# THE ROLE OF LIPID TRANSPORTER MFSD2A IN INNATE AND ADAPTIVE EFFECTOR T CELLS

by

Ann Renee Piccirillo

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This thesis was presented

by

**Ann Renee Piccirillo**

It was defended on

**April 20, 2016**

and approved by

**Thesis Advisor:**
Louise D'Cruz, PhD
Assistant Professor
Department of Immunology
School of Medicine
University of Pittsburgh

**Committee Member:**
F. Yesim Demirci, MD
Assistant Professor
Department of Human Genetics
Graduate School of Public Health
University of Pittsburgh

**Committee Member:**
Quasar Padiath, MBBS, PhD
Assistant Professor
Department of Human Genetics
Graduate School of Public Health
University of Pittsburgh

Louise D'Cruz, PhD

**THE ROLE OF LIPID TRANSPORTER MFSD2A IN INNATE AND ADAPTIVE EFFECTOR T CELLS**

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## ABSTRACT

Major facilitator superfamily domain containing 2a (MFSD2A) is a protein expressed by endothelial cells. It functions as a long chain fatty acid transporter, carrying fatty acids in the chemical form of lysophosphaditylcholine (LPC). It has recently been discovered that MFSD2A is the lipid transporter that carries docosahexaenoic acid (DHA) across the blood brain barrier into the brain. A role for MFSD2A in innate and adaptive T cells remains unclear. This is important to public health relevance because an understanding of how lipids are taken up and used during an activated effector T cell response is critical to fighting infection, immune disease, and even cancer. A search of the Immunological Genome Project (IMMGEN) Database reveals MFSD2A is upregulated in activated CD8 effector T cells exposed to *Listeria monocytogenes*, peaking at 12 hours post infection and decreasing over 48 hours. Preliminary microarray results of invariant natural killer T cells (iNKT), a form of innate cell, shows high upregulation of *MFSD2A* transcript upon *in vitro* activation with alpha-galactosylceramide. Results were validated using a real-time PCR approach in both activated iNKT and CD8 T cells. This was further supported by probing for MFSD2A protein analysis via western blot. *In vitro* activation studies using isolated CD8 T cells from C57BL/6 mice further support results by showing increased MFSD2A and LPC by flow cytometry analysis. Finally, a working *in vivo* congenic CD45.1 OT-I mouse infection model of *Listeria monocytogenes* tagged to OVA-peptide

recapitulates IMMGEN findings as MFSD2A is higher expressed at 12-72 hours post infection compared to wildtype OT-I control mice. These data all support the claim that MFSD2A and LPC play a critical role for lipid metabolism within the innate and adaptive effector T cell.

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# PREFACE

I would like to thank my thesis advisor, Louise D'Cruz, for taking a chance on a technician that just so happened to be a student and providing great mentorship throughout this process. I would like to thank the rest of my thesis committee for critical analysis of this work and ongoing encouragement with future work. Heather Buechel, Adolfo Frias, and Kaitlin Kiernan – thank you for being the best lab mates and for your help, advice, and comic relief whenever I needed it.

Thank you to my friends and family for your words of encouragement during this journey. A special thank you to Tyler for putting up with me, including late nights in the lab and long nights at my desk writing. In addition, thank you to my puppy Sidney for being happy and there for me no matter what. Thank you to my parents for instilling the work ethic in me that made this all possible. To all my family, friends, and past mentors – thank you for helping me to get to this point; I'm looking forward to what is yet to come.

# 1.0 BACKGROUND AND SIGNIFICANCE

## 1.1 INTRODUCTION TO EFFECTOR T CELLS

The immune system is composed of both fast-acting innate cells and delayed long-lasting adaptive cells that work together to keep the host free of pathogen. One innate cell type that helps to bridge the gap between innate and adaptive immune systems is the invariant natural killer T cell (iNKT). NKT cells undergo thymic development like other T cells but branch off at the double positive stage into a committed NKT lineage [1]. NKTs respond quickly to an immune response by using a semi-invariant CD1d-restricted T cell receptor (TCR); enabling rapid cytokine production and creating a memory-like NKT phenotype [1, 2]. Unlike traditional CD4 or CD8 T cells, NKT cells respond to lipid antigen. The first and most common NKT cell ligand originated from the marine sponge *Agelas mauritianus* [2]. It was found to be directly caused by alpha-branched galactosylceramide, now commonly referred to as aGalCer. Since their discovery, numerous subsets of NKT cells have constantly been discovered with a lineage based off of transcription factor differentiation and cytokine production [2]. Taking advantage of their unique and rapid effector phenotype, the potential use of NKT cells as cancer or autoimmune therapy is a continuously growing field. Figure 1 below shows the extensive differentiation of NKT cells, including their origin from double positive CD4 CD8 T cells that will be discussed below.

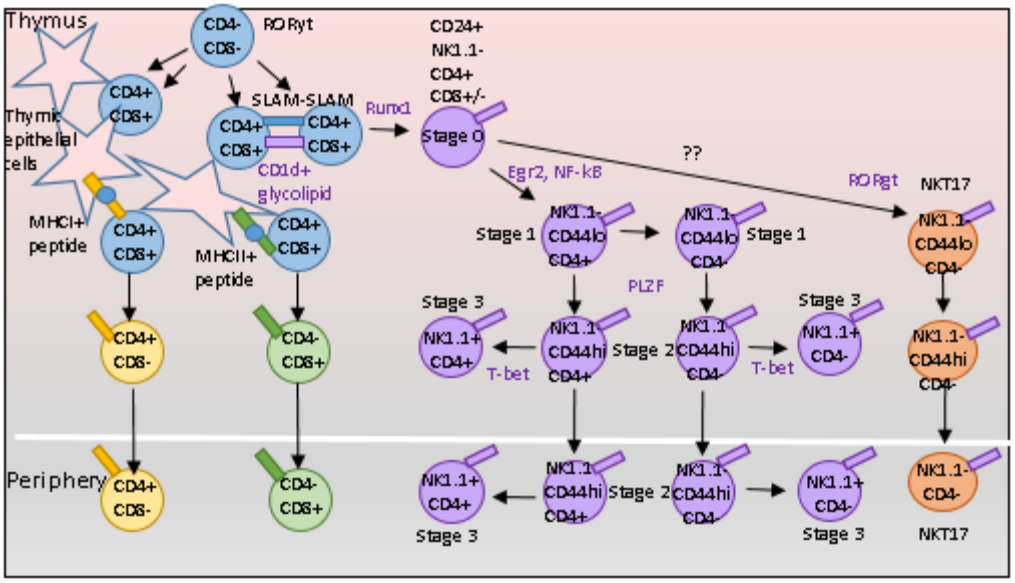



**Figure 1. NKT and CD4 CD8 T cell selection in the thymus**

**(adapted from Godfrey et al. )**

CD8 effector T cells also undergo thymic development (see above). CD8 T cells, unlike NKT cells, bind to glycoproteins bound to major histocompatibility complex I (MHC I), a surface receptor found on all nucleated cells [3, 4]. In order to become active, they require two stimuli. The first being the cell's TCR binding to MHC I. The second stimulus is the co-stimulus surface receptor CD28 on the T cell binding to CD80 CD86, however this can be enhanced or replaced by certain cytokines [5]. This usually occurs by means of an antigen presenting cell (APC), normally a dendritic cell in this case. One example of this 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 T cell.

## 1.2 EFFECTOR T CELL RESPONSE TO INFECTION IN MOUSE AND HUMAN

Upon infection, effector T cells undergo a transition from a quiescent, poor effector state, to metabolically active, proliferating cells that have the ability to produce cytokines rapidly[4, 5]. With such a large change in cellular state, it may not be surprising that this is accompanied by changes in gene expression at each stage of differentiation. Different pathogens can create different innate immune responses that go on to create an adaptive immune response that is infection-specific [4, 6]. Many of these signaling pathways are hot targets for vaccines and cancer therapy.

## 1.3 T CELLLIPIDOMICS AND METABOLISM

Naive T cells undergo major metabolic changes upon activation, infection, and cancer. Unactivated T cells are in a relatively quiescent state and predominately use oxidative phosphorylation (OXPHOS) as their metabolic means, whereas effector T cells primarily use glycolysis. A portion of these effector cells will differentiate into memory T cells that use both OXPHOS and lipolysis to fulfill energy requirements [6, 7]. This metabolic shift combined with increased energy demands for effector T cells indicates a need for diet and nutrition to properly generate an immune response. Research on the effector T cell immune response almost exclusively focuses on how these cells use metabolites to increase or decrease energy production [6-8]. Recent studies have focused on how activated effector T cells incorporate exogenous glycerol and glucose fuel sources to the synthesis of triacylglycerides and ATP [9, 10]. It is known that activated effector T cells can take up exogenous long chain fatty acids (LCFAs) from

their environment to assist with energy production [11]. There is, however, no knowledge on how effector T cells transport phospholipids such as lysophosphatidylcholine (LPC) and LCFAs across cellular membranes and what is regulating this process. This represents a huge gap in the understanding of effector T cell metabolism, especially in relation to how they use LCFAs to illicit an immune response.

## 1.4 INTRODUCTION TO LPC AND MFSD2A

LPC is a product of lysophospholipid (LPL) metabolism that is critical for maintaining cellular homeostasis with added therapeutic benefits such as wound healing and both autoimmunity and neurodegenerative treatment [12]. It is derived from polar surface phosphatidylcholine (PC) of lipoproteins or from cell membrane PC via phospholipase $A_2$ [13]. It can be either directly formed from 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. LPC acts as the chemical transporter of many species of fatty acids between cells, making it an important component to cellular metabolism. One such fatty acid is docosahexaenoic acid (DHA). In order to perform these actions, LPC requires a carrier molecule. This carrier molecule is Major Facilitator Superfamily Domain Containing 2a (MFSD2A).

MFSD2A is a 12 domain transmembrane protein. It has a well-established role as a lipid transporter that bears a close resemblance to the bacterial $Na^+$/melibiose supporter [14-16]. 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) [14, 17, 18].

It was first discovered as a fasting-induced gene that regulated PPARa and glucagon in the liver and brown adipose tissue (BAT), suggesting its role in lipid metabolism, growth, and thermogenesis [16, 17]. MFSD2A was subsequently found to be an important sodium-dependent tucinamycin transporter in humans and if knocked out in human cell lines, tunicamycin uptake would be significantly decreased [18]. This experiment supported MFSD2A's importance at the ER and role in cellular stress response. More recently, MFSD2A has been implicated in brain development and its important role as lipid transporter of DHA in the form of LPC across the blood brain barrier (BBB) [15, 19].

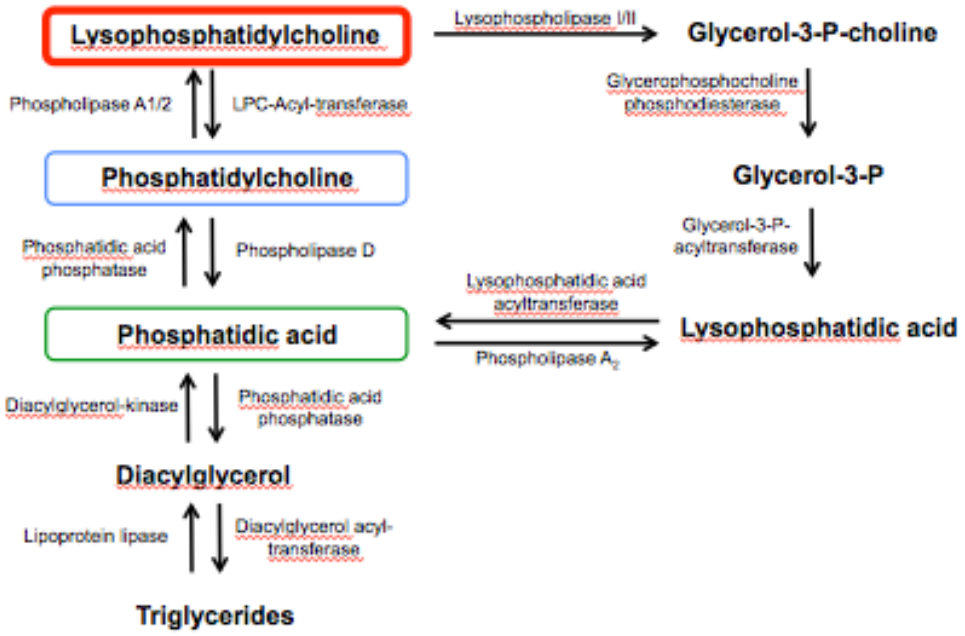



**Figure 2. Biochemical metabolism of LPC**

## 1.5 MFSD2A AND ITS RELATION TO DHA AND THE BRAIN

DHA has been well documented for its critical role in brain development [20-22]. 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 DHA in the form of LPC in a sodium- dependent manner [15].

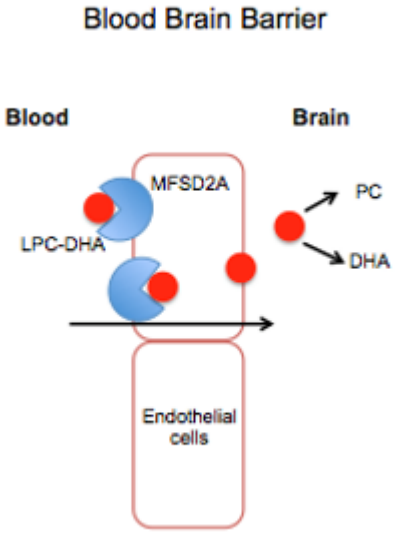



**Figure 3. MFSD2A/DHA interaction of BBB**

## 1.6 MFSD2A, IMMUNOLOGY, AND PUBLIC HEALTH

The mechanism by which effector T cells take up LCFAs and how it is regulated is still unknown. The potential role of MFSD2A as the carrier molecule of fatty acids into activated T cells by means of chemical form LPC would be a huge breakthrough in understand of effector T

cell metabolism. 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. The World Health Organization (WHO) and American Heart Association both recognize DHA as an important component to a healthy lifestyle. The research herein signifies an urgent need to understand and target how phospholipids, LCFAs, and DHA are imported into the effector T cell and how they impact the immune response to infection

## 1.7 OBJECTIVES

The main objective of this project is to uncover the role of MFSD2A in innate and adaptive effector T cell response. This has been accomplished using a multi-discipline approach of combining techniques including a literature review, molecular techniques, flow cytometry, and *in vitro* and *in vivo* mouse models under naïve and activated states. The following goals are used to determine the main objective:

1. Candidate gene studies on potential lipid transporter and effector T cell genes
2. A literature review for background on MFSD2A genotype and phenotype data
3. Molecular validation of MFSD2A results found in 1
4. *In vitro* activation analysis of MFSD2A in wildtype mouse
5. *In vivo* activation analysis of MFSD2A in transgenic mouse

The above goals are well illustrated in the figure below. The main objective of this project is to prove that MFSD2A with LPC are the means that effector T cells receive an influx of lipids upon activation and can then help to induce downstream effects to clear infection or tumors. A visual summary of experiments is summarized in the APPENDIX D flow chart.

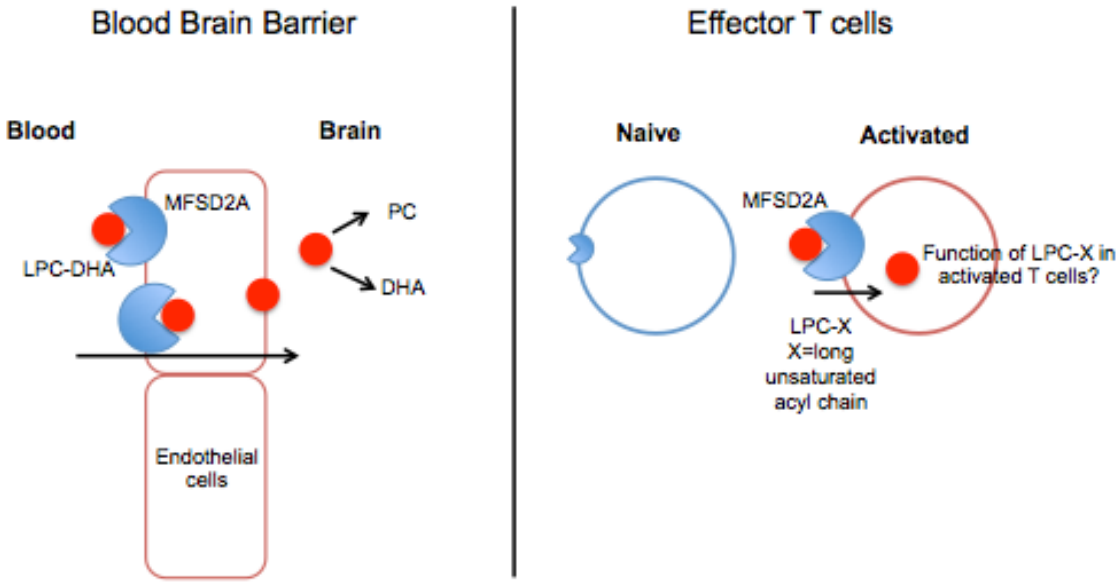



**Figure 4. Proposed role of MFSD2A and LPC in activated effector T cell**

Figure is shown relative to the known mechanism of MFSD2A and LPC in the brain to allow DHA to cross the BBB.

# 2.0 MATERIALS AND METHODS

## 2.1 MICROARRAY OF NKT TARGETS

A MoGene 1.0 mouse exon microarray chip (Affymetrix) was used to compare activated versus naïve NKT cells at 3 hours, 3 days, and 6 days. Cells from wildtype C57BL/6 mice (The Jackson Laboratory) were activated *in vivo* with aGC at 2 ug/mL.

## 2.2 DATABASE MINING OF MICROARRAY HITS

Top hits for fold-increase or decrease from microarray results from naïve NKT and 3 hour or 3 day activated NKT cells were further investigated by using the ImmGen database, a project of the Immunological Genome Project that can be found at www.immgen.org. A data browser search was performed using the Gene Skyline method to measure expression profile across a panel of immunologically relevant cell types and conditions. Specifically, activated T cells were searched for *MFSD2A*.

## 2.3 MFSD2A GENOTYPE AND PHENOTYPE LITERATURE SEARCH

MFSD2A was searched in both PubMed and OMIM databases. Almost all known data is exclusively related to brain function, development, and BBB. A database search of "MFSD2A and immunology" or "MFSD2A and T cells" contained no results.

## 2.4 NKT AND CD8 T CELL HARVESTING AND REAL-TIME PCR VALIDATION

Six 6-8 week old wild-type C57BL/6 mice were activated *in vivo* with either 2uM alpha-galactosylceramide or PBS for three hours then sacrificed. Spleen was harvested in PBS 2% FCS. Lymphocytes were extracted, pelleted, and counted. 20 million cells were stained for TCRb and CD1d-tetramer using antibodies from eBioscience and NIH Core Tetramer Facility at Emory University, respectively. iNKT cells were gated and collected on TCRb+ CD1d-tet+ using a BD FACS Aria cell sorter at the Flow Cytometry Core of the University of Pittsburgh. iNKT cells were sorted in Trizol (Invitrogen) and frozen at -80C until RNA extraction.

For CD8 T cells, 6-8 week old C57BL/6 mice were sacrificed and spleens harvested in PBS 2% FCS. Lymphocytes were extracted and counted. CD8 T cells were enriched using biotinylated CD8a antibody (eBioscience) and streptavidin-conjugated microbeads (Miltenyi Biotech, Inc.). CD8 T cells were counted and plated at $2x10^6$ cells/well in T cell media (RPMI, 10% FBS, 2% of each l-glutamine and pen-strep). Prior to plating, a 96 well plate was coated in anti-CD3 (eBioscience) for a minimum of 2 hours at 37C. Activated cells were cultured in wells coated with anti-CD3 and co-cultured with anti-CD28 (eBioscience) for 24, 48, and 72 hours. Cells were pelleted, resuspended in Trizol, and frozen at -80 until RNA extraction. Frozen Trizol

samples were thawed on ice. RNA was extracted using the Trizol Extraction Protocol For Sorted Cells found on the ImmGen database (https://www.immgen.org/Protocols/Total%20RNA%20Extraction%20with%20Trizol.pdf). Reverse transcription was performed using the All-in-One First Strand cDNA synthesis kit from Genecopoeia. Genomic DNA was removed using the DNA-free Removal Kit (Invitrogen). Real-time PCR was performed using the All-in-One qPCR kit (Genecopoeia) and a Roche LightCycler 96 SYBR Green analysis. Primers for *MFSD2A* were designed using a combination of NCBI Primer-BLAST and Primer Bank from Harvard University.

## 2.5 WESTERN BLOT FOR MFSD2A

Sample prep for CD8 T cells is the same as listed above. Cells were pelleted after 24, 48, and 72 hours of *in vitro* activation. Cells were incubated at 95C in SDS buffer containing b-mercaptoethanol for 5 minutes prior to Western blotting. Samples were ran on a 5-20% gradient gel (Bio-Rad) and transferred onto PVDF membrane on ice. Primary antibody for MFSD2A conjugated to anti-rabbit was purchased from Abcam. Membranes were visualized on Protein Simple FlouroChem machine (Biotechne). Quantification studies were performed using ImageJ software (NIH).

## 2.6 *IN VITRO* ANALYSIS FOR MFSD2A AND LPC

CD8 T cells were harvested as described above and stimulated with anti-CD3 anti-CD28 for 24, 48, and 72 hours. For each given timepoint, cells were co-cultured with TopFluor LPC (Avanti Polar Lipids) at 0.1uM. Cells were pelleted and stained for viability using Zombie Aqua viability dye (BioLegend). Cells were next surface stained for flow cytometry analysis using antibodies for CD8a, CD44, and CD62L (eBioscience). Cells were fixed and permeabilized using Cytofix/Cytoperm buffer kit (BD Biosciences). Permeabilized cells were stained with anti-MFSD2A (Abcam) conjugated to anti-rabbit PE to visualize. Stimulated CD8s were compared to naïve controls. TF-LPC is represented as GFP.

## 2.7 *IN VIVO* ANALYSIS FOR MFSD2A

The *in vivo* approach makes use of an adoptive transfer model distinguishable by CD45 common leukocyte antigen and OT-I transgenic CD8 T cells. The reasoning for using OT-I mice is that the transgenic Va2 Vb5 TCR is designed to recognize ovalbumin residues to study the role of peptides in the T cell's response to antigen. Mice naively express almost exclusive CD8+ MHC I T cells with a very low population of CD4+ MHC II T cells. The percent of CD8+ cells that express Va2 Vb5 TCR is roughly 20%.

$1x10^6$ transgenic OT-I Va2 CD8 T cells were adoptively transferred retrorbitally into congenically labeled CD45.1 C57BL/6 mice. On the following day, mice were injected IV with 5000 CFU *listeria*-OVA. Mice were bled from the submandibular vein at 1, 2, 3, 5, and 7 days into PBS 0.5% EDTA. A diagram of this model is in Appendix C. Lymphocytes were isolated

from whole blood and analyzed by flow cytometry as described above. Additionally, mice were stained for KLRG1 and CD127 to track the state of infection (effector versus effector memory).

The below figure is representative of FACS plots for typical infection timecourse. Total MFSD2A is measured directly from CD45.1-/CD8+/Va2+/CD45.2+ OT-I cells.

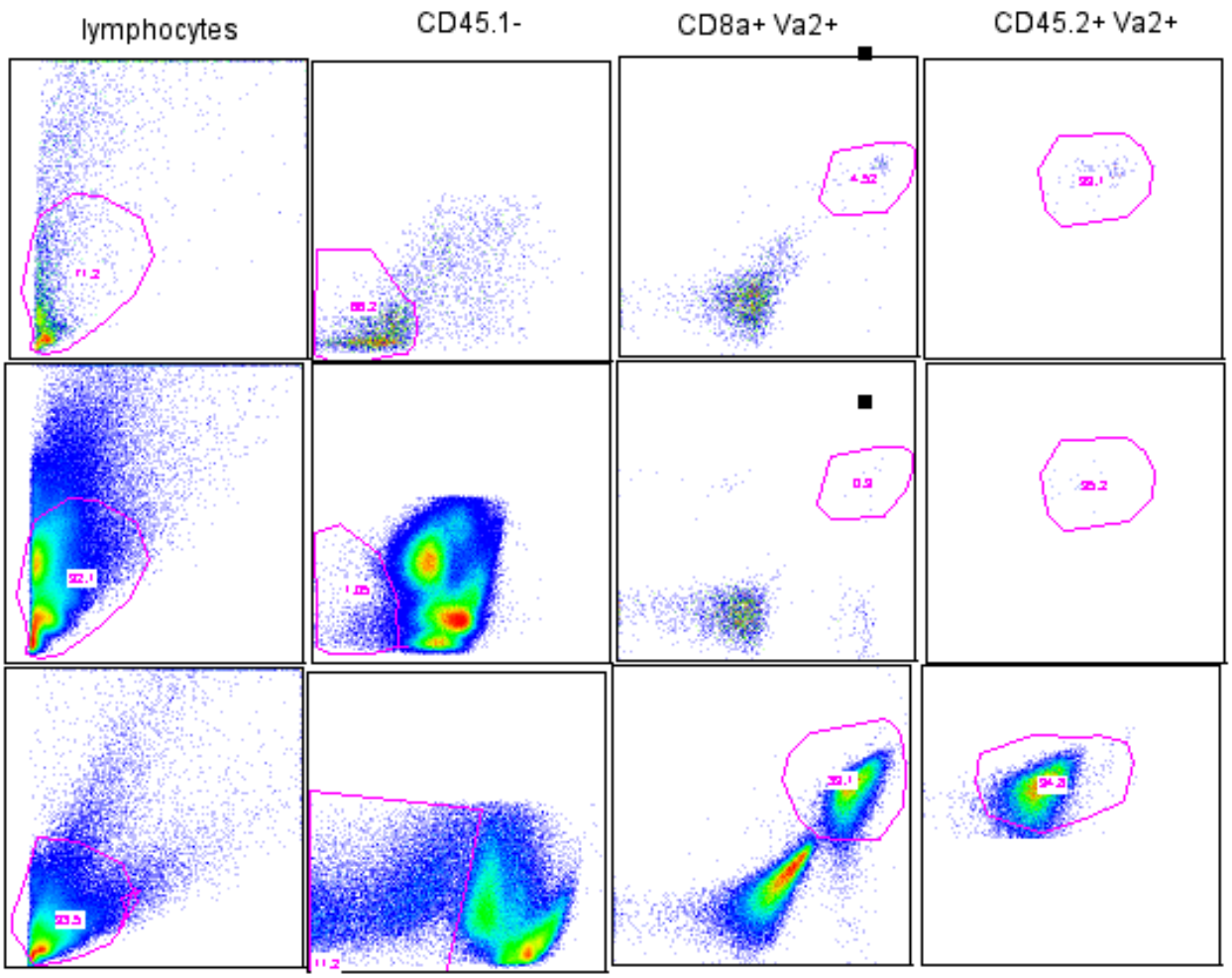



**Figure 5. *In vivo* model for MFSD2A FACS analysis**

The top of Fig. 5 is positive control naïve OT-I cells. Middle panel is day 3 early infection. Bottom panel is day 7 peak of infection. Gating schematic is as follows, going across: SSC FSC gate of total lymphocyte population from blood. Next, gate out CD45.1 recipient cells.

Then, gate on double positive CD8a Va2 cells. These are the donor OT-I cells. To confirm, regate this population on CD45.2 Va2 and it should be near 100% pure.

# 3.0 RESULTS

## 3.1 MICROARRAY RESULTS

Summarized results of top hits for genes upregulated in iNKT cells activated *in vivo* with 2 ug/mL aGalCer. Most results contain genes with a known association with the immune system and where therefore not further pursued.

**Table 1. iNKT microrray results summary**

| GeneSymbol | Day 0 | 3 hours | Day 3 | Day 6 | Function |
|---|---|---|---|---|---|
| Ccl3 | 74.79 | 9363.07 | 128.58 | 51.92 | CD8, macrophage, DC attractant |
| Nr4a2 | 90.24 | 4569.04 | 91.99 | 68.56 | IL-17 and IFNg production |
| Cd24a | 29.92 | 1408.34 | 35.40 | 117.29 | iNKT precursor marker |
| Cxcl9 | 70.63 | 1118.71 | 67.84 | 69.79 | chemokine; T cell trafficking |
| Nr4a3 | 194.97 | 6965.09 | 93.62 | 140.29 | steroid-thyroid hormone receptor |
| Nr4a1 | 260.10 | 5331.74 | 214.99 | 292.40 | T cell differentiation; cell death |
| Il2ra | 2024.47 | 7308.64 | 348.11 | 194.07 | Proliferation B and T cells |
| Egr3 | 217.11 | 1996.41 | 271.41 | 279.97 | Inflammation control; Proliferation |
| Sema7a | 73.87 | 2706.50 | 278.18 | 230.85 | Inflammation |
| Ifng | 1195.73 | 11303.52 | 746.93 | 560.11 | cytokine |
| Mfsd2a | 61.78 | 858.57 | 182.59 | 82.92 | fatty acid transport |

***MFSD2A* results highlighted in red. All genes normalized to *GAPDH*.**

## 3.2 DATABASE MINING RESULTS

Results from search of the ImmGen database for *MFSD2A*. Results are comparing transgenic OT-I CD8 T cells to naïve CD8 T cells for *MFSD2A*. This is an in vivo model of infection characterizing response to relative amount of OVA peptide tagged *Listeria monocytogenes* at given timepoints. Peak infection is represented at 8d. Memory timeframe is 45d. Note that expression in naïve is near 60 fold.

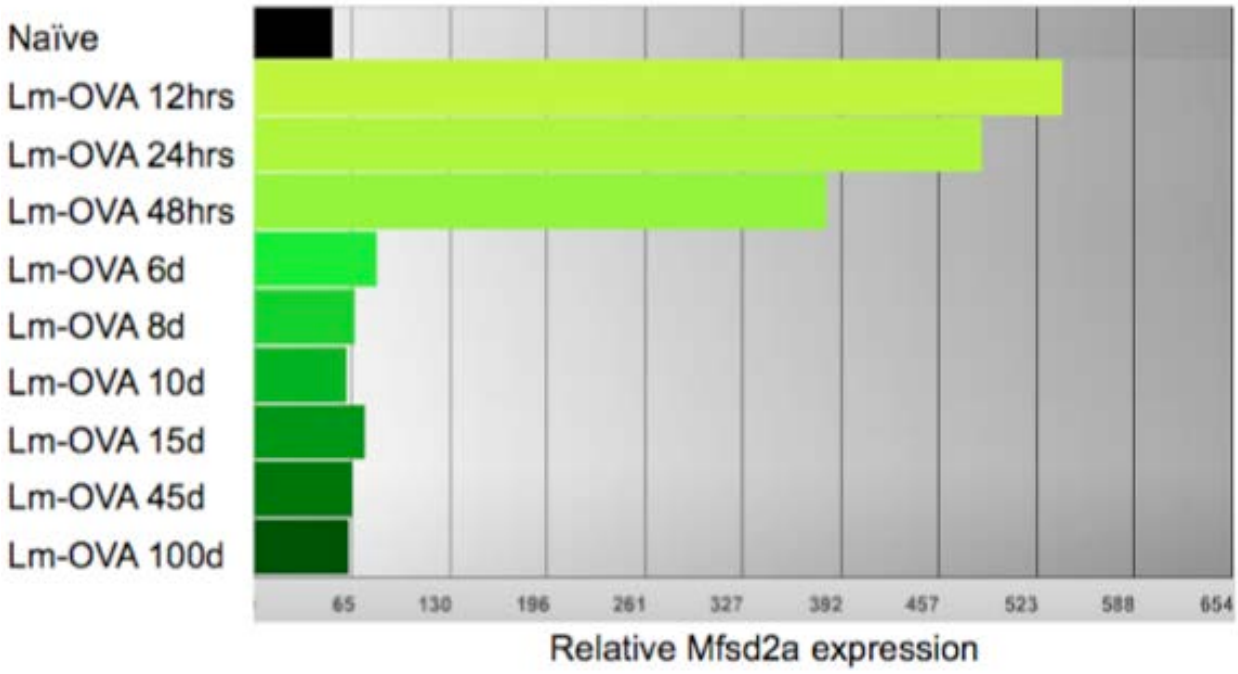



**Figure 6. IMMGEN database results for *MFSD2A***

**Relative amount of *MFSD2A* in naïve versus in vivo activated OT-I CD8 T cells. Lm-OVA; *Listeria monocytogenes*.**

## 3.3 MFSD2A GENOTYPING AND PHENOTYPING RESULTS

NCBI's PubMed biomedical database and OMIM (Online Mendelian Inheritance of Man) result in two hits, summarized in a total of three families with known MFSD2A point mutation syndromes. Families from Libya and Egypt suffer from a completely inactivating loss of MFSD2A, while the family from Pakistan has a partially inactivating loss of MFSD2A[14, 23]. Phenotypes are similar however a completely inactivating mutation results in lethality in childhood. All families studied were to some degree consanguineous with no other known mutations leading to phenotype.

**Table 2. MFSD2A phenotyping results**

| Family | Inheritance | Mutation | Exon | Phenotype | MFSD2A Function | Immune Investigation |
|---|---|---|---|---|---|---|
| Libya | Autosomal Recessive | g.40431162C>T C.1; c.497C>T | 5 | microcephaly, developmental delay, intellectual disability, hypotonia, hyperreflexia, seizures | inactivating | no |
| Egypt | Autosomal Recessive | g.40431005C>T C.1; c476C>T p.Thr159Met | 4 | | inactivating | no |
| Pakistan | Autosomal Recessive | g.39967632C>T C.1; c1016C>T p.Ser339Leu | 10 | progressive disease, microcephaly, ID, non-lethal | Partially inactivating | no |

## 3.4 REAL-TIME PCR RESULTS

NKT and CD8 T activated effector cells were analyzed for *Mfsd2a* expression relative to naïve effector cells and normalized to *GAPDH*. Cells were activated *in vitro* with anti-CD3 anti-CD28. Each graph is representative of three triplicate studies, n=3 for each.

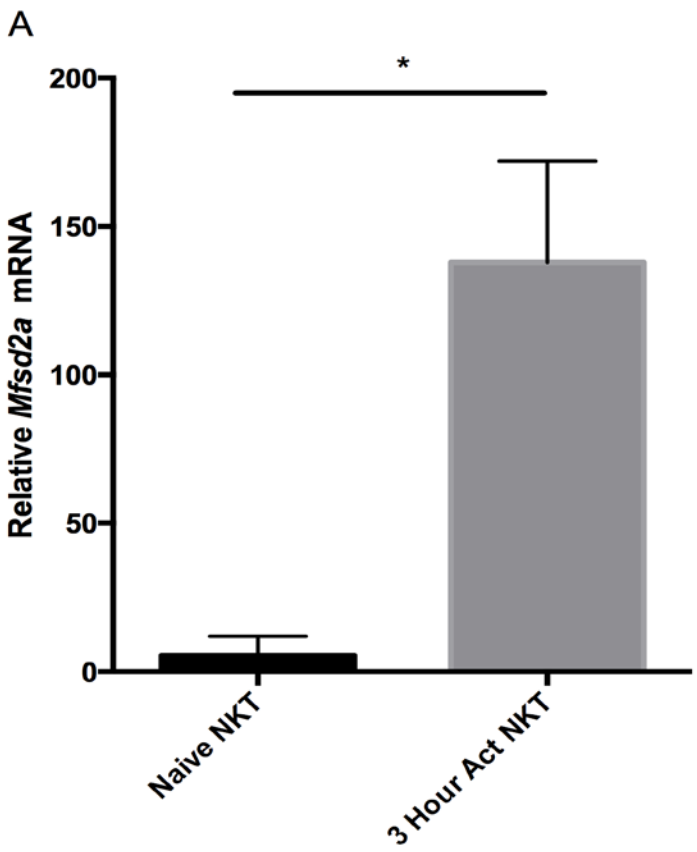



**Figure 7. Real-time PCR results for 3 hour activated NKT cells relative to naïve cells for Mfsd2a expression**

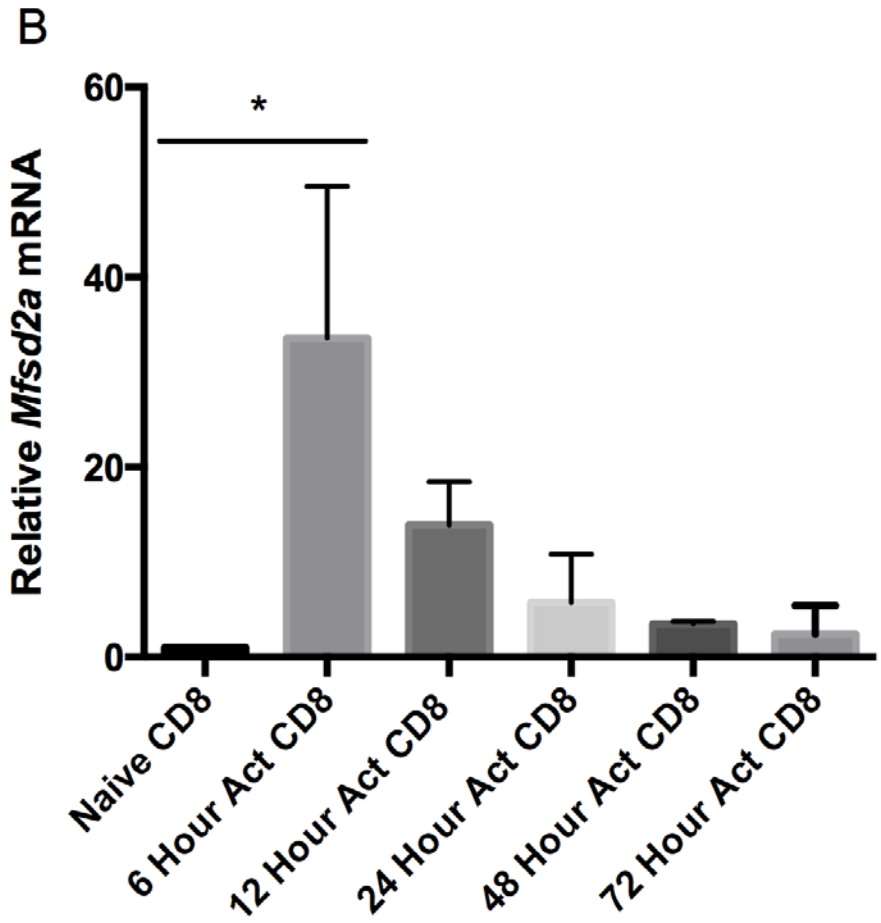



**Figure 8. Real-time PCR results for activated CD8 T cells relative to naïve cells for MFSD2A expression**

To validate the ImmGen data, cells were also activated *in vivo* using CD45.2 OT-I adoptive transfer lm-OVA model; n=1.

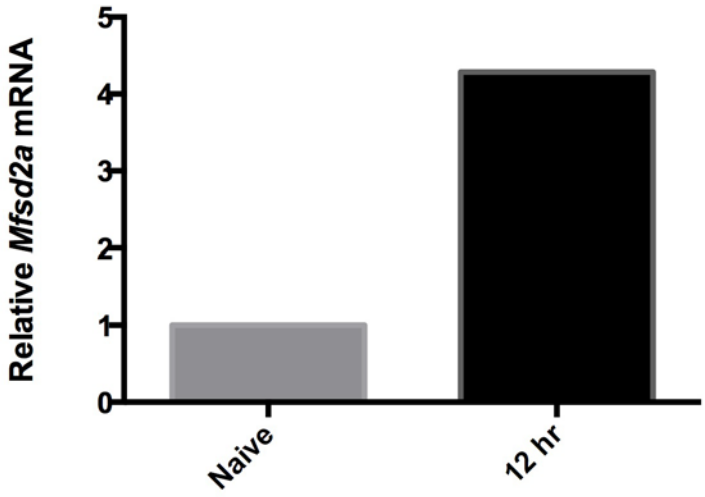



**Figure 9. Real-time PCR *MFSD2A in vivo* model in CD8 T cells**

## 3.5 WESTERN BLOTTING RESULTS

Western blotting protein analysis for MFSD2A relative to BETA-ACTIN. CD8 effector T cells were activated *in vitro* up until given time points, then immediately lysed and processed for analysis.

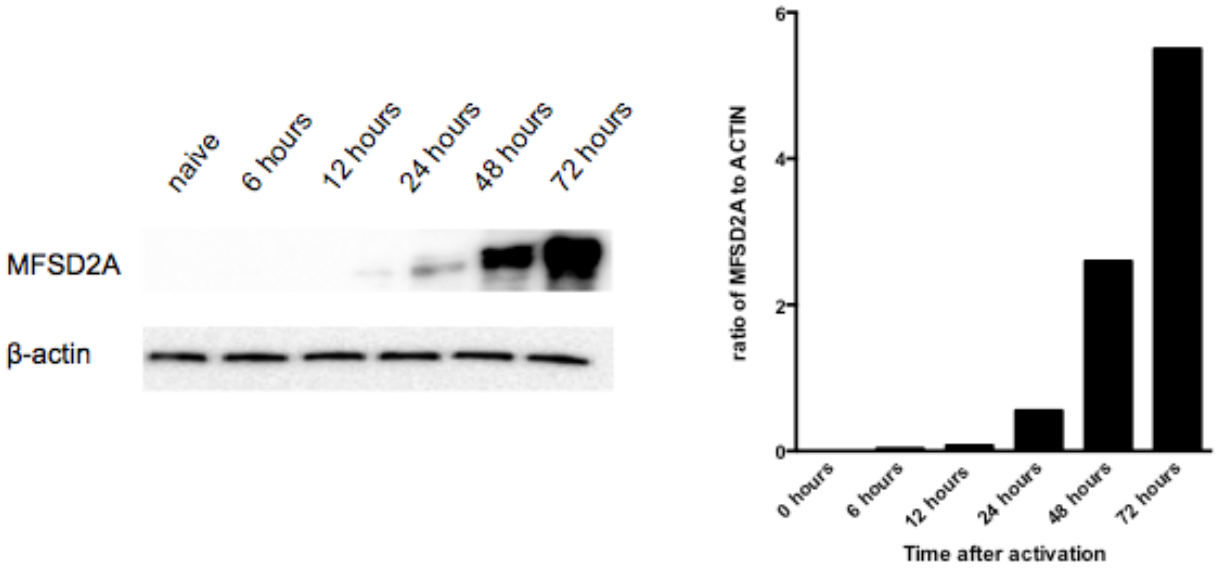



**Figure 10. Western Blotting protein analysis of CD8 T cells**

**Raw data (right) and quantified ratio of MFSD2A to BETA-ACTIN (left); n=1.**

## 3.6 *IN VITRO* RESULTS MFSD2A AND LPC

CD8 T cells from wildtype C57BL/6 mice were activated in vitro as mentioned previously with LPC being added the last four hours of culture. Cells were analyzed by flow cytometry for MFSD2A PE and LPC GFP.

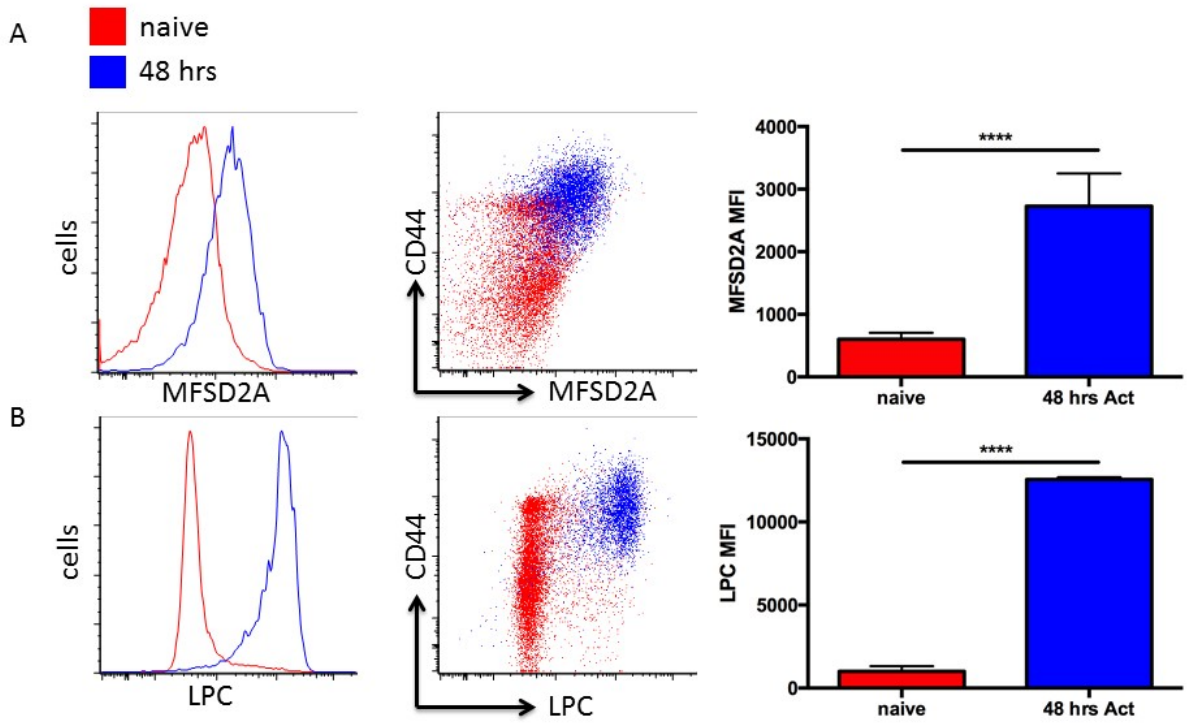



**Figure 11. *In vitro* analysis of MFSD2A and LPC**

Top of figure is data relevant to MFSD2A. Far left is representative histogram of mean MFI for MFSD2A for 48 hour activated compared to naïve CD8 T cells. Middle FACS plot is comparing MFSD2A to CD44 activation marker, illustrating an increase in MFSD2A in parallel to activation. Far right panel is quantification of MFSD2A; $p<0.0001$ using Student's T-test. Bottom half of the figure is for LPC GFP; analysis performed identically to MFSD2A; quantification $p<0.0001$ using Student's T-test. Figure is representative of triplicate experiments with each experiment n=3.

## 3.7 *IN VIVO* RESULTS MFSD2A

Below are representative flow cytometry FACS plots showing patterns of relative MFSD2A fluorescence intensity, comparing *in vivo* activated CD8 T OT-I transgenic mice that were adoptively transferred onto a congenic CD45.1 recipient that had been infected with 5000 CFU lm-OVA at day 3 and day 5 post-infection.

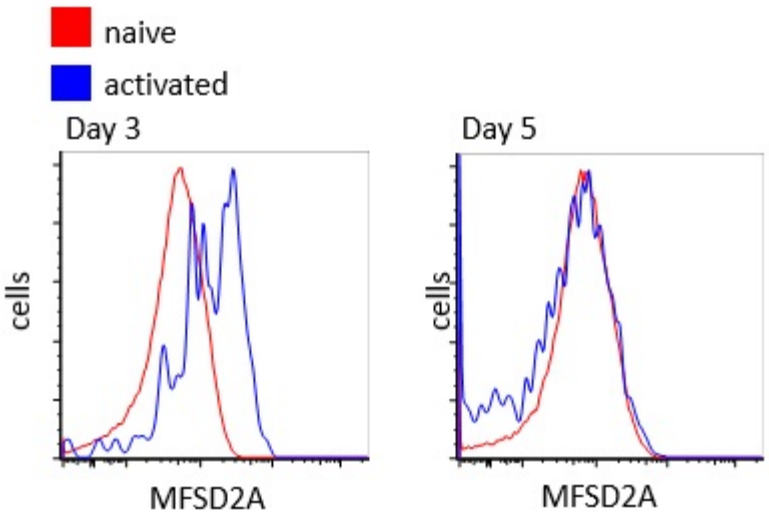



**Figure 12. MFI for MFSD2A *in vivo* activated OT-I CD8 T cells from blood**

Next, to better classify the amount of MFSD2A early on in infection, infected spleens were harvested at 12, 24, and 48 hours post-infection to investigate MFSD2A fluorescent intensity. CD8 OT-I cells were analyzed based on effector or naïve status based on cell surface markers, then these populations gated on MFSD2A.

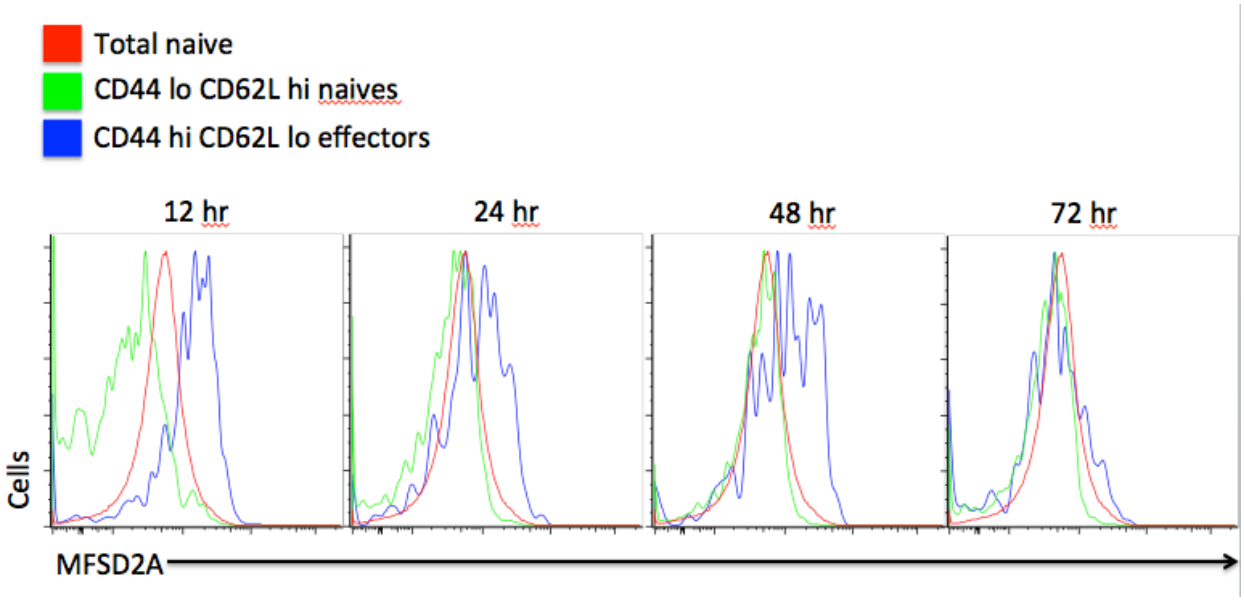



**Figure 13. MFSD2A intensity of CD8 T cells *in vivo* from spleen**

**Fluorescent intensity is highest in 12 hr CD44 hi CD62L lo effector CD8 T cells. Intensity decreases with time out to 72 hrs.**

# 4.0 DISCUSSION

Innate and adaptive effector T cells play a critical role in immunity, including clearance of infection and cancer, which makes finding out what makes them tick (i.e., metabolites, specifically lipidomics) very important in the hunt for cures and stepping up our bodies' defenses. Herein, it is proposed that MFSD2A and LPC could provide the potential mechanism that makes it capable for effector T cells to increase metabolic output during activation and successfully clear the system of infection. MFSD2A is clearly upregulated in both iNKT and CD8 T, innate and adaptive effector T cells respectively, upon activation. This is shown by our microarray and real-time PCR validation as well as western blotting protein analysis. These data are further supported by *in vitro* culture of mouse splenic CD8 T cells that after activation, expressed significantly higher levels of both fluorescent MFSD2A and LPC. This is recapitulated *in vivo* by using lm-OVA as an infection/tracking model.

There are a few potential pitfalls to the above approach. Protein levels seem to be highest at 72 hours, while mRNA levels peak around 12 hours, similar to the ImmGen data mentioned above. The delay in protein translation may be due to the time required for clonal expansion in order for the adaptive immune system to successfully illicit an immune response. Another area that needs to be addressed is repeating LPC flow cytometry measurements in the *in vivo* model. The reason this has not already been completed is because there is a lethality associated with I.V. LPC dosage and this problem has not been worked out as of print. Other gaps in the data include

lipidomic analysis for LPC GFP in activated and naïve effector T cell samples and LPC GFP microscopy work to complement the above data. Both of these missing links are being addressed and will be discussed in future endeavors.

Preliminary real-time PCR and *in vitro* data using human peripheral blood lymphocytes (HPBL) has shown a promising translation from mouse data into a human model, as MFSD2A and LPC both are significantly higher in wildtype activated versus naïve human CD8 T cells (n=3) (data not shown). More human data is to be performed and expanded on in the near future, including western blotting protein analysis and repeating *in vitro* work. If these data hold true, it would make sense to move forward with an *in vitro* human *MFSD2A* knock down model, such as shRNA technologies, to understand the effect on *MFSD2A* KD in humans. This would be of particular relevance for the families listed above with mutations in MFSD2A, as they may have a dysfunctional immune phenotype that has yet to be discovered.

Another experiment to perform in the very near future will be to test the effect of MFSD2A deletion in CD4 T cells, using a cre-lox model of deletion. Using CD4-cre mice, MFSD2A will be deleted in all types of T cells during the double positive stage of thymic development. This will therefore also knock out MFSD2A in CD8+ and NKT cells. We hypothesis that upon KO, these mice will suffer a decreased effector T cell response during infection and that this KO will directly lead to a poorer prognosis for these mice. MFSD2A flox mice were kindly donated from the Silver Lab at Duke-NUS Singapore and are currently being bred on a CD4-cre OT-I background.

Overall, accumulating data suggest that MFSD2A may be one of the missing links to understanding effector T cell metabolism during an immune response. Our data suggest this is true, but further experimentation needs to be performed, including in other model organisms, to

confirm that this is a real effect. Assuming data is conclusive, MFSD2A may be on the verge of becoming a critical player in effector T cell responses with an important role in general populations' public health.

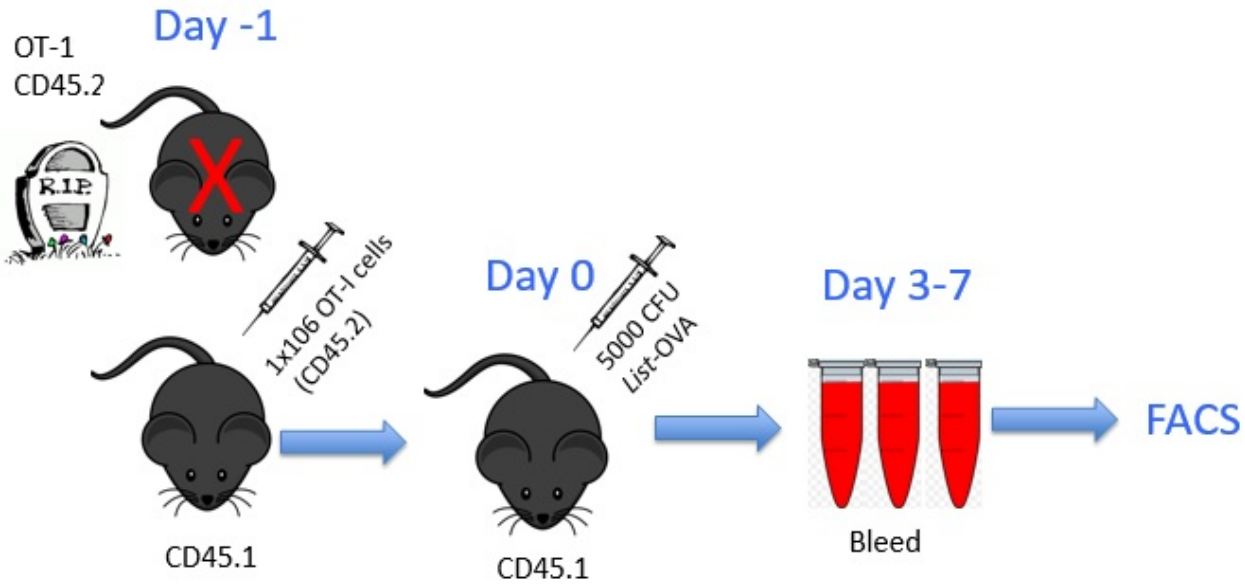
## APPENDIX A: LIST OF REAL-TIME PCR PRIMERS

| | | |
|---|---|---|
| Ccl3 | TGTACCATGACACTCTGCAAC | CAACGATGAATTGGCGTGGAA |
| Nr4a2 | GTGTTCAGGCGCAGTATGG | TGGCAGTAATTTCAGTGTTGGT |
| Cd24a | GGCTCTCTCTCTCTCTCTTCTAC | GGACTTGTGGTTGCCATTTG |
| Cxcl9 | AGAGTAGGGACCACAGACTATT | GCCTTTCTACCTCTCACACATAC |
| Nr4a3 | GCGCTGGTAGAACTGAGAAA | CAAGGAAGAGCTTGTCGATGA |
| Nr4a1 | GGCATGGTGAAGGAAGTTGTA | GGATGAGGGAAGTGAGAAGATTG |
| Il2ra | GAGCCTGCCCAACTTTTTGTG | TGTAGTCCCCAACTCATCTGTG |
| Egr3 | CCGGTGACCATGAGCAGTTT | TAATGGGCTACCGAGTCGCT |
| Sema7a | ACACACCGTGCTTTTCCATGA | CCTTTGTGGAGCCGATGTTC |
| IFNg | CGGCACAGTCATTGAAAGCCTA | GTTGCTGATGGCCTGATTGTC |
| MFSD2A | AGAAGCAGCAACTGTCCATTT | CTCGGCCCACAAAAAGGATAAT |

## APPENDIX B: LIST OF ANTIBODIES

| Antibody | Conjugate | Catalog # | Manufacturer |
|---|---|---|---|
| CD45.2 | BUV395 | 564616 | BD Biosciences |
| CD45.1 | APC | 17-0454-82 | eBioscience |
| CD8a | BV510 | 100752 | BioLegend |
| V**α**2 TCR | BV421 | 48-5812-82 | eBioscience |
| CD44 | PerCp-Cy5.5 | 45-0441-82 | eBioscience |
| CD62L | APC-Cy7 | 47-0629-42 | eBioscience |
| MFSD2A | - | ab105399 | Abcam |
| Anti-rabbit | PE | 12-4739-81 | eBioscience |

## APPENDIX C: DIAGRAM OF IN VIVO MODEL



## APPENDIX D: FLOW CHART OF EXPERIMENTS

## BIBLIOGRAPHY


1. Godfrey, D.I., S. Stankovic, and A.G. Baxter, *Raising the NKT cell family.* Nat Immunol, 2010. **11**(3): p. 197-206.
2. Bendelac, A., P.B. Savage, and L. Teyton, *The Biology of NKT Cells.* Annual Review of Immunology, 2007. **25**(1): p. 297-336.
3. Smith-Garvin, J.E., G.A. Koretzky, and M.S. Jordan, *T Cell Activation.* Annual Review of Immunology, 2009. **27**(1): p. 591-619.
4. Kenneth Murphy, P.T., Mark Walport, *Janeway's Immunobiology*. Vol. 7th edition. 2008, United States: Garland Science.
5. 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.
6. 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.
7. 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.
8. Pearce, E.L., et al., *Enhancing CD8 T-cell memory by modulating fatty acid metabolism.* Nature, 2009. **460**(7251): p. 103-107.
9. 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.

10. 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.

11. 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.

12. 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.

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

14. 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.

15. 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.

16. 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.

17. 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.

18. 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.

19. 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.

20. Horrocks, L.A. and Y.K. Yeo, *HEALTH BENEFITS OF DOCOSAHEXAENOIC ACID (DHA).* Pharmacological Research, 1999. **40**(3): p. 211-225.

21. 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.

22. 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.

23. 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.