: Regulation of Cholesterol Metabolism by Proteolysis of a Membrane-Bound Transcription Factor.* Cell, 1997. **89**(3): p. 331-340.

74. Chan, J.P., et al., *The lysolipid transporter Mfsd2a regulates lipogenesis in the developing brain.* PLOS Biology, 2018. **16**(8): p. e2006443.

75. Jeon, T.-I. and T.F. Osborne, *SREBPs: metabolic integrators in physiology and metabolism.* Trends in Endocrinology & Metabolism, 2012. **23**(2): p. 65-72.

76. Horton, J.D., J.L. Goldstein, and M.S. Brown, *SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver.* The Journal of Clinical Investigation, 2002. **109**(9): p. 1125-1131.

77. Shimano, H. and R. Sato, *SREBP-regulated lipid metabolism: convergent physiology — divergent pathophysiology.* Nature Reviews Endocrinology, 2017. **13**: p. 710.

78. Bantug, G.R., et al., *The spectrum of T cell metabolism in health and disease.* Nature Reviews Immunology, 2017. **18**: p. 19.

79. Buck, M.D., D. O'Sullivan, and E.L. Pearce, *T cell metabolism drives immunity.* The Journal of Experimental Medicine, 2015. **212**(9): p. 1345-1360.

80. MacIver, N.J., R.D. Michalek, and J.C. Rathmell, *Metabolic Regulation of T Lymphocytes.* Annual Review of Immunology, 2013. **31**(1): p. 259-283.

81. Almeida, L., et al., *Metabolic pathways in T cell activation and lineage differentiation.* Seminars in Immunology, 2016. **28**(5): p. 514-524.

82. Palmer, C.S., et al., *Glucose Metabolism Regulates T Cell Activation, Differentiation, and Functions.* Frontiers in Immunology, 2015. **6**(1).

83. Vander Heiden, M.G., L.C. Cantley, and C.B. Thompson, *Understanding the Warburg Effect: The Metabolic Requirements of Cell Proliferation.* Science, 2009. **324**(5930): p. 1029.

84. Ghesquière, B., et al., *Metabolism of stromal and immune cells in health and disease.* Nature, 2014. **511**: p. 167.

85. Delgoffe, G.M. and J.D. Powell, *Sugar, fat, and protein: new insights into what T cells crave.* Current Opinion in Immunology, 2015. **33**: p. 49-54.

86. O'Sullivan, D., et al., *Memory CD8+ T Cells Use Cell-Intrinsic Lipolysis to Support the Metabolic Programming Necessary for Development.* Immunity, 2014. **41**(1): p. 75-88.

87. Zhang, L., et al., *Mammalian Target of Rapamycin Complex 2 Controls CD8 T Cell Memory Differentiation in a Foxo1-Dependent Manner.* Cell Reports, 2016. **14**(5): p. 1206-1217.

88. Lochner, M., L. Berod, and T. Sparwasser, *Fatty acid metabolism in the regulation of T cell function.* Trends in Immunology. **36**(2): p. 81-91.

89. van der Windt, G.J.W., et al., *Mitochondrial Respiratory Capacity Is A Critical Regulator Of CD8(+) T Cell Memory Development.* Immunity, 2012. **36**(1): p. 68-78.

90. Howie, D., et al., *The Role of Lipid Metabolism in T Lymphocyte Differentiation and Survival.* Frontiers in Immunology, 2018. **8**(1949).

91. Ibitokou, S.A., et al., *Early Inhibition of Fatty Acid Synthesis Reduces Generation of Memory Precursor Effector T Cells in Chronic Infection.* The Journal of Immunology, 2018. **200**(2): p. 643.

92. Sukumar, M., et al., *Inhibiting glycolytic metabolism enhances CD8+ T cell memory and antitumor function.* The Journal of Clinical Investigation, 2013. **123**(10): p. 4479-4488.

93. van der Windt, G.J.W. and E.L. Pearce, *Metabolic switching and fuel choice during T-cell differentiation and memory development.* Immunological reviews, 2012. **249**(1): p. 27-42.

94. O'Connor, R.S., et al., *The CPT1a inhibitor, etomoxir induces severe oxidative stress at commonly used concentrations.* Scientific Reports, 2018. **8**(1): p. 6289.

95. Raud, B., et al., *Fatty acid metabolism in CD8+ T cell memory: Challenging current concepts.* Immunological Reviews, 2018. **283**(1): p. 213-231.

96. Raud, B., et al., *Etomoxir Actions on Regulatory and Memory T Cells Are Independent of Cpt1a-Mediated Fatty Acid Oxidation.* Cell Metabolism, 2018. **28**(3): p. 504-515.e7.

97. Divakaruni, A.S., et al., *Etomoxir Inhibits Macrophage Polarization by Disrupting CoA Homeostasis.* Cell Metabolism, 2018. **28**(3): p. 490-503.e7.

98. Macintyre, A.N., et al., *The Glucose Transporter Glut1 is Selectively Essential for CD4 T Cell Activation and Effector Function.* Cell metabolism, 2014. **20**(1): p. 61-72.

99. Wofford, J.A., et al., *IL-7 promotes Glut1 trafficking and glucose uptake via STAT5-mediated activation of Akt to support T-cell survival.* Blood, 2007. **111**(4): p. 2101-2111.

100. Sinclair, L.V., et al., *Control of amino-acid transport by antigen receptors coordinates the metabolic reprogramming essential for T cell differentiation.* Nature Immunology, 2013. **14**: p. 500.

101. Topham, D.J. and E.C. Reilly, *Tissue-Resident Memory CD8(+) T Cells: From Phenotype to Function.* Frontiers in Immunology, 2018. **9**: p. 515.

102. Schmitz, G. and K. Ruebsaamen, *Metabolism and atherogenic disease association of lysophosphatidylcholine.* Atherosclerosis, 2010. **208**(1): p. 10-18.

103. Boggs, K.P., C.O. Rock, and S. Jackowski, *Lysophosphatidylcholine and 1-O-Octadecyl-2-O-Methyl-rac- Glycero-3-Phosphocholine Inhibit the CDP-Choline Pathway of Phosphatidylcholine Synthesis at the CTP:Phosphocholine Cytidylyltransferase Step.* Journal of Biological Chemistry, 1995. **270**(13): p. 7757-7764.

104. Vance, D.E., *Role of phosphatidylcholine biosynthesis in the regulation of lipoprotein homeostasis.* Current Opinion in Lipidology, 2008. **19**(3): p. 229-234.

105. Munder, P.G., et al., *Lysophosphatidylcholine (lysolecithin) and its synthetic analogues. Immunemodulating and other biologic effects.* Springer Seminars in Immunopathology, 1979. **2**(2): p. 187-203.

106. Nguyen, L.N., et al., *Mfsd2a is a transporter for the essential omega-3 fatty acid docosahexaenoic acid.* Nature, 2014. **509**(7501): p. 503-506.

107. Drzazga Anna, S.A., and Koziolkiewiz Maria, *Lysophosphatidylcholine and Lysophosphatidylinosiol -- Novel Promissing Signaling Molecules and Their Possible*

Therapeutic Activity. Acta Poloniae Pharmaceutica -- Druge Research, 2014. **71**(6): p. 887-899.

108. Kabarowski, J.H., *G2A and LPC: Regulatory functions in immunity.* Prostaglandins & Other Lipid Mediators, 2009. **89**(3): p. 73-81.

109. Silva-Neto, M.A.C., A.H. Lopes, and G.C. Atella, *Here, There, and Everywhere: The Ubiquitous Distribution of the Immunosignaling Molecule Lysophosphatidylcholine and Its Role on Chagas Disease.* Frontiers in Immunology, 2016. **7**(62).

110. Horrocks, L.A. and Y.K. Yeo, *Health Benefits of Docosahexaenoic Acid (DHA).* Pharmacological Research, 1999. **40**(3): p. 211-225.

111. Bozzatello, P., et al., *Supplementation with Omega-3 Fatty Acids in Psychiatric Disorders: A Review of Literature Data.* Journal of Clinical Medicine, 2016. **5**(8).

112. Zibaeenezhad, M.J., et al., *Comparison of the effect of omega-3 supplements and fresh fish on lipid profile: a randomized, open-labeled trial.* Nutrition & Diabetes, 2017. **7**(12): p. 1.

113. Dyall, S.C., *Long-chain omega-3 fatty acids and the brain: a review of the independent and shared effects of EPA, DPA and DHA.* Frontiers in Aging Neuroscience, 2015. **7**(52).

114. Anderson, G.J., W.E. Connor, and J.D. Corliss, *Docosahexaenoic Acid Is the Preferred Dietary n-3 Fatty Acid for the Development of the Brain and Retina.* Pediatr Res, 1990. **27**(1): p. 89-97.

115. Wong, B.H., et al., *Mfsd2a Is a Transporter for the Essential ω-3 Fatty Acid Docosahexaenoic Acid (DHA) in Eye and Is Important for Photoreceptor Cell Development.* Journal of Biological Chemistry, 2016. **291**(20): p. 10501-10514.

116. Guemez-Gamboa, A., et al., *Inactivating mutations in MFSD2A, required for omega-3 fatty acid transport in brain, cause a lethal microcephaly syndrome.* Nat Genet, 2015. **47**(7): p. 809-813.

117. Prieto-Sánchez, M.T., et al., *Placental MFSD2a transporter is related to decreased DHA in cord blood of women with treated gestational diabetes.* Clinical Nutrition, 2017. **36**(2): p. 513-521.

118. McMurray, H.F., S. Parthasarathy, and D. Steinberg, *Oxidatively modified low density lipoprotein is a chemoattractant for human T lymphocytes.* The Journal of Clinical Investigation, 1993. **92**(2): p. 1004-1008.

119. Im, Y.-j., et al., *Multiple actions of lysophosphatidylcholine in human Jurkat T cells.* Acta Pharmacologica Sinica, 2006. **27**: p. 700.

120. Asaoka, Y., et al., *Role of lysophosphatidylcholine in T-lymphocyte activation: involvement of phospholipase A2 in signal transduction through protein kinase C.* Proceedings of the National Academy of Sciences of the United States of America, 1992. **89**(14): p. 6447-6451.

121. Hasegawa, H., et al., *Lysophosphatidylcholine enhances the suppressive function of human naturally occurring regulatory T cells through TGF-β production.* Biochemical and Biophysical Research Communications, 2011. **415**(3): p. 526-531.

122. Bendelac, A., P.B. Savage, and L. Teyton, *The Biology of NKT Cells.* Annual Review of Immunology, 2007. **25**(1): p. 297-336.

123. Godfrey, D.I., S. Stankovic, and A.G. Baxter, *Raising the NKT cell family.* Nat Immunol, 2010. **11**(3): p. 197-206.

124. Fox, L.M., et al., *Recognition of Lyso-Phospholipids by Human Natural Killer T Lymphocytes.* PLOS Biology, 2009. **7**(10): p. e1000228.

125. Salio, M. and V. Cerundolo, *Linking Inflammation to Natural Killer T Cell Activation.* PLOS Biology, 2009. **7**(10): p. e1000226.

126. Law, C.J., P.C. Maloney, and D.-N. Wang, *Ins and Outs of Major Facilitator Superfamily Antiporters.* Annual Review of Microbiology, 2008. **62**(1): p. 289-305.

127. Angers, M., et al., *Mfsd2a encodes a novel major facilitator superfamily domain-containing protein highly induced in brown adipose tissue during fasting and adaptive thermogenesis.* Biochemical Journal, 2008. **416**(3): p. 347-355.

128. Berger, J.H., M.J. Charron, and D.L. Silver, *Major Facilitator Superfamily Domain-Containing Protein 2a (MFSD2A) Has Roles in Body Growth, Motor Function, and Lipid Metabolism.* PLoS ONE, 2012. **7**(11): p. e50629.

129. Reiling, J.H., et al., *A haploid genetic screen identifies the major facilitator domain containing 2A (MFSD2A) transporter as a key mediator in the response to tunicamycin.* Proceedings of the National Academy of Sciences, 2011. **108**(29): p. 11756-11765.

130. Quek, D.Q.Y., et al., *Structural insights into the transport mechanism of the human sodium-dependent lysophosphatidylcholine transporter Mfsd2a.* Journal of Biological Chemistry, 2016.

131. Moritake, H., et al., *A mouse model reveals that Mfsd2a is critical for unfolded protein response upon exposure to tunicamycin.* Human Cell, 2017. **30**(2): p. 88-97.

132. Yang, Y.R., et al., *Mfsd2a (Major Facilitator Superfamily Domain Containing 2a) Attenuates Intracerebral Hemorrhage–Induced Blood–Brain Barrier Disruption by Inhibiting Vesicular Transcytosis.* Journal of the American Heart Association, 2017. **6**(7).

133. Ben-Zvi, A., et al., *Mfsd2a is critical for the formation and function of the blood-brain barrier.* Nature, 2014. **509**(7501): p. 507-511.

134. Zhao, Z. and Berislav V. Zlokovic, *Blood-Brain Barrier: A Dual Life of MFSD2A?* Neuron, 2014. **82**(4): p. 728-730.

135. Simopoulos, A.P., *Omega-3 fatty acids in health and disease and in growth and development.* The American Journal of Clinical Nutrition, 1991. **54**(3): p. 438-63.

136. Gázquez, A., M. Ruíz-Palacios, and E. Larqué, *DHA supplementation during pregnancy as phospholipids or TAG produces different placental uptake but similar fetal brain accretion in neonatal piglets.* British Journal of Nutrition, 2017. **118**(11): p. 981-988.

137. Hirschmugl, B., et al., *Relation of placental alkaline phosphatase expression in human term placenta with maternal and offspring fat mass.* International Journal of Obesity, 2018. **42**(6): p. 1202-1210.

138. Awad, H., et al., *MiR-155 deletion reduces ischemia-induced paralysis in an aortic aneurysm repair mouse model: Utility of immunohistochemistry and histopathology in understanding etiology of spinal cord paralysis.* Annals of Diagnostic Pathology, 2018. **36**: p. 12-20.

139. Wang, J.-Z., et al., *Mfsd2a-based pharmacological strategies for drug delivery across the blood–brain barrier.* Pharmacological Research, 2016. **104**: p. 124-131.

140. Sandoval, K.E., et al., *Mfsd2a and Glut1 Brain Nutrient Transporters Expression Increase with 32-Week Low and High Lard vs. Fish Oil Dietary Treatment in C57Bl/6 Mice.* Current Developments in Nutrition, 2018: p. nzy065-nzy065.

141. Milanovic, D., et al., *Short-Term Fish Oil Treatment Changes the Composition of Phospholipids While Not Affecting the Expression of Mfsd2a Omega-3 Transporter in the Brain and Liver of the 5xFAD Mouse Model of Alzheimer's Disease.* Nutrients, 2018. **10**(9): p. 1250.

142. Alakbarzade, V., et al., *A partially inactivating mutation in the sodium-dependent lysophosphatidylcholine transporter MFSD2A causes a non-lethal microcephaly syndrome.* Nat Genet, 2015. **47**(7): p. 814-817.

143. Harel, T., et al., *Homozygous mutation in MFSD2A, encoding a lysolipid transporter for docosahexanoic acid, is associated with microcephaly and hypomyelination.* neurogenetics, 2018.

144. Tiwary, S., et al., *Metastatic Brain Tumors Disrupt the Blood-Brain Barrier and Alter Lipid Metabolism by Inhibiting Expression of the Endothelial Cell Fatty Acid Transporter Mfsd2a.* Scientific Reports, 2018. **8**(1): p. 8267.

145. Chen, S., et al., *Association of polymorphisms and haplotype in the region of TRIT1 , MYCL1 and MFSD2A with the risk and clinicopathological features of gastric cancer in a southeast Chinese population.* Carcinogenesis, 2013. **34**(5): p. 1018-1024.

146. Ungaro, F., et al., *MFSD2A Promotes Endothelial Generation of Inflammation-Resolving Lipid Mediators and Reduces Colitis in Mice.* Gastroenterology. **153**(5): p. 1363-1377.e6.

147. Danese, S. and C. Fiocchi, *Ulcerative Colitis.* New England Journal of Medicine, 2011. **365**(18): p. 1713-1725.

148. Pu, W., et al., *Mfsd2a+ hepatocytes repopulate the liver during injury and regeneration.* Nature Communications, 2016. **7**: p. 13369.

149. Piccirillo, A., *The role of lipid transporter MFSD2A in innate and adaptive effector T cells*, in *Dept. of Human Genetics*. 2016: University of Pittsburgh.

150. Zhang, L. and P. Romero, *Metabolic Control of CD8⁺ T Cell Fate Decisions and Antitumor Immunity.* Trends in Molecular Medicine, 2018. **24**(1): p. 30-48.

151. Jones, N., et al., *Metabolic Adaptation of Human CD4+ and CD8+ T-Cells to T-Cell Receptor-Mediated Stimulation.* Frontiers in Immunology, 2017. **8**(1516).

152. Zhang, X., J. Liu, and X. Cao, *Metabolic control of T-cell immunity via epigenetic mechanisms.* Cellular And Molecular Immunology, 2017. **15**: p. 203.

153. Li, S., et al., *Metabolic Phenotypes of Response to Vaccination in Humans.* Cell, 2017. **169**(5): p. 862-877.e17.

154. Panagioti, E., et al., *Features of Effective T Cell-Inducing Vaccines against Chronic Viral Infections.* Frontiers in Immunology, 2018. **9**(276).

155. Mak, T.W., et al., *Glutathione Primes T Cell Metabolism for Inflammation.* Immunity. **46**(4): p. 675-689.

156. Organization, W.H., *Interim Summary of Conclusions and Dietary Recommendations on Total Fat & Fatty Acids*, in *FAO/WHO Exper Consultation on Fats and Fatty Acids in Human Nutrition*. 2008: Geneva.

157. *Omega-3 Fatty Acids*. 2018, National Institutes of Health: Office of Dietary Supplements.

158. Collot-Teixeira, S., et al., *CD36 and macrophages in atherosclerosis.* Cardiovascular Research, 2007. **75**(3): p. 468-477.