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Regulation of Immune Cell Activation and Functionby the nBMPp2 Protein andthe CD5 Co-Receptor

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A dissertation submitted to the faculty of
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in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

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ABSTRACT

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According to the centers for disease control and prevention (CDC) and the world health organization (WHO), heart disease and immune related diseases such as diabetes and cancer are among the leading causes of death around the world. Thus, the regulation of the function of immune cell plays a key role in health and disease. Calcium (Ca^{2+}) ions play a critical role in immune cell activation, function and in a robust immune response. Defects in Ca^{2+} signaling influences the development of cardiac disease, Alzheimer disease, immune cell metabolism, muscle dysfunction, and cancer. Each immune cell is unique in its activation and function, making it relevant to understand how activation of each type of immune cell is regulated. Here we describe the role of the nBMP2 protein in macrophage activation and function and the role of the CD5 co-receptor in helper T cell activation and function.

The nuclear bone morphogenetic protein 2 (nBMP2) is the nuclear variant of the bone morphogenetic protein 2 (BMP2), a growth factor important in heart development, neurogenesis, bone, cartilage and muscle development. To better understand the function of nBMP2, transgenic nBMP2 mutant mice were generated. These mice have a slow muscle relaxation and cognitive deficit caused in part by abnormal Ca^{2+} mobilization. Mutant nBMP2 mice also have an impaired secondary immune response to systemic bacterial challenge. Here we have further characterized macrophage activation and function from mutant nBMP2 mice before and after bacterial infection. We describe how nBMP2 influences the Ca^{2+} mobilization response and phagocytosis in macrophages, revealing a novel role of the nBMP2 protein in immune cell regulation.

CD5 is a surface marker on T cells, thymocytes, and the B1 subset of B cells. CD5 is known to play an important role during thymic development of T cells. CD5 functions as a negative regulator of T cell receptor (TCR) signaling and fine tunes the TCR signaling response. Here we describe our characterization of CD5 regulation of Ca^{2+} signaling in naïve helper T cells. We also outline our findings examining how CD5-induced changes in helper T cell activation influence other biological processes such as immune cell metabolism, the diversity of the gut microbiome, and cognitive function and behavior. Thus, this work elucidates the influence of the CD5 co-receptor on the functional outcomes in multiple systems when CD5 is altered.

Keywords: nBMP2, bone morphogenetic protein, CD5, co-receptor, calcium (Ca^{2+}), metabolism, behavior, TCR, T cell receptor, microbiome, macrophage, T helper cells

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ABBREVIATIONS

AMP - Adenosine monophosphate

AMPK - Adenosine monophosphate activated protein kinase

AMPK - AMP-activated protein kinase

APC - Antigen presenting cell

ATP - Adenosine triphosphate

AUC - Area under the curve

BCR - B cell receptor

BMD - bone marrow derived

BMP2 - Bone morphogenetic protein 2

Ca^{2+} - Calcium

CaMKK - Calmodulin-dependent protein kinase kinase

$CD5^{hi}$ - CD5 Higher expression levels

CD5KO/$CD5^{-/-}$ - CD5 Knock-out

CD5L - CD5 ligand

$CD5^{lo}$ - CD5 Lower expression levels

CD5WT - CD5 Wild Type

CDC - Center of disease control and prevention

CNS - Central nervous system

CRAC - Calcium release activated calcium channel

CREB - Cyclic-AMP-responsive-element-binding protein

CTLA-4 - Cytotoxic T-lymphocyte-associated protein 4

DAG - Diacylglycerol

ECS - *E. coli* supernatant

ER - Endoplasmic reticulum

Erk - Extracellular signal-regulated kinases

FAO - Fatty Acid Oxidation

FBS - Fetal Bovine Serum

FSC-A - Forward scatter area

FSC-W - Forward scatter width

GLUT1/3 - Glucose transporter 1/3

IACUC - Institutional Animal Care Unit

ICOS - Inducible T cell stimulator

IL-1 - Interleukin 1

IL-12 - Interleukin 12

IL-2 - Interleukin 2

IL-2R - Interleukin 2 receptor

INF- γ - Interferon gamma

IP3 - inositol 1, 4, 5 triphosphate

IP3R - IP3 Receptors in the ER

ITAMS - Tyrosine based activation motif

ITIMs - Tyrosine based inactivation motif

LPS - Lipopolysaccharide

LTA - Liphoteichoic acid

LTP - Long-term potentiation

MAP kinase - Mitogen activated protein

MHC - major histocompatibility complex

nBMP2 - Nuclear bone morphogenetic protein

nBmp2NLStm - Gene-targeted mutant mouse model nBMP2

NFAT - nuclear factor of activated T cells

NF-kB - nuclear factor-kB

NLS - Nuclear localization signal

OXPHOS - Oxidative phosphorylation

PD-1 - Programmed cell death protein 1

PI3K - Phosphatidylinositide 3 kinase

PLC - Phospholipase C

pMHC - peptide MHC complex

SAS - *S. aureus* supernatant

Self-pMHC - Self peptide MHC

SERCA - Sarco/endoplasmic reticulum Ca^{2+} ATPase

SOCE - Store-operated calcium channels

STIM1 - Stromal interaction molecule 1

SYK - Spleen tyrosine kinase

TCA- Tricarboxylic acid cycle

TCR - T cell receptor

TIL - Tumor infiltrating lymphocytes

TNF - Tumor necrosis factor

WHO - World Health Organization

ZAP-10 - Zeta chain associated protein 10

ECAR - Extra cellular acidification rate

OCR - Oxygen consumption rate

FCCP - Fluorocarbonyl cyanide phenylhydrazone

PRRS - Pattern-recognition receptors

PAPMS - Pathogen associated molecular patterns

CHAPTER 1: Calcium Signaling Role in the Immune Response

1.1 Introduction

The immune system protects the host from foreign pathogens and includes cells from both the innate and adaptive immune systems working together. While innate cells (e.g. macrophages) recognize and are activated by conserved pathogen associated molecular patterns (PAMPs) on infectious agents and help trigger a primary immune response, cells from the adaptive immune system (e.g. T and B lymphocytes) recognize and are activated by pathogen specific peptides[1,2]. These innate and adaptive systems are dependent on one another; in fact, it is normally the innate immune cells that initially activate cells of the adaptive immune system[3]. Phagocytes such as macrophages and dendritic cells are antigen presenting cells (APC) that carry out phagocytosis of foreign pathogens and play an important role in lymphocyte activation (e.g. T cells)[4]. T cells recognize foreign peptides presented by major histocompatibility complexes (MHC) on APCs via the T cell receptor (TCR), a protein complex that play an important role in signal transduction and leads to T cell activation[2,4]. Calcium (Ca^{2+}) signaling is a key second messenger that is involved in transmitting these activation signals and is essential for immune cell function and differentiation[5].

1.2 Activation of Immune Cells

Immune cells are activated upon recognition of foreign pathogens and can also be activated by altered self-proteins such as those found in cancer cells. Phagocytic cells such as macrophages and dendritic cells are first responders that act as sentinels to recognize their environment via surface receptors known as pattern-recognition receptors (PRRs), which recognize PAMPs on the surface of pathogens[1]. Upon PRR binding to a PAMP, that macrophage and dendritic cells are activated and intracellular signals trigger pathogen engulfment and pro-

inflammatory and antimicrobial responses. This is accomplished by secreting molecules such as cytokines and chemokines, processing and presenting antigen to T cells, and upregulating costimulatory molecules, all of which play an important role in the activation of the adaptive immune response[3,6].

The adaptive immune system includes T and B cells which expresses a diverse repertoire of receptors that play an important role in the recognition of self and non-self-peptides[2,7]. For T cell activation to occur, innate cells such as APCs engulf, process, and present the antigen-peptide. When this peptide is recognized by the TCR as foreign, together with co-stimulatory signals, the adaptive immune response is initiated. This starts with an increase in T cell intracellular Ca^{2+}, metabolic changes, and cytokine production that results in a T cell initiated immune response[4,5,8]. Deficiencies in innate and adaptive immune cells are known to increase susceptibility to infection, autoimmunity and cancer[9]. For instance, diabetes mellitus, a metabolic disorder, has been associated with dysregulation of innate immunity (neutrophil function), humoral immunity, and an aberrant T cell response[10-12]. Hence, proper activation and interactions of the innate and adaptive immune cells are key for an efficient immune surveillance (Fig. 1A). Defective pathogen recognition, intracellular signaling, or metabolic function result in defective immune surveillance (Fig. 1B).

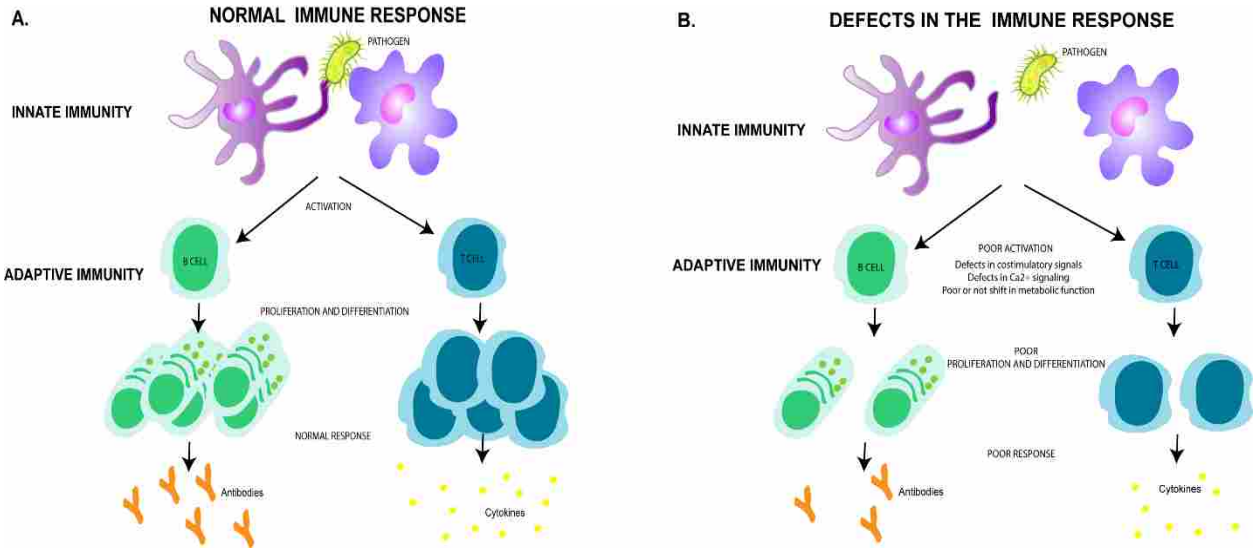

Figure 1. Schematic representation of normal and defective immune response.
A. In a normal immune response, innate immune cells (e.g. macrophages or dendritic cells) recognize foreign pathogens and engulf them. They then process and present antigen to T cells. Upon activation lymphocytes proliferate and differentiate and provide protection via antibodies and cytokines. B. Defective immune responses downregulate innate and adaptive immune responses to foreign pathogens. Defects may be due to ineffective phagocytosis, deficiencies of co-stimulatory signals, calcium signaling defects, and altered metabolism resulting in impaired antibody and cytokine production.

1.3 Calcium Signaling

Calcium (Ca^{2+}) is a universal second messenger involved in many aspects of cellular life such as cell activation, motility, and cell death[13]. Mobilization of a cellular Ca^{2+} signal involves multiple intracellular proteins and channels important in Ca^{2+} regulation; for example, the calcium release activated calcium channel (CRAC) on the surface of the cell and adenosine triphosphate (ATP)-driven pumps, and exchanger channels found in intracellular organelles such as the mitochondria, endoplasmic reticulum (ER), and nucleus all play a critical role in regulating the Ca^{2+} signal to initiate gene transcription specifically tailored to the pathogen (Fig. 2)[13].

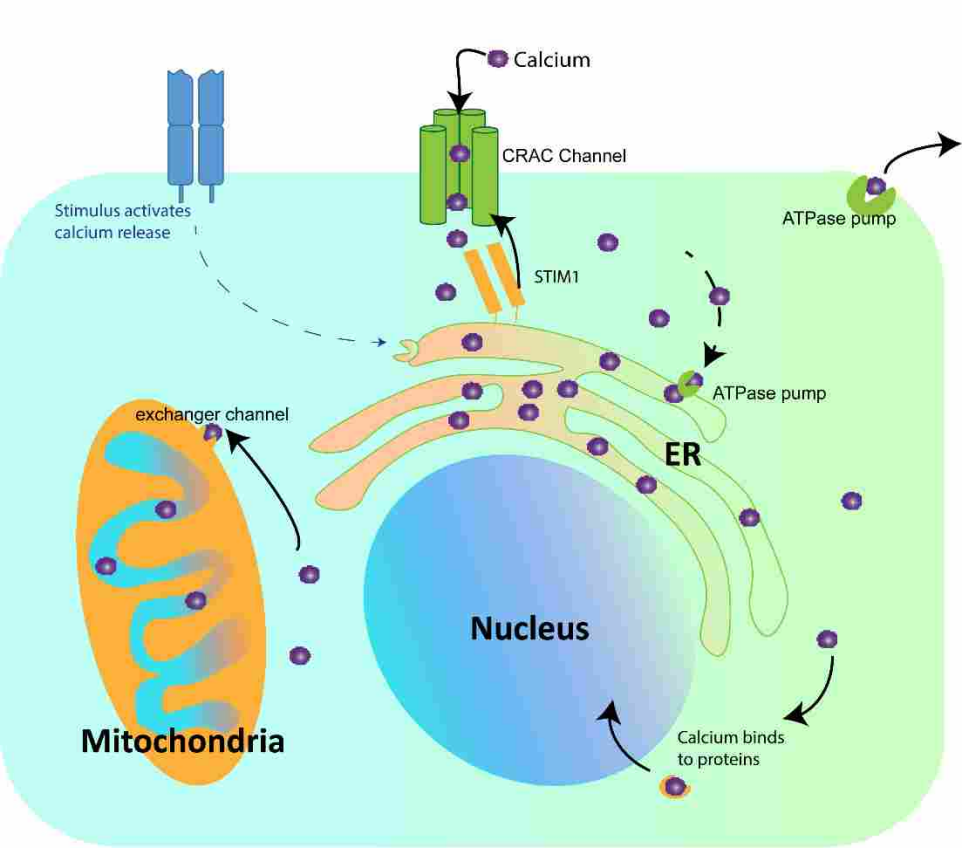

Figure 2. Schematic representation of the main structures involved in calcium homeostasis. This model shows an overview of the calcium transporter system within the cell (endoplasmic reticulum, mitochondria and nucleus).

Intracellular Ca^{2+} levels are important in both the innate and adaptive immune response[14]. As cells are stimulated, intracellular Ca^{2+} levels play an important role in immune cells fate, as it influences their activation and function[15]. The importance of Ca^{2+} signaling in T cells and macrophages will be discussed more in depth separately in this chapter.

1.4 Macrophage Ca^{2+} Signaling

Macrophages are immune cells that engulf pathogens and apoptotic or dead cells and activate the adaptive immune system by processing and presenting MHC bound peptides to T cells. In macrophages, there has been less characterization of the Ca^{2+} pathway compared to T cells[16,17]. However, it is known that Ca^{2+} is important for macrophage activation. It has been

suggested that the actin cytoskeleton has a central role in mediating both the physical interaction of the macrophage with the T cell, and the subsequent signaling pathways leading to T cell activation[18].

In macrophages, engagement of the Fc receptors result in a robust influx of Ca^{2+} from the extracellular space[19]. In a similar manner to T cells, Ca^{2+} influx is due to store operated Ca^{2+} entry (SOCE) that is activated by a reduction of Ca^{2+} in the ER stores and activation of the CRAC channel via STIM1 (Fig.3)[20]. It has been shown that murine macrophages lacking *Stim1* expression had severely compromised FcγR II/III mediated Ca2+ influx and they seemed to be impaired in a number of *in vivo* models of autoantibody and immune complex induced macrophage function[21].

Ca^{2+} signaling also appears to play an important role in Fc-receptor mediated phagocytosis and cell migration[22,23]. In order for phagocytosis to initiate, pathogens, dead cells, or foreign particles need to be opsonized by antibodies, complement, fibronectin, and mannose-binding lectin and bind to opsonic receptors such as the FcγR found in the membrane of the phagocyte, and that appears to be regulated by Ca^{2+} [24-26]. It is not clear what the pathways involved are, however there have been studies suggesting that intracellular Ca^{2+} elevations during phagocytosis might regulate other function within the phagocyte such as cytoskeletal rearrangements, endolysosomal fusion and the oxidative burst[24,25,27].

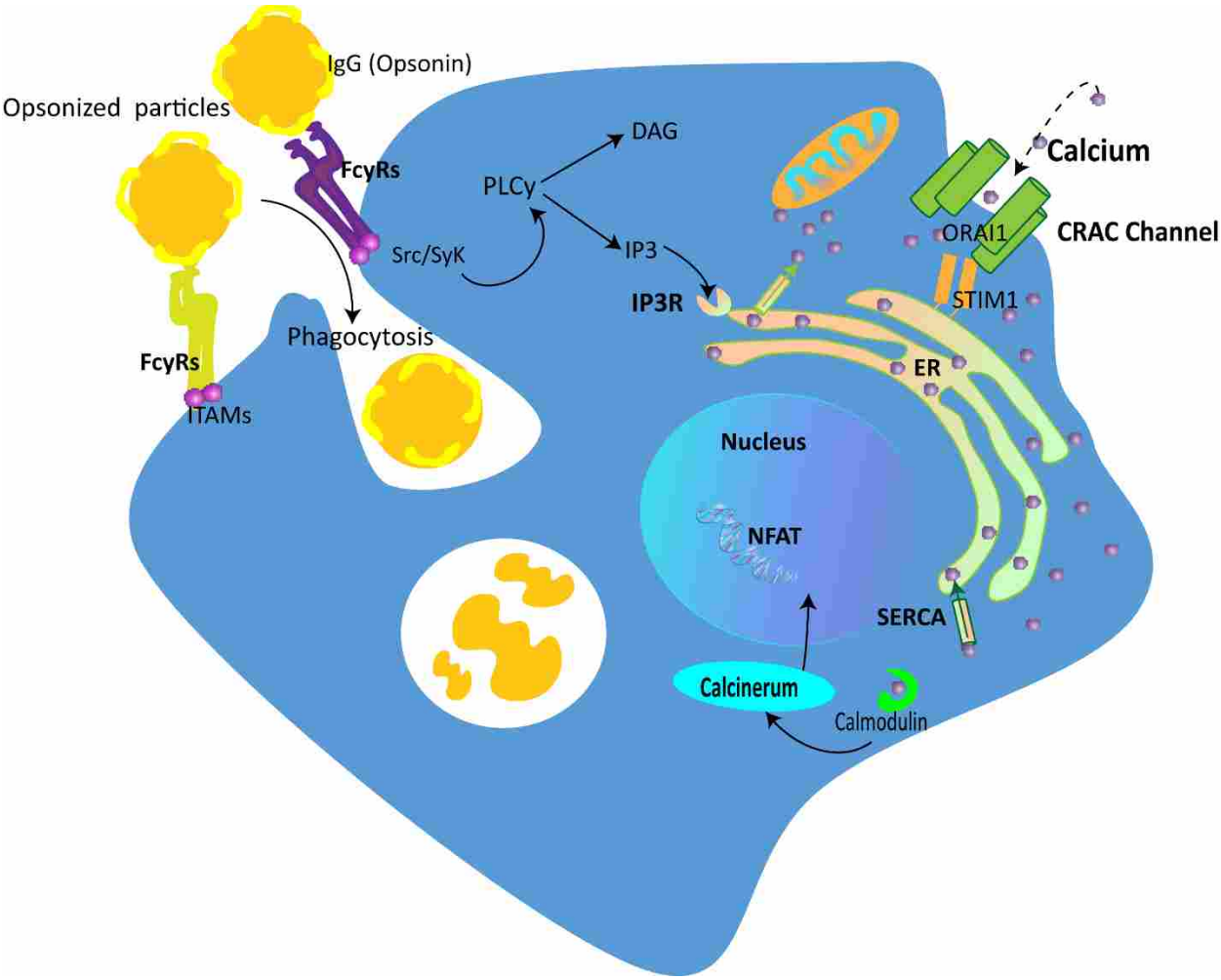

Figure 3. Phagocytic receptors involved in Ca^{2+} signaling.
Initiation of signal transduction in macrophages occurs upon activation of Fc receptors, increasing the intracellular Ca^{2+} concentration in the cytoplasm[24,25,27,28]

1.5 nBMP2

Bone morphogenetic protein 2 (BMP2) is a secreted growth factor that modulates many essential physiological and developmental processes through extracellular signaling. The Bridgewater lab identified a novel nuclear variant of BMP2, nuclear BMP2 (nBMP2), which is translated from an alternative start codon downstream of the signal peptide sequence, eliminating the N-terminal signal peptide that targets the protein to the secretory pathway, instead allowing cytoplasmic translation and subsequent translocation to the nucleus by means of a bipartite nuclear localization signal (NLS) (Fig.4)[29].

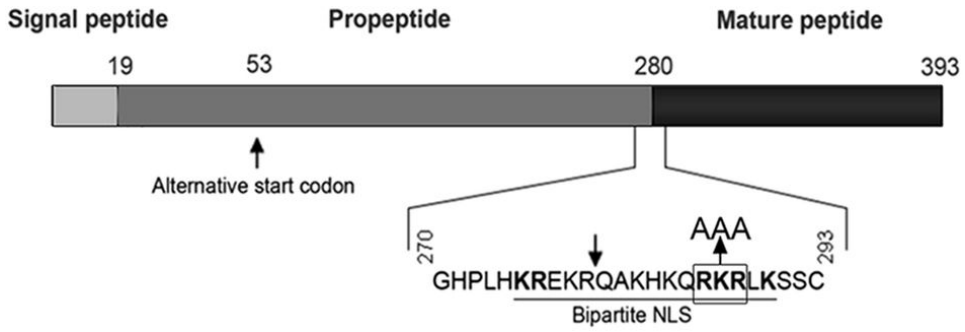

Figure 4. The nBMP2 is translated from a downstream alternative start codon.
The signal peptide sequence (in light gray), in the alternative initiation codon allows translation in the cytosol rather than in the ER, with subsequent nuclear localization directed by a bipartite NLS. The three amino acids (RKR) were changed to alanines (AAA) to prevent nuclear localization of nBMP2 in the mutant mouse model (nBmp2NLStm). Figure reprinted from "The BMP2 nuclear variant, nBMP2, is expressed in mouse hippocampus and impacts memory" by Cordner RS et al, 2017[30]

In order to better understand the function of nBMP2 in the nucleus, the Bridgewater lab generated a gene-targeted mutant mouse model (nBmp2NLStm) in which the RKR sequence was replaced with AAA, thereby inhibiting the translocation of nBMP2 to the nucleus (Fig. 4)[31]. Mice with this nBMP2 dysfunction (the nBmp2NLStm mutant) showed altered functions of skeletal muscle, neurological function and immune response in mice over 6 months of age (Fig. 5)[30-32]. Dysfunction in the skeletal muscle was demonstrated by a significant increase in the time required for relaxation following a stimulated twitch contraction (Fig. 5A)[31]. Muscle relaxation after contraction is mediated by the active transport of Ca^{2+} from the cytoplasm to the sarcoplasmic reticulum by sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA), and enzyme activity assays revealed that SERCA activity in skeletal muscle from nBmp2NLStm mice was

reduced to approximately 80% of wild type[31]. These results suggest that nBMP2 plays a role in the establishment or maintenance of intracellular Ca^{2+} transport pathways in skeletal muscle.

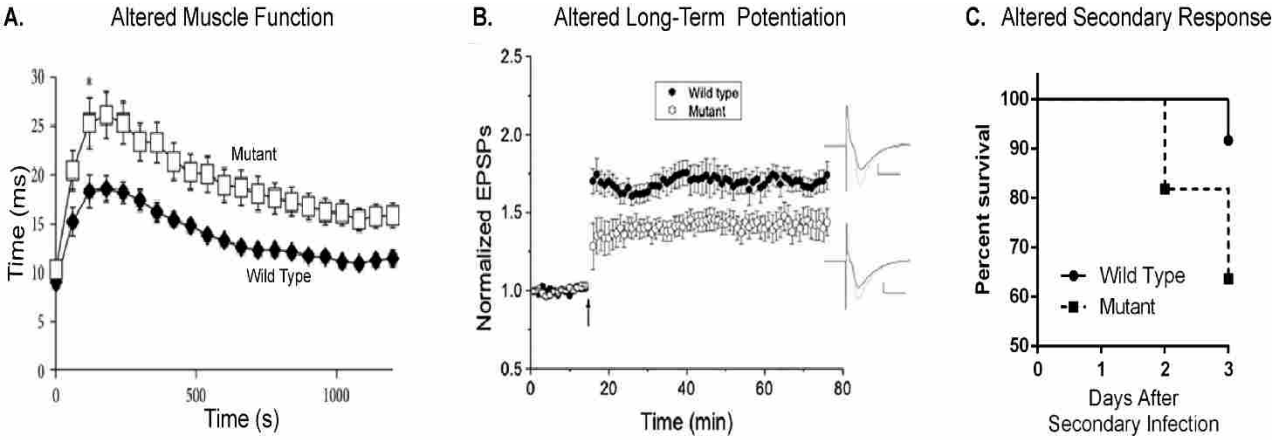

Figure 5. nBmp2NLStm mice have altered functions related to Ca^{2+} mobilization
A. Mutant mice have defective muscle relaxation contraction. Figure reprinted from "A Novel Bone Morphogenetic Protein 2 Mutant Mouse, nBmp2NLStm, Displays Impaired Intracellular Ca^{2+} Handling in Skeletal Muscle" by Bridgewater L., et al, 2013[31] B. Long-term potentiation (a form of synaptic plasticity dependent on Ca^{2+} signaling) was defective in mutant mice. Figure reprinted from "The BMP2 nuclear variant, nBMP2, is expressed in mouse hippocampus and impacts memory" by Cordner RS et al, 2017[30] C. Mutant mice had a higher mortality than wild type when challenged to a secondary infection Figure reprinted from "Targeted Mutation of Nuclear Bone Morphogenetic Protein 2 Impairs Secondary Immune Response in a Mouse Model" by Olsen DS., et al, 2015[32]

Neurological function was also affected in nBMP2 mutant mice. Immunohistochemistry assays in the hippocampus revealed that nBMP2 was not preset in the nuclei of CA1 neurons[30]. The nBMP2 mutant mice also presented significant cognitive deficits related to hippocampal dysfunction and its electrophysiological measurement suggested a dysregulation in the Ca^{2+} transport (Fig. 5B)[30].

Similarly, nBMP2 mutant mice had an impaired secondary immune response to bacterial infection (*Staphylococcus aureus*)[32]. The mutant mice had smaller spleens than WT and histological analysis of splenic cells after secondary infection suggested mutant spleens had fewer hemosiderin-laden macrophages than wild type spleens. We hypothesized that mutant macrophages had a decreased ability to engulf damaged red blood cells, and we found that the

mutant mice did have a deficiency in phagocytosis function this was due to an impaired Ca^{2+} mobilization response (Fig. 5C)[32].

1.6 T cell Ca^{2+} Signaling

T cells are important because they help orchestrate the immune response and provide essential cell mediated cytotoxicity[33,34]. Engagement of the TCR to the peptide and major histocompatibility complex (pMHC) determines the magnitude of the response and the fate of the T cell[35]. This interaction triggers the Ca^{2+} signaling pathway, important in T cell activation and the immune response[5,36].

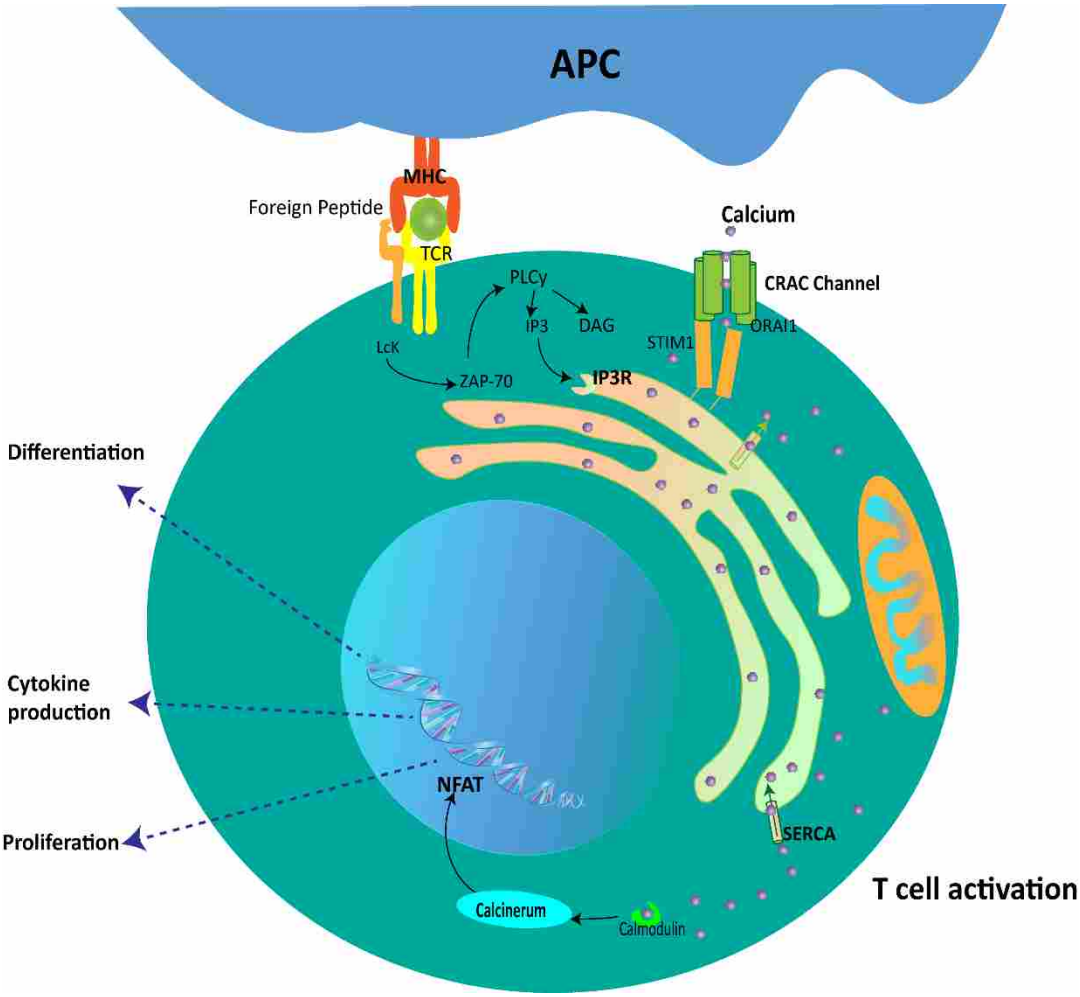

Figure 6. T cell Ca^{2+} signaling cascade.
TCR-pMHC engagement initiates a signaling cascade that leads to T cell activation, differentiation, proliferation and function. The major steps involving Ca^{2+} signaling are shown.

Normally in naïve T cells, the intracellular Ca^{2+} concentration is maintained lower than the extracellular Ca^{2+} concentration and as a T cell is activated upon interaction of the TCR-pMHC, intracellular Ca^{2+} levels increase[5]. This Ca^{2+} increase is the result of a cascade of phosphorylation events by kinases such as ZAP70, LCK, PLCγ1 and others kinases, cleavage of IP3 and diacylglycerol (DAG), and binding of IP3 to IP3R on the ER (Figure 6)[37,38]. Binding of IP3 to the IP3R causes Ca^{2+} to exit from the ER into the cytoplasm, followed by recognition of Ca^{2+} depletion in the ER by stromal interaction molecule 1 (STIM 1). STIM 1 then activates the CRAC channel on the cell surface and causes a large influx of Ca^{2+} from outside the cell. The Ca^{2+} then binds to cytoplasmic binding proteins such as calmodulin and then calcineurin, traffics to the nucleus and activates transcription factors such as nuclear factor of activated T cells (NFAT), nuclear factor-κB (NF-κB), or cyclic-AMP-responsive-element-binding protein (CREB)[5]. Thus, Ca^{2+} signaling patterns, amplitude and duration each play an important role in regulating T cell activation and effector functions[18,37-39].

1.7 CD5 T cell co-receptor

CD5 is a transmembrane glycoprotein that functions as a co-receptor that regulates the intensity of the TCR-pMHC interaction. CD5 expression in T cells in set during thymic development and it is linked to the TCR signal strength (high avidity) interaction to self-peptide MHC (self-pMHC)[40]. The higher the TCR affinity for self-pMHC, the higher the CD5 expression levels in T cells. TCRs play a critical role in $CD4^+$ T cell activation and function. Previous studies have reported CD5 has a costimulatory function, as CD5 high ($CD5^{hi}$) expression levels in T cells increase intracellular Ca^{2+}, inositol triphosphate, interleukin-2 (IL-2) secretion, and IL-2R expression, which are associated with higher phosphorylation of TCRζ at

baseline and ERK upon activation and enhance TCR-mediated activation and T cell proliferation[41,42].

1.7.1 CD5lo (LLO118) and CD5hi (LLO56)

To further examine CD5 expression levels in helper T cell function, our lab uses two transgenic mouse lines LLO118 and LLO56 with TCRs specific for the same immune dominant epitope of *Listeria monocytogenes* ($LLO_{190-205}$)[43]. LLO118 and LLO56 T cells have similar TCRs (Vα2; Vβ2), but they differ by 15 amino acids found mainly in the CDR3 region of their TCR sequences. They also differ in their *in vivo* immune responses (LLO118 has a better primary response and LLO56 has a better secondary response) and in their CD5 expression levels [LLO118 has low levels of CD5 ($CD5^{lo}$) and LLO56 has high levels of CD5 ($CD5^{hi}$)][42,43]. While much has been learned about Ca^{2+} signaling in lymphocytes and its relationship to T cell activation and function, there are still many questions regarding how Ca^{2+} signaling is important in helper T cell memory formation, and how Ca^{2+} mobilization can affect other biological processes such as metabolism, behavior and even gut microbiota diversity.

1.8 Summary of Research Chapters

Chapter 2 is a published research paper in which we characterize the role of nBMP2 in the Ca^{2+} response of bone marrow derived (BMD) macrophages and splenic macrophages from nBMP2 mutant mice before and after bacterial infection. We show that nBMP2 is in the nuclei of WT BMD and splenic macrophages, while the nBMP2 protein is significantly decreased in the nuclei of mutant nBMP2 macrophages. We also analyzed the Ca^{2+} mobilization response and its effect on macrophage engulfment. Our findings suggest that the Ca^{2+} mobilization response and engulfment levels in BMD macrophages are similar before bacterial infection. However, after a secondary bacterial challenge, splenic macrophages from mutant nBMP2 mice exhibited a

dysregulated Ca^{2+} response and decreased levels of engulfment. These observations suggest that nBMP2 plays an important role in macrophage activation and function. It also highlights the importance of further addressing the mechanisms of nBMP2 function in other cells in the future.

Chapter 3 is a published review paper describing the importance of the CD5 co-receptor in Ca^{2+} signal transduction of the TCR-pMHC interaction in T cells upon activation. Co-receptors are surface molecules found on T cells that play an important role in sustaining or limiting the strength of the signals that modulate the effects of the TCR-pMHC interaction. Here we compare the function of the CD5 co-receptor with PD-1 and CTLA-4, two well studied co-receptors with similar regulatory functions. CD5 expression levels in T cells correlates with the Ca^{2+} mobilization response in T cells, suggesting that CD5 could be a novel checkpoint used to modulate T cell function and metabolism. Metabolism can be altered by Ca^{2+} mobilization, which also could help in the modulation of T cell inflammatory responses with many potential effects on health and disease.

Chapter 4 is a published research paper that characterizes calcium mobilization levels in helper T cells with different CD5 expression levels. CD5 is a co-receptor that plays an important role in regulating T cell signaling and fate during thymocyte education, and its surface expression on mature single positive thymocytes correlates with the TCR signal strength for positive selecting self-ligands. CD5 also plays a role in T cell function after thymic development is complete. Peripheral T cells with higher CD5 expression respond better to foreign antigen than those with lower CD5 expression and CD5 high T cells are enriched in memory populations. In our study, we examined the role of CD5 expression and calcium mobilization in the primary response of T cells using two *Listeria monocytogenes* specific T helper cells (LLO118 and LLO56). These T cells recognize the same immunodominant epitope ($_{LLO190-205}$) of *L.*

monocytogenes and have divergent primary and secondary responses as well as different levels of CD5 expression. We characterized the role of CD5 expression and calcium influx in these CD5 high and CD5 low T cells over the course of 8 days. We found significantly different calcium signaling levels in naïve and day 3 post-stimulation LLO56 and LLO118 T helper cells. To further investigate the role CD5 expression plays in calcium mobilization, we measured the calcium influx in T cells from LLO118-CD5 knockout mice versus those from LLO56-CD5 knockout mice. We found that CD5 expression is important in regulating calcium mobilization in the CD5 high naïve LLO56 T cells during the initial response to antigen, and as CD5 levels decrease over time, its role in regulating calcium also decreases.

Chapter 5 describes how CD5, a co-receptor that influences Ca^{2+} regulation, has an effect in T cell metabolism. We hypothesized that CD5 deficient T cells have different bioenergetic demands that affect metabolic pathways key to T cell activation and function. We evaluated the effects of the CD5 co-receptor on metabolism by measuring the metabolic profiles of CD5KO and wild type T cells. Our preliminary data suggests that CD5KO T cells have higher mitochondrial respiration than wild type T cells and we are examining the mitochondrial mass in CD5KO naïve T cells. Thus, CD5 may play an important role in metabolic programing in T cells and could potentially be useful in modulating the T cell response in the tumor microenvironment.

Chapter 6 describes our efforts to understand how the CD5 co-receptor, a T cell response modulator, influences mice cognitive behavior and gut microbiota diversity. The immune system and the gut microbiota often work in synergy, and previous work has shown that alterations in T cell metabolism can influence behavior and microbial diversity[44]. Here we outline our work to examine the influence of CD5 on behavior and gut microbial diversity. For this study we

compared wild type mice (CD5WT) and the CD5 co-receptor knock-out (CD5KO). We found significant differences between CD5KO and CD5WT mice in marble burying rates, elevated plus maze behavior, and open field activity. These behavioral test results suggest CD5 deficient mice have altered cognitive function and higher levels of fear and anxiety-like behavior. We also quantified the gut microbiota diversity of these mice (CD5KO vs. CD5WT) and found significant differences in microbiota populations. Thus, our data suggests that the CD5 co-receptor plays a critical role in mice cognitive behavior and gut microbiota.

Chapter 7 is the final chapter, here we summarize the main findings for nBMP2 and CD5. We also propose future research plans to further characterize the importance of nBMP2 in the Ca^{2+} signaling pathway and to examine the influence of CD5 in the regulation of cognitive function, metabolism, and diversity of the gut microbiota.

1.9 Summary of Appendices

Appendix I contains an abstract published in the *Journal of Immunology* regarding the influence of the CD5 co-receptor in T cells metabolism and in cognitive behavior. The work for this abstract was presented as an oral and poster presentation in the annual conference of The American Association of Immunologist in Austin, TX in May of 2018.

Appendix II has a list of the presentations done during the time of my PhD.

Finally, Appendix III contains a compilation of the work I published during my dissertation (both as a first author and as a co-author).

CHAPTER 2: The Nuclear Variant of Bone Morphogenetic Protein 2 (nBMP2) is Expressed in Macrophages and Alters Calcium Response

The content of this chapter was published in *Scientific Reports*. Tellez Freitas, C. M. *et al.* "The nuclear variant of bone morphogenetic protein 2 (nBMP2) is expressed in macrophages and alters calcium response." *Scientific Reports* 9, 934, doi:10.1038/s41598-018-37329-5 (2019). It has been formatted for this dissertation, but it is otherwise unchanged.

Abstract

We previously identified a nuclear variant of bone morphogenetic protein 2 (BMP2), named nBMP2, that is translated from an alternative start codon. Decreased nuclear localization of nBMP2 in the nBmp2NLStm mouse model leads to muscular, neurological, and immune phenotypes—all of which are consistent with aberrant intracellular calcium (Ca^{2+}) response. Ca^{2+} response in these mice, however, has yet to be measured directly. Because a prior study suggested impairment of macrophage function in nBmp2NLStm mutant mice, bone marrow derived (BMD) macrophages and splenic macrophages were isolated from wild type and nBmp2NLStm mutant mice. Immunocytochemistry revealed that nuclei of both BMD and splenic macrophages from wild type mice contain nBMP2, while the protein is decreased in nuclei of nBmp2NLStm mutant macrophages. Live-cell Ca^{2+} imaging and engulfment assays revealed that Ca^{2+} response and phagocytosis in response to bacterial supernatant are similar in BMD macrophages isolated from naïve (uninfected) nBmp2NLStm mutant mice and wild type mice, but are deficient in splenic macrophages isolated from mutant mice after secondary systemic infection with *Staphylococcus aureus*, suggesting progressive impairment as macrophages respond to infection. This direct evidence of impaired Ca^{2+} handling in nBMP2 mutant macrophages supports the hypothesis that nBMP2 plays a role in Ca^{2+} response.

2.1 Introduction

Our group has reported the existence of a nuclear variant of the growth factor bone morphogenetic protein 2 (BMP2), designated nBMP2 [45]. This variant protein is produced by translation from an alternative downstream start codon that eliminates the N-terminal endoplasmic reticulum signal peptide, thus preventing the protein's delivery to the secretory pathway. Instead, nBMP2 is translated in the cytoplasm and translocated to the nucleus by means of an embedded bipartite nuclear localization signal (NLS)[45]. Using immunohistochemistry, we have detected nBMP2 in skeletal muscle nuclei and in the nuclei of CA1 neurons in the hippocampus [30,31].

To examine the function of nBMP2, we generated a mutant mouse strain (nBmp2NLStm) in which a three-amino acid substitution in the NLS inhibits translocation of nBMP2 to the nucleus while still allowing normal synthesis and secretion of the conventional BMP2 growth factor 2. The mice appear overtly normal and are fertile. They do, however, lack nBMP2 in myonuclei, and electrophysiological studies revealed that skeletal muscle relaxation is significantly slowed after stimulated twitch contraction, a process that is regulated by intracellular Ca^{2+} transport. Consistent with impaired intracellular Ca^{2+} transport, sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) activity is decreased in skeletal muscle [31]. The mutant mice also lack nBMP2 in CA1 hippocampal neurons, and electrophysiological studies revealed reduced long-term potentiation (LTP) in the hippocampus [30]. LTP is dependent on intracellular Ca^{2+} transport and is thought to be the cellular equivalent of learning and memory [46-48]. Behavioral tests revealed that the nBMP2 mutant mice have impaired object recognition memory [30].

Intracellular Ca^{2+} elevation also regulates the activation and differentiation of several different types of immune cells including T cells, B cells, dendritic cells, and macrophages [14,19,49,50]. To see if nBmp2NLStm mutants had compromised immune response, mice were challenged by systemic infection with *Staphylococcus aureus*. While the mutants' immune response to a primary infection appeared normal, their immune response to a secondary infection challenge 30 days later resulted in higher levels of bacteremia, increased mortality, and failure of spleens to enlarge normally [32]. Although we did not observed differences in the total number of macrophages in spleen, thymus, or lymph node from wild type compared to mutant mice, we did observe that after the secondary infection, spleen from nBmp2NLStm mutant mice showed fewer hemosiderin-laden macrophages than spleen from wild type mice [32]. Macrophages in the spleen accumulate hemosiderin by phagocytosing damaged red blood cells and hemoglobin, which would be present in the blood stream of *S. aureus*-challenged mice due to the hemolysins that *S. aureus* expresses [51-53]. The observation of fewer hemosiderin-laden macrophages in the spleens of mutant mice after a secondary infection suggested to us that macrophage phagocytic activity might be impaired in the absence of nBMP2, potentially providing us with an accessible cell type in which to directly test our hypothesis that intracellular Ca^{2+} response is disrupted in the absence of nBMP2.

To interrogate if nBMP2 might play a role in Ca^{2+} response, we isolated macrophages from wild type and nBmp2NLStm mutant mice. These macrophages included bone marrow derived (BMD) macrophages from uninfected mice, and splenic macrophages from mice that had undergone primary and secondary infections with *S. aureus*[54]. Live-cell Ca^{2+} imaging as well as bead engulfment assays were performed to measure intracellular Ca^{2+} response and phagocytic activity. These analyses revealed deficient Ca^{2+} response and phagocytosis in splenic

macrophages isolated from mutant mice after secondary systemic infection with *S. aureus*, but not in BMD macrophages from naïve mice, suggesting that as nBmp2NLStm mutant cells respond to infection over time, Ca^{2+} response is progressively impaired.

2.2 Materials and Methods

2.2.1 Research Animals

This study was carried out in strict accordance with recommendations in the Guide for the Care and Use of Laboratory Animals [55]. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Brigham Young University (protocol numbers 15-0107 and 15-0603).

Mice were housed in a temperature-controlled (21–22°C) room with a 12:12 hour light-dark cycle and fed standard rodent chow and water *ad libitum*. The nBmp2NLS^{tm} mice were constructed on a Bl6/129 background, as described [31]. The homozygous wild type and mutant mice used in this study were obtained by breeding heterozygotes, and genotyping was performed as previously described [56]. All experiments were performed with male mice at least 6 months of age.

2.2.2 BMD and Splenic Macrophage Isolation

BMD macrophages were obtained from femurs and tibias of wild type and nBmp2NLS^{tm} mutant mice and were matured in culture at 37°C with 5% CO_2 for 7 days in macrophage medium (DMEM (HyClone), 10% fetal bovine serum (FBS) (HyClone), 20% supernatant from L929 mouse fibroblast as a source of macrophage colony-stimulating factor (M-CSF), 5% heat inactivated horse serum (Sigma), 1 mM sodium pyruvate (Gibco by Life Technologies), 1.5 mM L-glutamine (Thermofisher), 10 u/ml penicillin, 10 μg/ml streptomycin (Gibco by Life Technologies)) prior to plating for immunocytochemistry, Ca^{2+} imaging or engulfment assays.

Spleens from wild type and nBmp2NLS^{tm} mutant mice were homogenized in phosphate buffered saline (PBS). The homogenate was filtered, pelleted at 450 x g for 5 min, suspended in lysis buffer (155 mM NH_2Cl, 10 mM $KHCO_3$, 0.1 mM EDTA) on ice for 3-5 min to lyse erythrocytes, and then washed with 37°C macrophage media and plated in macrophage medium in 6-well plates. After 3 days of culture at 37°C in 5% CO_2, medium was replaced to remove non-adherent cells [57]. On day 4, 100 ng/ml lipopolysaccharide (LPS) was added to the culture medium to stimulate differentiation, and cells were incubated for 3-4 more days[58]. Differentiated cells were then plated for immunocytochemistry, Ca^{2+} imaging, or engulfment assays.

2.2.3 Immunocytochemistry

Immunocytochemistry was performed using BMD and splenic macrophages. Following macrophage isolation and 7-day differentiation as described above, cells were plated on coverslips that were pre-treated with 0.025% HCl in PBS for 20 min to facilitate cell attachment. Cells were cultured for 1-2 days to reach 70-90% confluence, then fixed at 37°C in 4% paraformaldehyde for 10 min. Epitopes were exposed through antigen retrieval using 5% sodium citrate and 0.25% Tween-20 in ddH2O, pH 6.0, at 95°C for 10 min. Cells were permeabilized using 0.1% Triton X-100 then blocked for 1.5 hour at room temperature (RT) using SEA BLOCK blocking buffer (ThermoFisher Scientific, 37527). The samples were then probed with 1:50 anti-BMP2 antibody (Novus Biologicals, NBP1-19751) diluted in 10% SEA BLOCK blocking buffer in 0.1% Tween-20/PBS (PBS-T), overnight at 4°C. The probed slides were then stained with anti-rabbit Alexa Fluor 488 (ThermoFisher Scientific, A-11034) for 1 hour at RT. Afterwards, nuclei were stained by incubating the slides in 1:5000 DAPI in PBS-T for 15 min., then slides were mounted using Prolong^{TM} Gold Antifade Mountant (Life Technologies, P10144) and cured overnight prior to microscopic imaging. Cells were imaged

using a Leica TCS-SP8 confocal microscope with 63X magnification, using the same laser intensities for all samples. Appropriate laser lines were used such as 405 nm for DAPI and 488 nm for BMP2-Alexa Fluor 488.

Comparison of nuclear BMP2 staining intensity between wild type and mutant cells was performed on tiff versions of confocal microscope images using ImageJ to create tracings of DAPI-stained regions and to calculate the mean pixel intensity of nBMP2 staining within each nucleus. Mean nuclear staining intensity was calculated for each image, and groups were compared using an unpaired, two-tailed t-test in GraphPad Prism. (Fig 1B).

2.2.4 S. aureus Bacterial Infections

S. aureus ATCC strain 12600 was cultured in tryptic soy broth liquid culture alternating with standard streak plating on mannitol salt agar (Thermo Fisher Scientific) for counting. To prepare bacteria for injections, 100 µl of overnight liquid culture was transferred into a new 15 ml broth culture and grown until OD_{600} reached 1.0, then pelleted and resuspended in 15 ml of PBS with 20% glycerol, aliquoted, and stored at -80˚C for 3 weeks before injection. Frozen stock concentration was verified one day before the infection by thawing a single aliquot and performing standard serial dilution plate counts. On the day of infection, *S. aureus* was diluted from the frozen stock to the desired concentration in PBS, and mice received a 200 µl retroorbital injection using a 1 ml syringe and 27-gauge needle. The injected volume contained a priming dose of 1×10^4 CFU/g body weight on day 0 (primary infection), and a dose of 3×10^5 CFU/g body weight on day 35 (secondary infection). Macrophages were harvested three days later.

2.2.5 Bacterial Supernatant Preparation

Bacterial supernatant obtained from *E. coli* K12 and *S. aureus* 12600 was used to stimulate Ca^{2+} fluxes in BMD and splenic macrophages [59,60]. A single colony was picked from an agar plate and inoculated into liquid broth overnight culture. The next day, 1 ml of the overnight culture was inoculated into 15 ml liquid broth and incubated with shaking at 37°C until culture reach an OD_{600} of 1-1.3. Cells were then pelleted by centrifugation at 1,800 x g for 12 min at 4°C, and supernatant was collected.

2.2.6 Calcium Imaging

BMD and splenic macrophages were isolated and matured in culture for 7 days as described above, then seeded on 8-chambered coverglasses (Nunc 155411, Thermo Scientific) and incubated overnight in macrophage medium at 37°C in 5% CO_2. For BMD macrophages, 10 ng/ml LPS from E. coli O55:B5 (Sigma) was included in the overnight incubation to activate cells. The next day, cells were loaded with 3 μM Fura-2AM (Invitrogen) in Ringers solution containing Ca^{2+} to be used as an extracellular source during the Ca^{2+} imaging assay (150 mM NaCl, 10mM glucose, 5 mM HEPES, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, pH 7.4) for 30 min at 37°C in 5% CO_2, washed with Ringers solution, then incubated for another 30 minutes at 37°C in Ringers solution. Calcium imaging was performed at room temperature using an Olympus IX51 inverted microscope equipped with a xenon arc lamp. Fura-2AM loaded macrophages were excited using 340 nm and 380 nm excitation filters, and images of 340 nm, 380 nm, and transmitted light were capture using a florescence microscope camera (Q Imaging Exi Blue) with a 20x objective (N.A. 0.75) at 3-sec intervals. At the 2-min time point in each imaging protocol, 20 μl of bacterial supernatant was added to stimulate Ca^{2+} flux. Ionomycin (1 μM final concentration) was added at the 10-min time point as a positive control. 10-20

representative cells were selected as regions of interest in each frame, and F340:F380 ratios were calculated and analyzed using CellSens software from Olympus. Each individual cell's fluorescence was normalized to its first recorded value according to the equation (F-Fo)/Fo, where F is the fluorescence at the specific time point, and Fo is the fluorescence value at time 0 [60,61].

2.2.7 Engulfment Assay

BMD and splenic macrophages were isolated and matured in culture for 7 days as described above, then seeded in 12-well culture plates for flow cytometry-based engulfment assays [62-68]. 100% FBS was used to resuspend 2.0 μm phycoerythrin-conjugated polychromatic red latex microspheres (Polysciences, Inc.) to prevent beads from sticking to the cell membranes during engulfment [62]. The ~10^9 particles/ml concentration was chosen to ensure that beads were not a limiting factor in phagocytosis rates [62]. Macrophages were then activated by adding LPS from E. coli O55:B5 (Sigma) to a final concentration of 10 ng/ml and incubated for 1 hour at 37°C and 5% CO_2. Media was removed and cells were rinsed with cold PBS, then collected and analyzed by flow cytometry using an Attune flow cytometer (Applied Biosystems by Life technologies). Cells were pre-treated with anti-CD16/32 antibodies (14-0161-85 eBioscience) to prevent non-specific antibody binding, then surface stained with APC-conjugated anti-CD11b antibodies (17-0112-82 eBioscience) and FITC-conjugated anti-F4/80 FITC antibodies (11-4801-82 eBioscience) to identify mature macrophages. Doublets were removed based on forward scatter width (FSC-W)/forward scatter area (FSC-A), and the F4/80 and CD11b double positive population was selected. From within this gate, engulfing macrophages were distinguished from non-engulfing macrophages based on phycoerythrin fluorescence, and

macrophages could be further distinguished based on the engulfment of one, two, or three or more beads. Results were analyzed using FlowJo software (Tree Star).

2.2.8 Data Analysis

All assays were performed as at least three independent repeats, each in triplicate. Area under the curve (AUC) was determined using GraphPad Prism. Statistical significance was assessed using unpaired two-tailed Students T test in GraphPad Prism.

2.3 Results

2.3.1 The nuclear variant nBMP2 is expressed in BMD and splenic macrophages from wild type mice

To determine whether nBMP2 is expressed in macrophages, BMD macrophages and splenic macrophages were isolated from naïve (uninfected) wild type and nBmp2NLStm mutant mice and differentiated in vitro, and immunocytochemistry was performed using an anti-BMP2 antibody that binds to both BMP2 and nBMP2. Consistent with our prior observation of impaired immune response in nBmp2NLStm mutant mice[32], nBMP2 was detected in the nuclei of wild type BMD (Fig. 7a) and splenic (Fig. 7b) macrophages. As expected, nBMP2 was significantly decreased in macrophage nuclei from nBmp2NLStm mutant mice (Fig. 7a and 7b, mutant). ImageJ software quantification of immunofluorescence images showed that the density of nuclear BMP2 staining was significantly more intense in wild type compared to mutant macrophages in both BMD macrophages ($p = 0.0005$) and splenic macrophages ($p < 0.0001$) (Fig. 8). BMP2 staining was visible throughout the cytoplasm of both wild type and mutant macrophages, as expected, given that nBMP2 is synthesized in the cytosol before being translocated to the nucleus and that the conventional BMP2 growth factor is synthesized in the rough ER and translocated through the Golgi before being secreted from the cell.

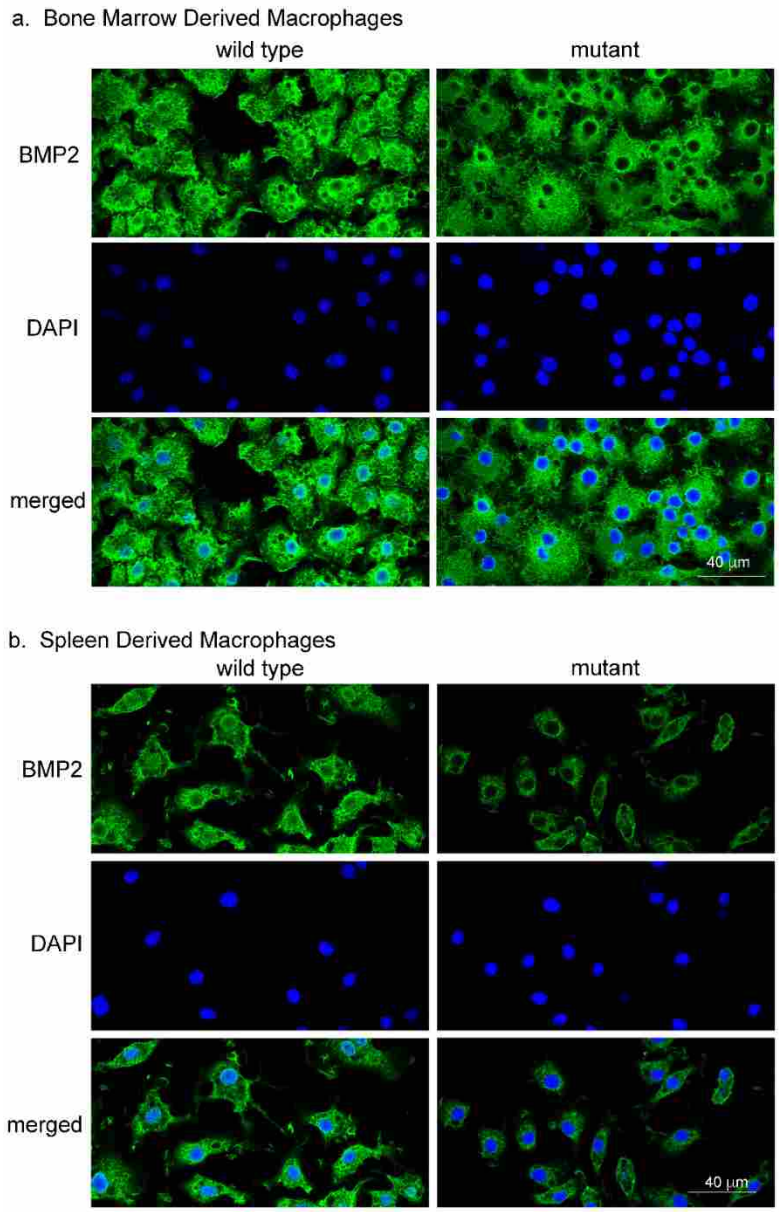

Figure 7. BMD macrophages and splenic macrophages express nBMP2, which is decreased in the nuclei of nBmp2NLS^{tm} mutant macrophages.
(a) BMD macrophages and (b) splenic macrophages were stained with anti-BMP2 antibody (green) and counterstained with DAPI (blue), demonstrating that nBMP2 is expressed and localized to the nucleus in wild type macrophages, and that nuclear translocation of nBMP2 is inhibited in mutant macrophages. BMP2 labeling within the cytoplasm is present in both wild type and mutant cells as expected, because the targeted mutation allows translation of nBMP2 in the cytoplasm but inhibits nuclear translocation, and it allows normal synthesis and secretion of conventional BMP2.

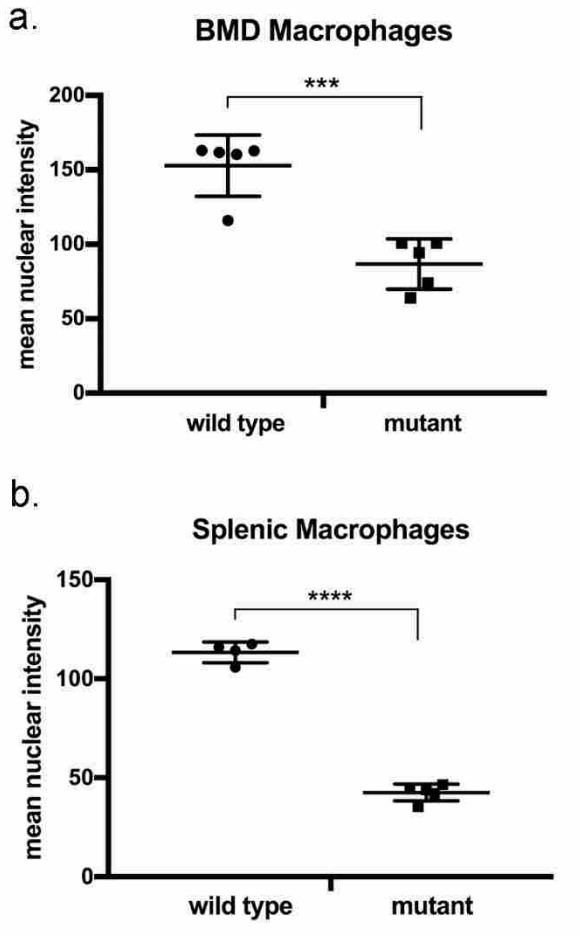

Figure 8. Quantification of nBMP2 nuclear staining intensity.
Five images each were analyzed for wild type and mutant BMD macrophages and for mutant splenic macrophages. Four images were analyzed for wild type splenic macrophages. Each image contained between 10 and 93 cells, and the number of cells analyzed per group ranged from 100 to 337. ImageJ was used to outline DAPI-stained regions and quantify BMP2 immunostaining as the sum of pixel intensities within each nucleus. The mean density of BMP2 immunostaining was then calculated for all nuclei in an image. An unpaired, two-tailed t-test was performed to compare nuclear staining between wild type and mutant cells. For BMD wild type vs. mutant macrophages, $p = 0.0005$. For splenic wild type vs mutant macrophages, $p < 0.0001$.

2.3.2 BMD macrophages from uninfected nBmp2NLS^{tm} mutant mice and wild type mice have similar Ca^{2+} response

Naïve BMD macrophages isolated from femurs and tibias of uninfected mice were matured and activated *in vitro* then plated for live-cell Ca^{2+} imaging. Plated cells were loaded with Fura-2AM, a UV-excitable ratiometric calcium indicator that changes its excitation in

response to Ca^{2+} binding; Fura-2AM emits at 380 nm when Ca^{2+} is not bound, and at 340 nm when Ca^{2+} binds to the dye. The fluorescence ratio (F340/F380), increases as cytosolic Ca^{2+} levels increase [69]. At the 2 min time point, supernatant from *Escherichia coli* (ECS) cultures was added to stimulate Ca^{2+} flux (Fig. 9a) [60,70,71]. Following this stimulation, there were no observable differences between naïve mutant and wild type BMD macrophages in peak Ca^{2+} response (Fig. 9b) or sustained Ca^{2+} levels (Fig. 9c).

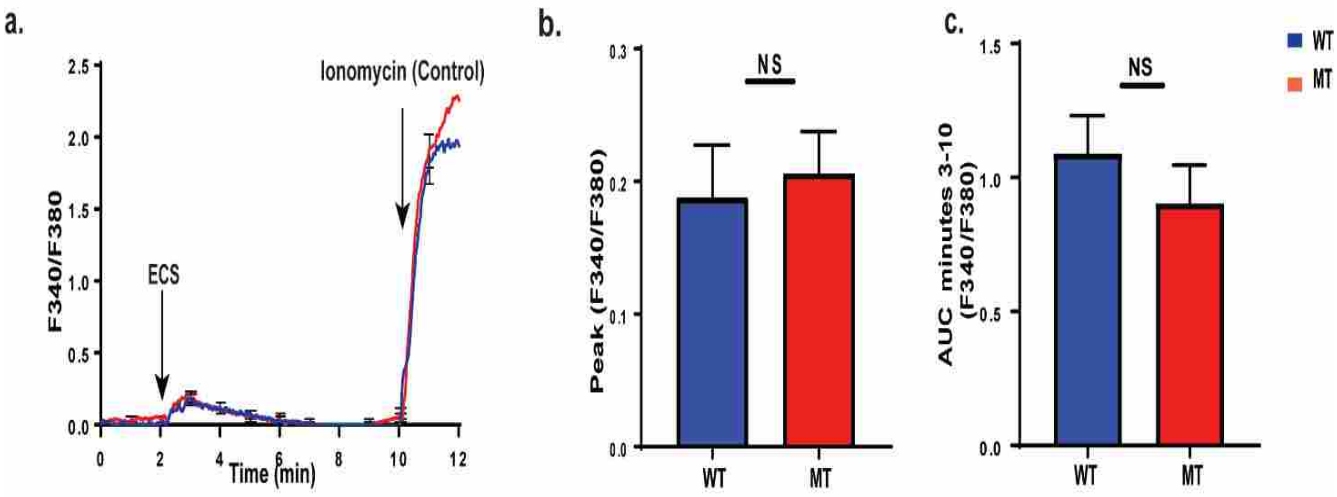

Figure 9. Naïve bone marrow derived (BMD) macrophages from nBmp2NLS^{tm} mutant mice and wild type mice have a similar Ca^{2+} response.
Naïve BMD macrophages from wild type (WT) and nBmp2NLS^{tm} mutant (MT) mice were loaded with Fura-2AM for live-cell Ca^{2+} imaging. During imaging, cells were stimulated at 2 min with *E. coli* supernatant (ECS), then at 10 min with ionomycin as a positive control. (a) Average curves showing intracellular Ca^{2+} response in wild type and nBmp2NLS^{tm} mutant BMD macrophages. Fluorescence ratios (F340/F380) were measured at 3 sec intervals from 0-12 min (n = 38 cells). Error bars (s.e.m.) are shown at one min intervals. (b) Average (± s.e.m.) of peak Ca^{2+} influx (F340/F380) in wild type and nBmp2NLS^{tm} mutant BMD macrophages (n = 38 cells). (c) Area under the curve (AUC) of F340/F380 ratios from minutes 3 to 10 min shows sustained intracellular Ca^{2+} levels (n = 38 cells). NS, not significant.

2.3.3 Splenic macrophages isolated from nBmp2NLS^{tm} mutant mice after secondary infection show impaired Ca^{2+} response

In our prior study, immune deficiencies in nBMP2NLS^{tm} mice were detectable only after the mice received a secondary infection with *S. aureus* [32]. Because our current experiments

revealed no significant differences in Ca^{2+} response in naïve BMD macrophages from mutant compared to wild type mice, we decided to replicate the *in vivo* conditions of our previous work by examining splenic macrophage harvested from mice after a secondary infection with *S. aureus*, and by using *S. aureus* supernatant as the stimulus to trigger Ca^{2+} flux [32]. Although *S. aureus* is a gram positive bacteria that does not produce LPS, it does produce liphoteichoic acid (LTA), which is similarly able to activate macrophages[72,73]. Thirty-five days after primary systemic *S. aureus* infections, mice were given a second injection of *S. aureus*, and splenic macrophages were isolated 3 days later.

After one week *in vitro* maturation, splenic macrophages were loaded with Fura-2AM for live-cell Ca^{2+} imaging experiments. *S. aureus* supernatant (SAS) was used to stimulate Ca^{2+} flux at the 2-min time point (Fig. 10a). Compared to the lack of a difference in naïve BMD macrophages, it is particularly striking that peak Ca^{2+} response was significantly decreased ($p=0.0335$) in mutant splenic macrophages after secondary infection (Fig. 10b). Sustained Ca^{2+} levels as measured by the area under the curve (AUC) from minutes 3-10 was also significantly decreased ($p=0.0008$) (Fig. 10c).

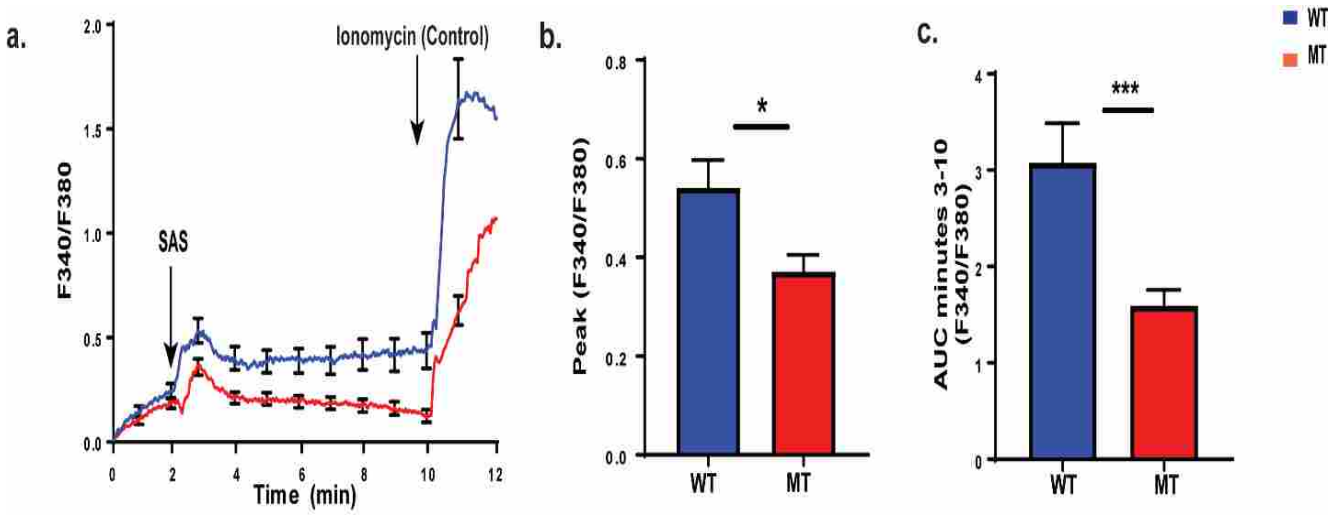

Figure 10. Splenic macrophages collected from nBmp2NLS^{tm} mutant mice after secondary infection have an impaired Ca^{2+} response.
Splenic macrophages from wild type (WT) and nBmp2NLS^{tm} mutant (MT) mice were loaded with Fura-2AM for live-cell Ca^{2+} imaging. During imaging, cells were stimulated at 2 min with *S. aureus* supernatant (SAS), then at 10 min with ionomycin as a positive control. (a) Average curves showing intracellular Ca^{2+} response in wild type and nBmp2NLS^{tm} mutant splenic macrophages. Fluorescence ratios (F340/F380) were measured at 3 sec intervals from 0-12 min (n = 44 cells). Error bars (s.e.m.) are shown at one min intervals. (b) Average ± s.e.m. of peak Ca^{2+} influx (F340/F380) in wild type and nBmp2NLS^{tm} mutant splenic macrophages shows a significant difference (n = 44 cells). (c) AUC of F340/F380 ratios from minutes 3 to 10 min shows a significant difference in sustained intracellular Ca^{2+} levels (n = 44 cells). * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.0001$.

2.3.4 BMD macrophages from uninfected nBmp2NLS^{tm} mutant mice and wild type mice show similar phagocytic activity

To test phagocytic activity of naïve BMD macrophages (meaning macrophages that were isolated from uninfected mice) from nBmp2NLS^{tm} mutant compared to wild type mice, we measured fluorescent bead engulfment by CD11b and F4/80 positive cells with flow cytometry (Fig. 11a)[62-68]. We observed no differences in the phagocytic activity of naïve BMD macrophages from nBmp2NLS^{tm} mutant compared to wild type mice (Fig. 11b, 11c, 11d and 11e).

Splenic macrophages from nBmp2NLS^{tm} mutant mice show impaired phagocytic activity

To test phagocytic activity in macrophages isolated from mice after secondary infection, splenic macrophages were isolated from wild type and nBmp2NLS^{tm} mutant mice 3 days after mice received a second systemic infection with *S. aureus*, and fluorescent bead engulfment was measured as described above. While differences between wild type and mutant macrophages did not reach significance when subgroups that engulfed 1, 2, or 3 or more beads were analyzed individually (Fig. 12a, 12b and 12c), there was a significant reduction in overall mutant phagocytic activity ($p = 0.0176$) when the subgroups were pooled (Fig. 12d). These data suggest a possible relationship between the decreased Ca^{2+} response and reduced phagocytosis in nBmp2NLS^{tm} mutant splenic macrophages.

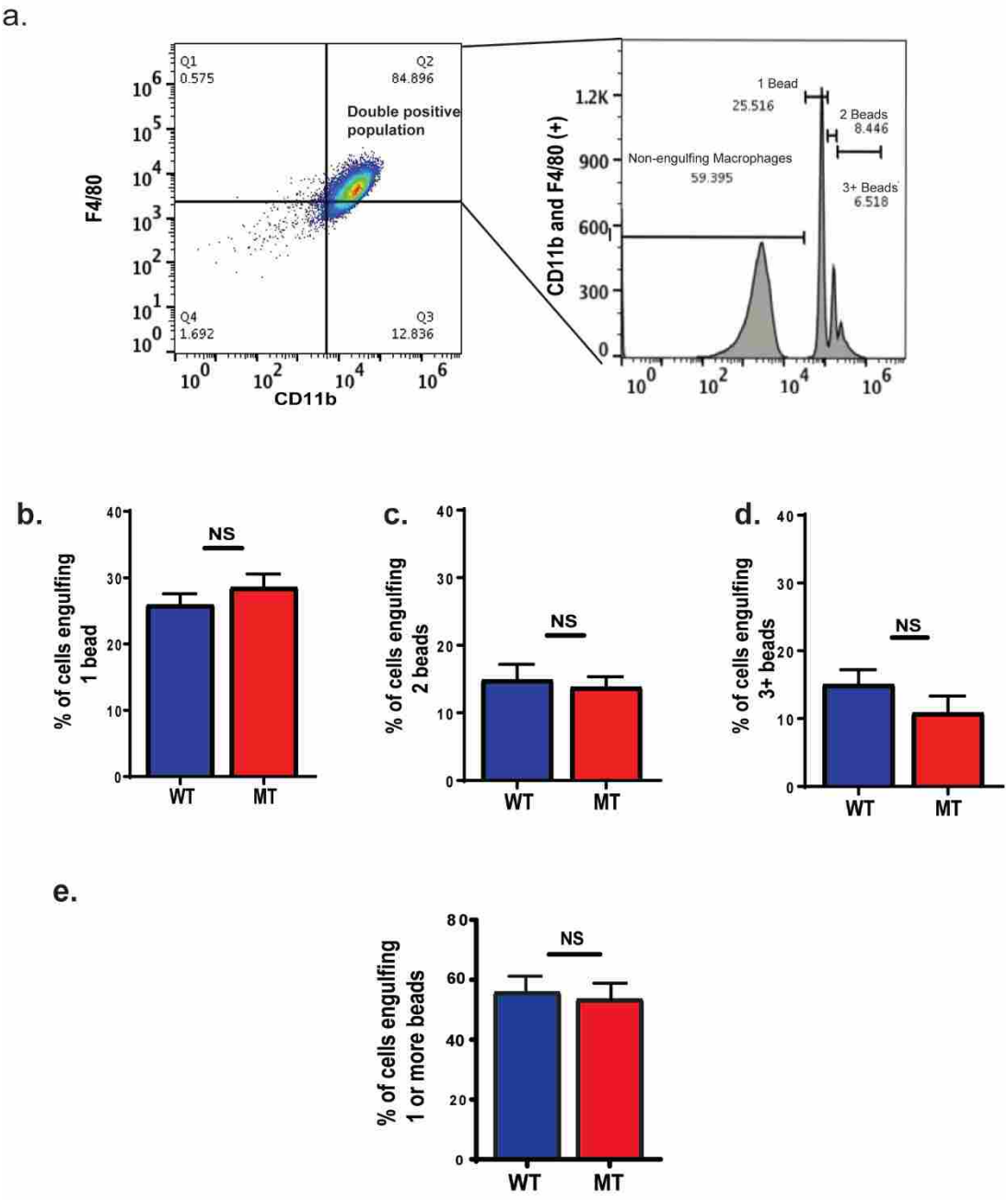

Figure 11. Naïve bone marrow derived (BMD) macrophages from nBmp2NLS^{tm} mutant mice and wild type mice show similar phagocytic activity.
After incubation with fluorescent microspheres, macrophages were analyzed by flow cytometry. (a) A representative analysis is shown. The F4/80 and CD11b double positive population was selected, and from this gate a histogram was produced to identify macrophages that had engulfed 1, 2, or 3 or more beads. The percentages of total double positive cells represented within each peak are indicated. (b) Percent of cells engulfing 1 bead, (c) percent of cells engulfing 2 beads, and (d) percent of cells engulfing 3 or more beads. (e) Percent of cells engulfing one or more beads. N=3 pairs of wild type and 3 pairs of mutant mice. NS, not significant.

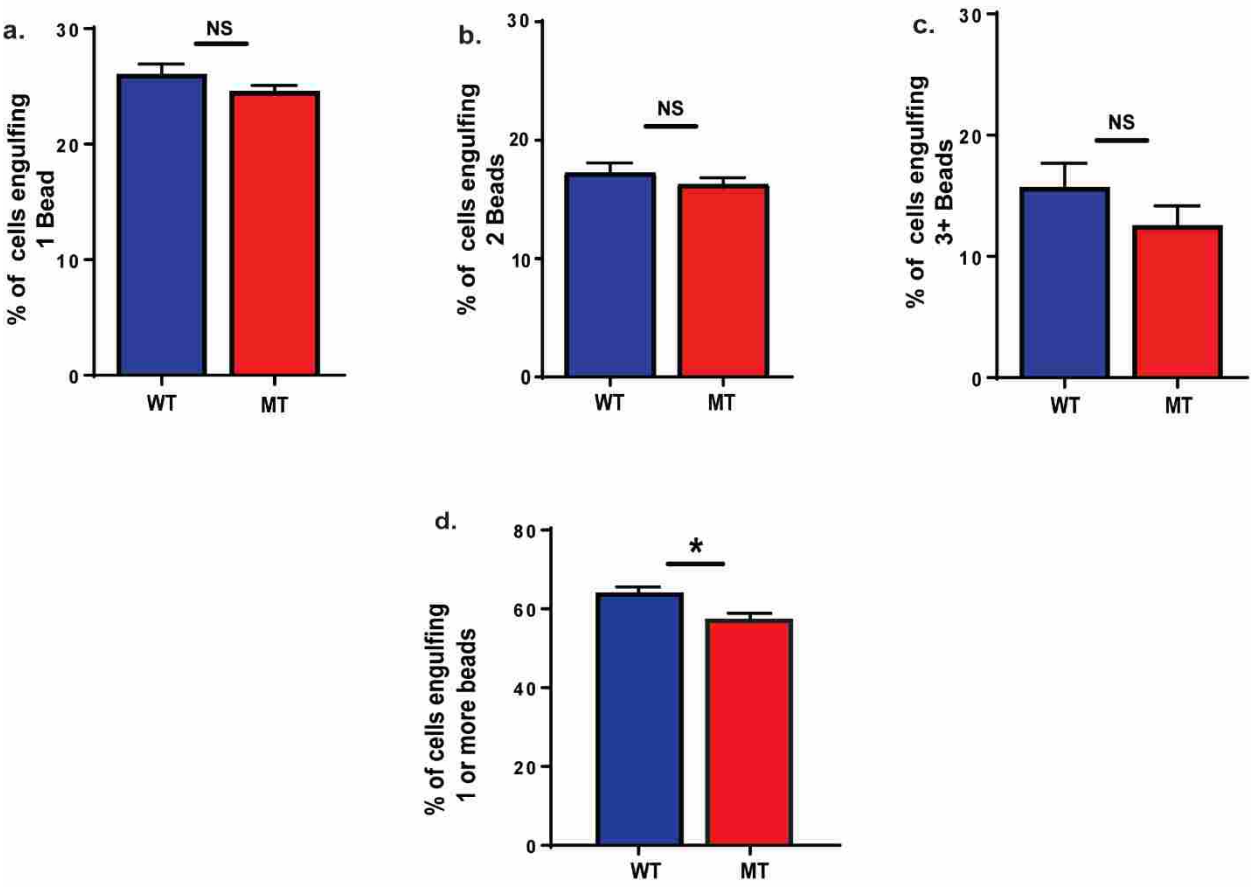

Figure 12. Splenic macrophages collected from nBmp2NLS^{tm} mutant mice after secondary infection show impaired engulfment activity.
After incubation with fluorescent microspheres, macrophages were analyzed by flow cytometry as described in Figure 3. (a) Percent of cells engulfing 1 bead, (b) percent of cells engulfing 2 beads and, (c) percent of cells engulfing 3 or more beads. (d) Percent of cells engulfing one or more beads. N=3 pairs of wild type and 3 pairs of mutant mice. NS, not significant. * = $p < 0.05$.

2.4 Discussion

The role of BMP2 in macrophages is unknown and remains an area of active research. BMP2 has been reported to be constitutively expressed in M1 (inflammatory) macrophages [74]. Other studies have shown that BMP2 expression is upregulated as macrophages shift toward the pro-healing/anti-inflammatory M2 phenotype [75]. BMP2 secretion by macrophages promotes migration of vascular smooth muscle cells, and macrophages in the intestinal muscularis secrete BMP2 to signal enteric neurons [76,77]. Reports of BMP2 expression by hematopoietic cells, in

particular macrophages, are relevant to this study because nBMP2 can be produced from the same mRNA as the conventional secreted BMP2 growth factor—any time BMP2 mRNA or BMP2 growth factor is detected, the potential for nBMP2 synthesis exists [78]. Accordingly, we have demonstrated by immunofluorescence that both BMD macrophages and splenic macrophages express the nuclear variant of BMP2, nBMP2, and that nBMP2 is decreased in the nuclei of macrophages from nBmp2NLS^{tm} mutant mice.

Previously, we demonstrated that deficiency of nBMP2 in the nucleus impairs secondary immune response as evidenced by diminished spleen enlargement, poor clearance of *S. aureus* from the bloodstream, and increased mortality after secondary infection [32]. We have also shown that deficiency of nBMP2 in myonuclei is correlated with slowed skeletal muscle relaxation after contraction, and deficiency of nBMP2 in the nuclei of hippocampal neurons is correlated with learning/memory deficits [56]. Each of these phenotypes is consistent with deficiencies in intracellular Ca^{2+} transport, but until now, no direct measurements of intracellular Ca^{2+} have been performed in cells from nBmp2NLS^{tm} mutant mice. The discovery that macrophages express nBMP2 (Fig. 7) provided an accessible cell type in which to directly address the question of whether nBMP2 plays a role in intracellular Ca^{2+} response.

We found that intracellular Ca^{2+} response was impaired in mutant splenic macrophages after secondary infection with *S. aureus*, but not in mutant BMD macrophages isolated from uninfected mice, even though both macrophage types expressed nBMP2. Recent work has revealed that innate immune cells can undergo memory-like adaptive responses to increasing pathogen load, and the deficient Ca^{2+} response in splenic macrophages after secondary infection might represent a failure of those adaptive responses [79,80]. Alternatively, it may be that the effects of nBMP2 deficiency in the nucleus are simply cumulative, causing a Ca^{2+}-handling

phenotype that becomes progressively more severe as cells differentiate and mature. A progressive phenotype is consistent with our previously reported observation that hippocampal long-term potentiation (LTP) was normal in 3-week-old nBmp2NLS^{tm} mutant mice but deficient in 3-month-old mice [56]. Progressive impairment of intracellular Ca^{2+} response has received attention recently as a potential mechanism for both brain and muscle aging [81-83], suggesting that nBMP2 dysfunction could contribute to premature aging or aging-related diseases.

Deficiency of nBMP2 in the nucleus also produced a significant decrease in the total phagocytic activity of splenic macrophages from nBmp2NLS^{tm} mutant mice, suggesting that mutant cells may be less effective at clearing pathogens from the blood stream. This is consistent with prior studies suggesting that intracellular Ca^{2+} mobilization plays a role in macrophage phagocytic activity. For example, impaired Ca^{2+} response in macrophages from Trpm4 (-/-) mutant mice led to decreased phagocytic activity, resulting in bacterial overgrowth and translocation to the bloodstream [84]. Intracellular Ca^{2+} levels increase during Fcɣ receptor (FcR)-mediated phagocytosis [22,85,86], and the loss of CaMKK2, a calcium-dependent kinase, left macrophages unable to phagocytose bacteria or synthesize cytokines in response to bacterial lipopolysaccharide (LPS) [87].

Although evidence supports the involvement of Ca^{2+} response in macrophage phagocytic activity, the scale of the decreased phagocytosis by splenic macrophages observed in our study seems insufficient to account for the markedly increased mortality of nBmp2NLS^{tm} mutant mice after secondary infection [56]. We cannot rule out the possibility that the bead engulfment assay did not fully reflect the severity of phagocytosis impairment in splenic macrophages. Liver macrophages also play a role in bacterial clearance, and it is possible that the absence of nBMP2 in the nucleus affects their function more severely [88,89]. In addition, the absence of nBMP2 in the

nucleus might affect other immune system cell types besides macrophages, and it is possible that another cell type, or perhaps several cell types together, account for the increased mortality of nBmp2NLS^{tm} mutant mice after secondary infection [56]. Indeed, BMP2 (and therefore potentially nBMP2) is expressed by a specialized endothelial population in the early embryo, termed hemogenic endothelium, that gives rise to hematopoietic stem cells [90]. The absence of nBMP2 at the earliest stages of hemogenesis could therefore impact a wide range of immune cell types. BMP2 is also expressed in human cord blood cells, including those that express CD34, a hematopoietic progenitor cell antigen [91], and acute bleeding triggers upregulation of BMP2 expression in hematopoietic stem cells [92]. BMP2 expression is also found in mature B cells, where it is upregulated in response to infection with *Aggregatibacter actinomycetemcomitans* [93]. It is possible, therefore, that nBMP2 impacts the activation or function of other immune cell types in addition to macrophages, and the combined functional deficits account for the increased mortality in nBmp2NLS^{tm} mutant mice after secondary infection.

It will be important, in future work, to elucidate the molecular mechanisms underlying the Ca^{2+} response differences between macrophages from wild type and nBMP2 mutant mice. Differences may stem from impaired uptake or release of Ca^{2+} from endoplasmic reticulum stores, as suggested by the decreased SERCA activity observed in skeletal muscle of nBMP2 mutant mice[31]. Alternatively, transport of Ca^{2+} could be impaired at the macrophage cell membrane, consistent with observations that increasing extracellular Ca^{2+} levels can improve phagocytosis [94,95]. Neurons and muscle cells are excitable cells and are therefore equipped with a different set of ion channels and transporters than are macrophages, and so it will be important to examine molecular details of the Ca^{2+} handling defect in all three cell types. This work has thus opened the way for future studies into the molecular interactions and activities of nBMP2.

Questions about how nBMP2 functions from inside the nucleus to affect Ca^{2+} response also remain to be answered. The novel protein nBMP2 was first identified from among nuclear proteins that had been isolated using DNA affinity chromatography, but subsequent experiments failed to show direct binding of nBMP2 to DNA, and the amino acid sequence of nBMP2 contains no predicted DNA-binding domain [78]. It is possible that nBMP2 interacts indirectly with DNA through a transcription factor, and future studies of nBMP2's impact on the expression of genes involved in Ca^{2+} signaling will be informative.

In summary, this study supports our working hypothesis that aberrant intracellular Ca^{2+} response is the mechanism that unites the otherwise disparate muscle, neurological, and immune phenotypes observed in nBmp2NLS^{tm} mutant mice [30-32,96-98]. In doing so, this study has paved the way for future work to elucidate the precise molecular nature of the Ca^{2+} signaling disruptions in nBMP2 mutant cells and to understand how nBMP2's interactions in the nucleus impact Ca^{2+} signaling.

2.5 Acknowledgements

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CHAPTER 3: T cell Calcium Signaling Regulation by Co-receptor CD5

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Abstract

Calcium influx is critical for T cell effector function and fate. T cells are activated when T cell receptors (TCRs) engage peptides presented by antigen-presenting cells (APC), causing an increase of intracellular calcium (Ca^{2+}) concentration. Co-receptors stabilize interactions between the TCR and its ligand, the peptide-major histocompatibility complex (pMHC), and enhance Ca^{2+} signaling and T cell activation. Conversely, some co-receptors can dampen Ca^{2+} signaling and inhibit T cell activation. Immune checkpoint therapies block inhibitory co-receptors, such as cytotoxic T-lymphocyte associated antigen 4 (CTLA-4) and programmed death 1 (PD-1), to increase T cell Ca^{2+} signaling and promote T cell survival. Similar to CTLA-4 and PD-1, the co-receptor CD5 has been known to act as a negative regulator of T cell activation and to alter Ca^{2+} signaling and T cell function. Though much is known about the role of CD5 in B cells, recent research has expanded our understanding of CD5 function in T cells. Here we review these recent findings and discuss how our improved understanding of CD5 Ca^{2+} signaling regulation could be useful for basic and clinical research

3.1 Introduction

T cells are a critical component of the adaptive immune system. T cell responses are influenced by signals that modulate the effects of the T cell receptor (TCR) and peptide-major

histocompatibility complex (pMHC) interaction and initiate the transcription of genes involved in cytokine production, proliferation and differentiation [99-101]. T cell activation requires multiple signals. First, the TCR engages the pMHC leading to tyrosine phosphorylation of CD3 and initiation of the Ca^{2+}/Calcineurin/ Nuclear factor of activated T cells (NFAT) or PKCθ/Nuclear factor-κ-light chain enhancer of activated B cells (NF-κB) or Mitogen-activated protein kinase (MAP kinase)/AP-1 pathways [102-104]. Second, cell surface costimulatory molecules, such as co-receptor CD28, amplify TCR-pMHC complex signals and promote stronger intracellular interactions to prevent T cell anergy [105,106]. Finally, cytokines such as interleukin-12 (IL-12), interferon α (INFα) and interleukin-1 (IL-1) promote T cell proliferation, differentiation and effector functions [104].

Co-receptors such as CD4 and CD8 interact with MHC molecules and additional co-receptors interact with surface ligands present on antigen presenting cells (APCs) to regulate T cell homeostasis, survival, and effector functions with stimulatory or inhibitory signals [107]. Altering co-receptor levels, balance, or function dramatically effects immune responses and their dysfunction is implicated in autoimmune diseases [108]. Stimulatory co-receptors such as CD28, Inducible T-cell COStimulator (ICOS), 4-1BB, OX40, glucocorticoid induced TNF receptor (GITR), CD137 and CD77 promote T cell activation and protective responses [109]. Co-receptor signaling is initiated by the phosphorylation of tyrosine residues located in immunoreceptor tyrosine-based activation motifs (ITAMs) or immunoreceptor tyrosine based inhibitory motifs (ITIMs) [105,110]. The phosphorylated tyrosines serve as docking sites for spleen tyrosine kinase (Syk) family-members such as zeta-chain-associated protein kinase 10 (ZAP-70) and Syk which activate the phospholipase C γ (PLCγ), RAS, and extracellular signal regulated kinase (ERK) pathways in addition to mobilizing intracellular Ca^{2+} stores [111].

One of the best described T cell co-receptors, CD28, is a stimulatory T cell surface receptor from the Ig superfamily with a single Ig variable-like domain which binds to B7-1 (CD80) and B7-2 (CD86) [100]. Ligand binding phosphorylates CD28 cytoplasmic domain tyrosine motifs such as YMNM and PYAP and initiates binding and activation of phosphatidylinositide 3 kinase (PI3K) which interacts with protein kinase B (Akt) and promotes T cell proliferation and survival [99]. CD28 also activates the NFAT pathway and mobilizes intracellular Ca^{2+} stores through association with growth factor receptor-bound protein 2 (GRB2) and the production of phosphatidylinositol 4-,5-bisphosphate (PIP2), the substrate of PLCγ1, respectively [100,112]. Blocking stimulatory co-receptors suppresses T cell effector function. For example, blocking stimulatory CD28 with anti-CD28 antibodies promotes regulatory T cell function and represses activation of auto- and allo-reactive T effector cells after organ transplantation [106,113].

T cells also have inhibitory co-receptors which regulate T cell responses [106]. The best characterized are Ig superfamily members cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed cell death protein 1 (PD-1) [106,114]. CTLA-4 binds CD80 and CD86 with greater avidity than CD28, and its inhibitory role refines early phase activation signals for proliferation and cytokine production [114-117]. PD-1, another CD28/B7 family member, regulates late phase effector and memory response [118]. Inhibitory co-receptors such as CTLA-4 and PD-1, known as “immune checkpoints”, block the interaction between CD28 and its ligands altering downstream secondary T cell activation signals [117]. Therefore, blocking CTLA-4 or PD-1 promotes effector T cell function in immunosuppressive environments [117,119].

There are also a number of co-receptors that have differential modulatory properties. For example, CD5, a lymphocyte glycoprotein expressed on thymocytes and all mature T cells, has contradictory roles at different time points. CD5 expression is set during thymocyte development

and decreases the perceived strength of TCR-pMHC signaling in naïve T cells by clustering at the TCR-pMHC complex and reducing TCR downstream signals such as the Ca^{2+} response when its cytoplasmic pseudo-ITAM domain is phosphorylated [120-123]. The CD5 cytoplasmic domain has four tyrosine residues (Y378, Y429, Y411 and Y463), and residues Y429 and Y441, are found in a YSQP-(x8)-YPAL pseudo ITAM motif while other tyrosine residues make up a pseudo-ITIM domain [121]. Phosphorylated tyrosines recruit several effector molecules and may sequester activation kinases away from the TCR complex, effectively reducing activation signaling strength [121]. Recruited proteins include Src homology-2 protein phosphatase-1 (SHP-1), Ras GTPase protein (rasGAP), CBL, casein kinase II (CK2), zeta-chain-associated protein kinase 70 (ZAP70), and PI3K which are involved in regulating both positive and negative TCR-induced responses [124-126]. For example, ZAP-70 phosphorylates other substrates and eventually recruits effector molecules such as PLC gamma and promotes Ca^{2+} signaling and Ras activation which stimulates the ERK pathway and leads to cellular activation [111,127]. Conversely, SHP1 inhibits Ca^{2+} signaling and PKC activation via decreased tyrosine phosphorylation of PLCγ [111,124,128,129]. Further, Y463 serves as a docking site for c-Cb1, a ubiquitin ligase, which is phosphorylated upon CD3-CD5 ligation and leads to increased ubiquitylation and lysosomal/proteasomal degradation of TCR downstream signaling effectors and CD5 itself [130]. Thus, CD5 has a mix of downstream effects that both promote and inhibit T cell activation. Curiously, recent work suggests that in contrast to its initial inhibitory nature, CD5 also co-stimulates resting and mature T cells by augmenting CD3-mediated signaling [61,123,131,132].

Ca^{2+} is an important second messenger in many cells types, including lymphocytes, and plays a key role in shaping immune responses. In naïve T cells, intracellular Ca^{2+} is maintained at low levels, but when TCR-pMHC complexes are formed, inositol triphosphate (IP3) initiates

Ca^{2+} release from intracellular stores of the endoplasmic reticulum (ER) which opens the Ca^{2+} release activated Ca^{2+} channels (CRAC) and initiates influx of extracellular Ca^{2+} through store operated Ca2+ entry (SOCE) [5,14,19,133-135]. The resulting elevation of intracellular Ca^{2+} levels activates transcription factors involved in T cell proliferation, differentiation and cytokine production (e.g. nuclear factor of activated cells (NFAT)) [5,19]. Thus, impaired Ca^{2+} mobilization affects T cell development, activation, differentiation and function [136,137]. Examples of diseases with impaired Ca^{2+} signaling in T cells include systemic lupus erythematosus, type 1 diabetes mellitus, and others [138,139].

In this review, we will focus on CD5 co-receptor signaling and its functional effects on T cell activation. First, we will discuss how the inhibitory co-receptors CTLA-4 and PD-1 modulate T cell function. Then we will compare CTLA-4 and PD-1 function to CD5 function, examine recent findings that expand our understanding of the role of CD5, and assess how these findings apply to T cell Ca^{2+} signaling. Finally, we will consider CD5 Ca^{2+} signaling regulation in T cells and its potential physiological impact in immunometabolism, cell differentiation, homeostasis, and behavior.

3.2 Roles of Negative Regulatory T cell Co-receptors

3.2.1 CTLA-4

Cytotoxic T-lymphocyte antigen-4 (CTLA-4, CD152) inhibits early stages of T cell activation by recruiting inhibitory proteins such as SHP-2 and type II serine/threonine phosphatase PP2A that interfere with T cell synapse signaling [119,140-142]. CTLA-4 binds B7, a protein on activated APCs, with higher affinity than the stimulatory co-receptor CD28; the resulting balance between inhibitory and stimulatory signals controls T cell activation or anergy [117,143]. In naïve T cells, CTLA-4 is located in intracellular vesicles which localize at TCR binding

sites following antigen recognition and intracellular Ca^{2+} mobilization [117,144]. Like CD28, CTLA-4 aggregates to the central supramolecular activation complex (cSMAC) where it then extrinsically controls activation by decreasing immunological synapse contact time [145-147]. This suppresses pro-activation signals by activating ligands (B7-1 and B7-2) and induces the enzyme Inoleamine 2,3-dioxygenase (IDO) which impairs Ca^{2+} mobilization and suppresses T cell activation, ultimately altering IL-2 production and other effector functions in T cells [145,148,149]. CTLA-4 also stimulates production of regulatory cytokines such as transforming growth factor beta (TGF-β) which inhibit APC presentation and T cell effector function [141,146,147]. Compared to effector T cells (T_{eff}), CTLA-4 is highly expressed in regulatory T cells (T_{reg}) and plays a role in maintaining T_{reg} homeostasis, proliferation and immune responses [114,150,151]. Total or partial CTLA-4 deficiency inhibits T_{reg} ability to control cytokine production and can cause immune dysregulation [150,152-154]. Thus, CTLA-4 has an important role in the T_{reg} suppressive response [153]. Additionally, CTLA-4 mutations are associated with autoimmune diseases as thoroughly reviewed by Kristiansen et al [155].

The loss of CTLA-4 results in removal of CTLA-4 competition with CD28 for B7-1 and B7-2 and is implicated in autoimmunity and cancer [113,156]. Because CTLA-4 inhibits TCR signaling, CTLA-4 deficiency leads to T cell overactivation as measured by increased CD3ζ phosphorylation and Ca^{2+} mobilization [157]. Thus, modulating CTLA-4 signaling is an attractive target for immunotherapies that seek to boost or impair early TCR signaling for cancer and autoinflammatory diseases [158,159]. For example, Ipilimunab, an IgG1 antibody-based melanoma treatment, is a T cell potentiator that blocks CTLA-4 to stimulate T cell proliferation and stem malignant disease progression by delaying tumor progression and has been shown to significantly increase life expectancy [117,160,161]. Additionally, Tremelimumab, a non-complement

fixing IgG2 antibody, has been tested alone or in combination with other antibodies such as Durvalumab (a PD-1 inhibitor) and improves antitumor activity in patients with non-small cell lung cancer (NSCLC), melanoma, colon cancer, gastric cancer and mesothelioma treatment [162-167].

3.2.2 PD-1

Programmed cell death protein-1 (PD-1, CD279) is a 288-amino acid (50-55 KDa) type I transmembrane protein and a member of the B7/CD28 immunoglobulin superfamily expressed on activated T cells, B cells and myeloid cells [117,168,169]. PD-1 has two known ligands, PD-L1 and PD-L2, which inhibit T cell activation signals [170]. Like CTLA-4, PD-1 also inhibits T cell proliferation and cytokine production (INF-γ, TNF and IL-2) but is expressed at a later phase of T cell activation [117]. PD-1 has an extracellular single immunoglobulin (Ig) superfamily domain and a cytoplasmic domain containing an ITIM and an immunoreceptor tyrosine-based switch motif (ITSM) subunit critical for PD-1 inhibitory function [171]. Upon T cell activation, PD-1 is upregulated and initiates ITIM and ITSM tyrosine interaction with SHP-2 which mediates TCR signaling inhibition by decreasing Erk phosphorylation and intracellular Ca^{2+} mobilization [172,173]. PD-1 can block the activation signaling pathways PI3K-Akt and Ras-Mek-Erk, which inhibit or regulate T cell activation [172,174]. Thus, engagement of PD-1 by its ligand affects intracellular Ca^{2+} mobilization, IL-2 and TNF-α production, supporting PD-1 inhibitory role in TCR strength mediated signals [175].

PD-1 signaling also affects regulatory T cell (T_{reg}) homeostasis, expansion, and function [176]. T_{reg} activation and proliferation are impacted by PD-1 expression which enhances their development and function while inhibiting T effector cells [168,177]. PD-1, PD-L and T_{regs} help terminate immune responses [178]. Thus, PD-1 deficiency results not only in increased T cell

activation, but in the breakdown of tolerance and the development of autoimmunity in diseases such as multiple sclerosis and systemic lupus erythematosus [178-182]. PD-1 and its ligands protect tissues from autoimmune attacks by regulating T cell activation and inducing and maintaining peripheral tolerance [183,184]. Studies done in PD-1 deficient mice observed the development of lupus-like glomerulonephritis and arthritis, cardiomyopathy, autoimmune hydronephrosis, and Type I diabetes, among other ailments [185,186]. PD-1 protects against autoimmunity and promotes T_{reg} function. [178]. Enhancing T_{reg} response with a PD-L1 agonist shows therapeutic potential for asthma and other autoimmune disorders [178,187]. Because PD-1 specifically modulates lymphocyte function, effective FDA-approved monoclonal antibodies targeting PD-1 are clinically available (i.e. Pembrolizumab and Nivolumab) to treat advanced malignancies [118]. Not only does blocking PD-1 decrease immunotolerance of tumor cells, it also increases cytotoxic T lymphocyte anti-tumor activity [118].

3.3 CD5: A Contradictory Co-receptor

3.3.1 Overview of CD5 signaling and Ca^{2+} mobilization in T cells

CD5, known as Ly-1 antigen in mice or as Leu-1 in humans, is a type I transmembrane glycoprotein (67 kDa) expressed on the surface of thymocytes, mature T cells, and a subset of B cells (B-1a) [188,189]. Although CD5 was discovered over 30 years ago, it was only in the last decade that CD5 gained attention as a key T cell activation regulator [190,191]. CD5 expression is set in the thymus during positive selection and correlates with how tightly the thymocyte TCR binds to self-peptide-MHC (self-pMHC); greater TCR affinity for self-peptide leads to increased CD5 expression in double positive (DP) thymocytes [192]. In other words, DP thymocytes that receive strong activation signals through their TCR express more CD5 than those DP thymocytes that receive weak TCR signals [192]. CD5 knock out mice ($CD5^{-/-}$) have a defective negative and

positive selection process, and therefore their thymocytes are hyper-responsive to TCR stimulation with increased Ca^{2+} mobilization, proliferation and cytokine production [121,190]. On the other hand, because of the increased TCR avidity for self-pMHC, mature T cells with high CD5 expression ($CD5^{hi}$) (peripheral or post-positive selection T cells) respond to foreign peptide with increased survival and activation compared to mature T cells with low CD5 expression ($CD5^{lo}$) [132,193]. Therefore, CD5 is a negative regulator of TCR signaling in the thymus and modulates mature T cell response in the periphery [40,121,132,192].

While CTLA-4 and PD-1 belong to the immunoglobulin (Ig) family, CD5 belongs to group B of the scavenger receptor cysteine-rich (SRCR) superfamily and contains three extracellular SRCR domains [128,188,194]. The cytoplasmic tail of CD5 contains several tyrosine residues which mediate the negative regulatory activity independent of extracellular engagement [192,195,196]. As CD5 physically associates with TCRζ/CD3 complex upon TCR and pMHC interaction, the tyrosine residues in both TCRζ and CD5 are phosphorylated by tyrosine kinases associated with the complex [128,197-201]. This interaction is so intrinsic to T cell signaling that CD5 expression levels are proportional to the degree of TCRζ phosphorylation, IL-2 production capacity, and Erk phosphorylation which are critical for CD3-mediated signaling [42,131]. It is unknown whether post-translational modifications, such as conserved domain 1 and domain 2 glycosylations impact CD5 signaling [202,203]. CD5 is present in membrane lipids rafts of mature T cells where, upon activation, it helps augment TCR signaling, increases Ca^{2+} mobilization, and upregulates ZAP-70/LAT (linker for activation of T cells) activation [204-206]. This suggests that CD5 is not only a negative regulator in thymocytes, but also appears to positively influence T cell immune response to foreign antigen [207,208]. See Figure 13.

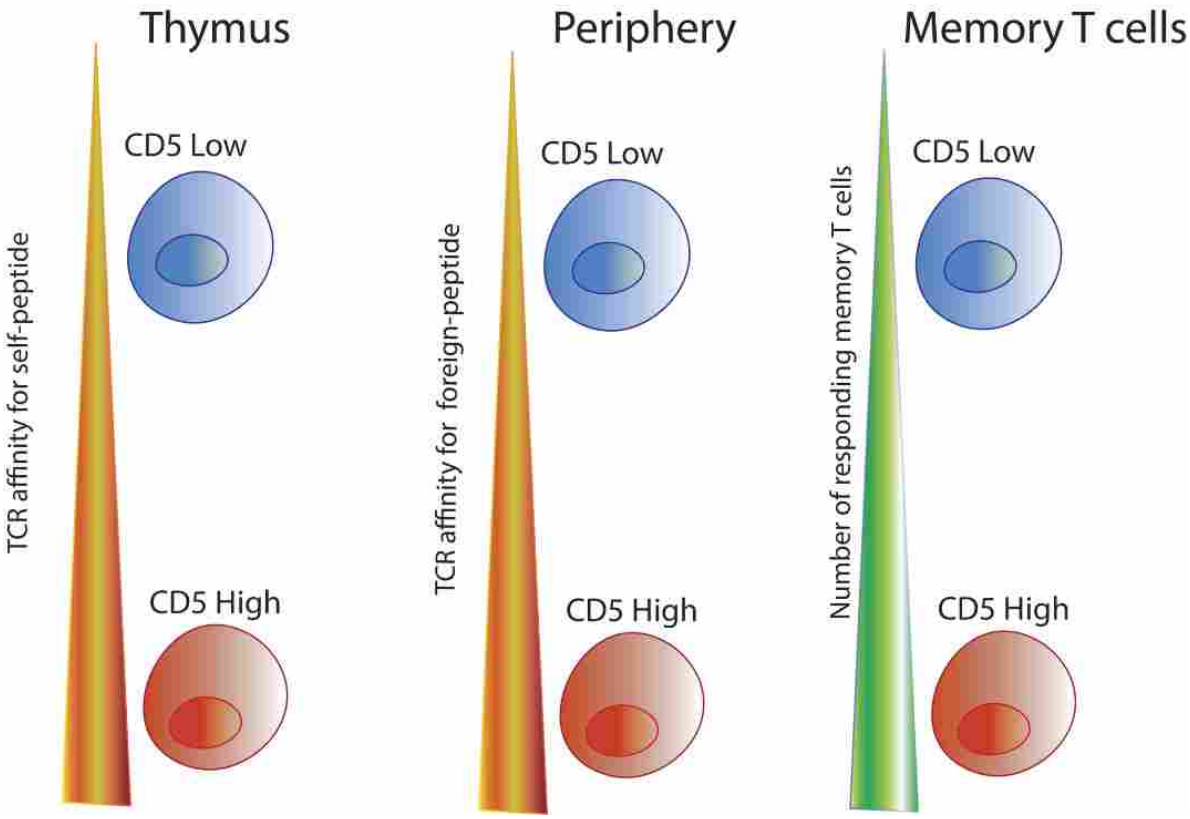

Figure 13. Effects of CD5 on different stages of T cell development.
CD5 expression on thymocytes is directly proportional to the signaling intensity of the TCR:self-pMHC interaction. In the periphery, T cells with higher CD5 levels ($CD5^{hi}$) are better responders to foreign-peptide. Long-lived memory cells populations are enriched for $CD5^{hi}$ T cells [40,132,209].

3.3.2 CD5 as a Ca^{2+} Signaling Modulator

As previously mentioned, CD5 expression levels are set in the thymus during T cell development and are maintained on peripheral lymphocytes [207]. CD5 expression in T cells plays an important role during development and primes naïve T cells for responsiveness in the periphery [42,43,61]. $CD5^{hi}$ T cells have the highest affinity for self-peptides and respond with increased cytokine production and proliferation to infection [35,193,210].

Our laboratory works with two TCR transgenic mouse lines with different levels of CD5 expression: LLO56 ($CD5^{hi}$) and LLO118 ($CD5^{lo}$) [42,43,207]. While LLO56 ($CD5^{hi}$) and LLO118 ($CD5^{lo}$) have similar affinity for the same immunodominant epitope ($LLO_{190-205}$) from *Listeria*

monocytogenes, on day 7 of primary response LLO118 ($CD5^{lo}$) has approximately three times the number of responding cells compared to LLO56 ($CD5^{hi}$), and conversely, on day 4 during secondary infection LLO56 ($CD5^{hi}$) has approximately fifteen times more cells than LLO118 ($CD5^{lo}$) [43]. This difference is not due to differential proliferative capacity, rather LLO56 ($CD5^{hi}$) has higher levels of apoptosis during the primary response [43]. Thus, LLO56 $CD5^{hi}$ and LLO118 $CD5^{lo}$ capacity to respond to infection appears to be regulated by their CD5 expression levels [207]. LLO56 ($CD5^{hi}$) thymocytes have greater affinity for self-peptide, which primes them to be highly apoptotic [43].

Recently we reported that in response to foreign peptide, LLO56 ($CD5^{hi}$) naïve T cells have higher intracellular Ca^{2+} mobilization than LLO118 ($CD5^{lo}$), which correlates with increased rate of apoptosis of LLO56 ($CD5^{hi}$), as Ca^{2+} overloaded mitochondria release cytochrome c which activates caspase and nuclease enzymes, thus initiating the apoptotic pathways [61,211,212]. LLO56 ($CD5^{hi}$) naïve T cell increased Ca^{2+} mobilization also provides additional support to the idea that $CD5^{hi}$ T cells have an enhanced response to foreign peptide [61,212]. This supports previous research that found that upon T cell activation increased CD5 expression is correlated with greater basal TCRζ phosphorylation, increased Erk phosphorylation, and more IL-2 production [193].

Thus, unlike CTLA-4 and PD-1 that are expressed only on activated T cells in the periphery during early and late phases of immune response, respectively, CD5 is set during T cell development, and influences T cells both during thymic development and during post-thymic immune responses [117,193] (See Fig. 14). CD5 not only has an important inhibitory role in the thymus, but also appears to positively influence the T cell population response; for example, more CD5hi T cells populate the memory T cell repertoire because CD5hi naïve T cells have a

stronger primary response [132,213].CD5 fine-tunes the sensitivity of TCR signaling to pMHC, altering intracellular Ca2+ mobilization and NFAT transcription, key players in T cell effector function [117,157,214]. As Ca2+ signaling plays a key role in T cell activation and function, controlling Ca^{2+} mobilization in T cells through CD5 expression could influence diverse areas of clinical research including metabolism, cancer treatments and even cognitive behavior.

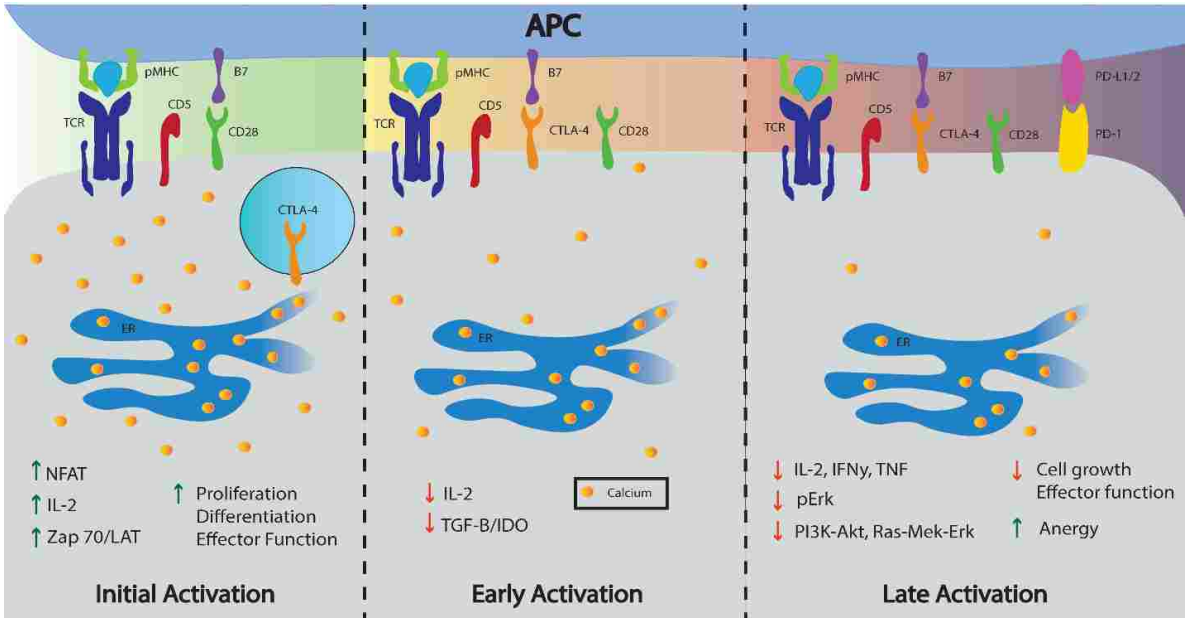

Figure 14. Inhibiting co-receptors modulate T cell activation.
CD5 is present in naïve T cells and localizes to the TCR-pMHC complex during activation. Initial activation cascades signal for the release of CTLA-4 from vesicles to the cell surface while the transcription factor NFAT transcribes PD-1. CTLA-4 provides inhibitory signals during early activation while PD-1 is expressed later and inhibits later stages of T cell activation. The initial Ca^{2+} mobilization is decreased by CTLA-4 and PD-1 downstream signals.

3.4 Physiological Impact of CD5 Expression in T cells

3.4.1 Metabolism

Naive T cells are in a quiescent state and rely on oxidative phosphorylation (OXPHOS) to generate ATP for survival [215,216]. Upon TCR-pMHC interaction, T cells undergo metabolic reprograming to meet energetic demands by switching from OXPHOS to glycolysis [217]. Glycolysis is a rapid source of ATP and regulates posttranscriptional production of INF-γ, a

critical effector cytokine [218]. Following the immune response, most effector T cells undergo apoptosis while a subset become quiescent memory T cells. Memory T cells have lower energetic requirements and rely on OXPHOS and Fatty Acid Oxidation (FAO) to enhance mitochondrial capacity for maintenance and survival [219].

Ca^{2+} signaling is a key second messenger in T cell activation and Ca^{2+} ions also modulate T cell metabolism through CRAC channel activity and NFAT activation [8,101]. During TCR-pMHC binding Ca^{2+} is released from the endoplasmic reticulum where it is absorbed by the mitochondria and initiates an influx of extracellular Ca^{2+} [101]. First, the rise of cytoplasmic Ca^{2+} activates stromal interaction molecule 1 (STIM1) located on the ER membrane to interact with the CRAC channel located on the cell membrane [220]. The release of the ER store and resulting extracellular Ca^{2+} influx increases the intracellular Ca^{2+} concentration and promotes AMPK (adenosine monophosphates activated protein kinase) expression and CaMKK (calmodulin-dependent protein kinase kinase) activity [101,220,221]. AMPK senses cellular energy levels through the ratio of AMP to ATP and generates ATP by inhibiting ATP-dependent pathways and stimulating catabolic pathways [222]. This indirectly controls T cell fate as AMPK indirectly inhibits mTOR (mammalian target of rapamycin complex) [223]. Because mTOR coordinates the metabolic cues that control T cell homeostasis, it plays a critical role in T cell fate [224]. T cells that are TSC1 (Tuberous sclerosis complex 1) deficient show metabolic alterations through increased glucose uptake and glycolytic flux [225].

The rise of cytoplasmic Ca^{2+} also encourages mitochondria to uptake cytoplasmic Ca^{2+} through the mitochondrial Ca^{2+} uniporter (MCU) [226]. This MCU uptake increases Ca^{2+} influx by depleting Ca^{2+} near the ER which further activates the CRAC channels and promotes STIM1 oligomerization [101,227-229]. Ca^{2+} uptake in the mitochondria also enhances the function of

the tricarboxylic acid cycle (TCA), which generates more ATP through OXPHOS [230,231]. OXPHOS is maintained by a glycolysis product, phosphoenolpyruvate (PEP), which sustains TCR-mediated Ca^{2+}-NFAT signaling by inhibiting the ER SERCA pump, thus promoting T cell effector function [232,233]. Downregulation of calmodulin kinase, CaMKK2, which controls NFAT signaling decreases glycolytic flux, glucose uptake, and lactate and citrate metabolic processes [87]. Ca^{2+} may also orchestrate the metabolic reprogramming of naïve T cells by promoting glycolysis and OXPHOS through the SOCE/calcineurin pathway which controls the expression of glucose transporters GLUT1/GLUT3, and transcriptional co-regulator proteins important for the expression of electron transport chain complexes required for mitochondria respiration [8].

Co-receptor stimulation plays a pivotal role in T cell metabolism and function. A decrease in T cell Ca^{2+} signaling represses glycolysis and affects T cell effector function [230]. PD-1 and CTLA-4 depress Ca^{2+} signaling and glycolysis while promoting FAO and antibodies against CTLA-4 and PD-1 increase Ca^{2+} mobilization and glycolysis during T cell activation [234,235]. Like CTLA-4 and PD-1, CD5 modulatory function has the potential to influence T cell metabolism. Analysis of gene families modulated by CD5 in B cells found that CD5 upregulates metabolic related genes including VEFG, Wnt signaling pathways genes, MAPK cascade genes, I-kB/NF-kB cascade genes, TGF β signaling genes, and adipogenesis process genes [236]. Therefore, proliferation differences correlated with CD5 expression in T cells may be caused by improved metabolic function as $CD5^{lo}$ T cells seem to be more quiescent than $CD5^{hi}$ T cells [237]. Although not much is known about how CD5 alters metabolic function in T cells, signaling strength differences of $CD5^{hi}$ and $CD5^{lo}$ T cell populations correlate with intracellular Ca^{2+} mobilization during activation and influence their immune response [42,43,61]. This implies that different metabolic processes may be initiated which would influence proliferation, memory cell

generation, and cytokine production. Fig. 15 summarizes how Ca^{2+} may be mobilized in $CD5^{hi}$ and $CD5^{lo}$ naïve T cells and the role Ca^{2+} may play on metabolism.

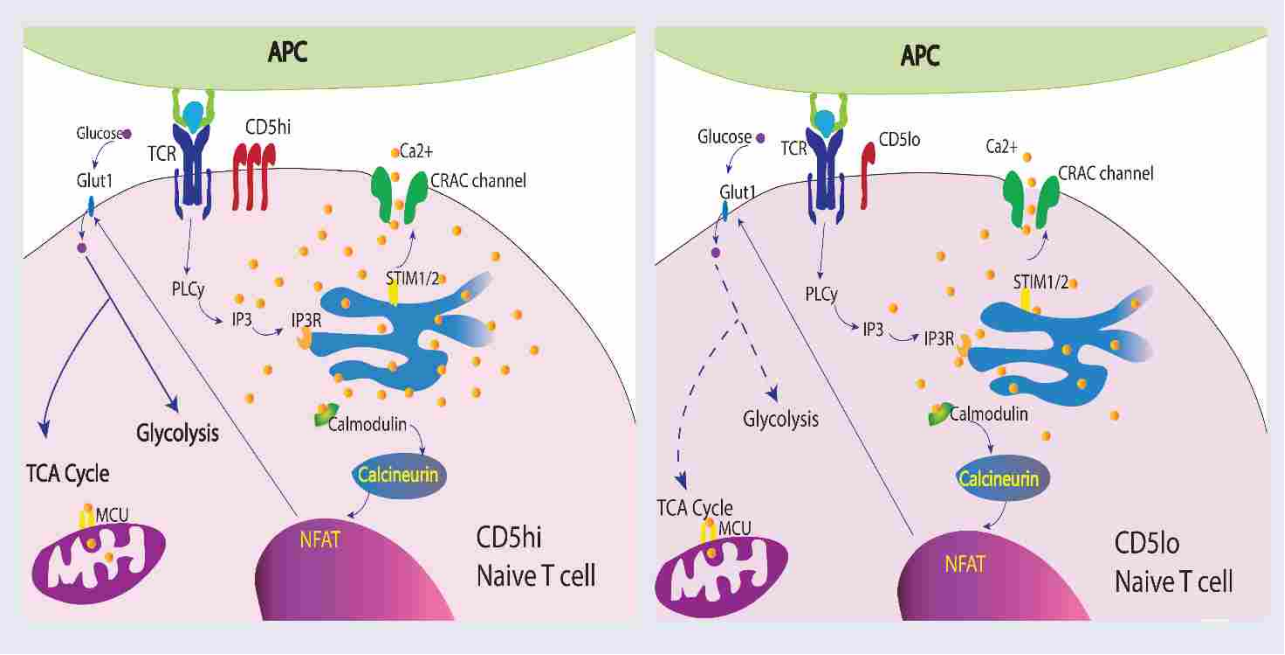

Figure 15. CD5 expression levels in naïve T cells may influence T cell metabolism and function. Differential levels of CD5 results in differences in Ca^{2+} mobilization in naïve T cells. $CD5^{hi}$ naïve T cells have higher Ca^{2+} influx than $CD5^{lo}$ naïve T cells upon TCR:pMHC interaction [61]. Ca^{2+} signaling plays a significant role in T cell activation and influences metabolism and T cell function. Differential Ca^{2+} mobilization, and expression of calcineurin and NFAT affects glycolysis and mitochondrial respiration, suggesting CD5 expression may affect metabolic reprograming during T cell activation [8].

3.4.2 Neuroimmunology

The field of neuroimmunology examines the interplay between the immune system and the central nervous system (CNS) [238]. The adaptive immune system does influence the CNS as cognition is impaired by the absence of mature T cells [239]. In wild type mice, there is an increase in the number of T cells present in the meninges during the learning process, in stark contrast to mice with T helper 2 cytokine deficiencies (such as IL-4 and IL-13) who have decreased T cell recruitment and impaired learning [240]. Furthermore, regulation of T cell activation and cytokine

production critically assists neuronal function and behavior, suggesting that manipulation of T cells could be a potential therapeutic target in treating neuroimmunological diseases [241,242].

T cells go through several microenvironments before reaching the CNS [243]. Many of the signal interactions present in these microenvironments affect T cell function and involve changes in intracellular Ca^{2+} levels [243,244]. In experimental autoimmune encephalitis (EAE), a model for human multiple sclerosis, autoreactive T cells have Ca^{2+} fluctuations throughout their journey to the CNS [243]. Prior to reaching the CNS, T cells interact with splenic stroma cells that do not display the cognate auto-antigen and this interaction produces short-lived low Ca^{2+} mobilization spikes [243]. Following entrance into the CNS T cells encounter autoantigen-presenting cells and have sustained Ca^{2+} mobilization which results in NFAT translocation and T cell activation [243,245]. EAE mice display reduced social interaction and cognition demonstrating that autoimmune response impairs neuronal function and organismal behavior [246].

Inhibitory T cell co-receptors are implicated in CNS dysregulation and disease. Varicella zoster virus (VZV) infection is characterized by lifelong persistence in neurons. VZV increases the expression of CTLA-4 and PD-1 in infected T cells which reduces IL-2 production and increases T cell anergy [247,248]. PD-1 deficient mice ($Pdcd1^{-/-}$) have increased T cell activation, leading to greater intracellular Ca^{2+} mobilization, and as previously discussed, increased glycolysis [179]. PD-1 deficiency causes elevated concentration of aromatic amino acids in the serum, specifically tryptophan and tyrosine, which decreases their availability in the brain where they are important for the synthesis of neurotransmitters such as dopamine and serotonin; consequently, there is an increase in anxiety-like behavior and fear in $Pdcd1^{-/-}$ mice [179]. Therefore, increased T cell activation caused by PD-1 deficiency can affect brain function and thus, effects cognitive behavior [179].

3.4.3 Cancer

T cells are critical components of the immune response to cancer. Helper T cells directly activate killer T cells to eradicate tumors and are essential in generating a strong antitumor response alone or in concert with killer T cells by promoting killer T cell activation, infiltration, persistence, and memory formation [249-254]. Tumor-specific T cells may not mount a robust response towards cancerous cells because the tumor microenvironment has numerous immunosuppressive factors; cancerous cells also downregulate cell surface co-stimulatory and MHC proteins which suppresses T cell activation [255-259]. Potent anti-tumor immune checkpoint blockade therapies using CTLA-4 and PD-1 monoclonal antibodies augment T cell response by suppressing the co-receptors inhibitory signals, thereby promoting increased Ca^{2+} mobilization, glycolysis and activation [260,261]. CTLA-4 monoclonal antibodies such as ipilimumab (Yervoy) and tremelimumab block B7-interaction and have been used to treat melanoma [141,262,263]. The monoclonal antibody pembrolizumab is highly selective for PD-1 and prevents PD-1 from engaging PD-L1 and PD-L2, thus enhancing T cell immune response [117,264,265]. Further research will address whether combining anti-CTLA-4 and anti-PD-1 antibodies will improve cancer treatments [117].

As previously mentioned, Ca^{2+} is critical for T cell activation and immune response. Manipulating Ca^{2+} signaling to enhance T cell-directed immune response against cancer is an intriguing notion, yet the means to target the Ca^{2+} response of specific cells without tampering with the metabolic processes of other cells remains elusive [266]. Anti-tumor activity of tumor infiltrating lymphocytes (TIL), is inversely related to CD5 expression [191]. CD5-levels in naïve T cells are constantly tuned in the periphery by interactions with self pMHC complexes to maintain homeostasis; therefore, CD5 expression on TILs can be downregulated in response to low

affinity for cancer-antigens [267-269]. Thus, the majority of TILs are $CD5^{lo}$ which increases their reactivity while $CD5^{hi}$ TILs do not elicit a Ca^{2+} response and become anergic and are unable to eliminate malignant cells [191,269]. While downregulation of CD5 on TILs enhances anti-tumor T cell activity, $CD5^{lo}$ T cells are also more likely to experience activation induced cell death (AICD) as CD5 protects T cells from overstimulation [121]. To maximize TIL effectiveness, the inhibitory effects of CD5 could be blocked by neutralizing monoclonal-antibodies or soluble CD5-Fc molecules combined with soluble FAS-Fc molecules to reduce the inherent AICD [121,270,271]. Soluble human CD5 (shCD5) may have a similar effect but avoid targeting issues by blocking CD5-mediated interaction via a "decoy receptor" effect. Mice constitutively expressing shCD5 had reduced melanoma and thyoma tumor cell growth and increased numbers of $CD4^+$ and $CD8^+$ T cells [272]. Wild type mice treated with an injection of recombinant shCD5 also had reduced tumor growth [272]. Finally, CD5-deficient mice engrafted with B16-F10 melanoma cells have slower tumor growth compared to wild type C57BL/6 mice [273]. This evidence suggests that CD5, along with PD-1 and CTLA-4, may be a potential target to specifically modulate T cell Ca^{2+} mobilization in an immunosuppressive tumor setting.

3.4.4 Microbiome

The gut microbiome, including the bacteria and their products, forms a dynamic beneficial symbiosis with the immune system influencing host genes and cellular response. The gut microbiome shapes and directs immune responses while the immune system dictates the bacterial composition of the gut microbiome [274]. As the gut is the major symbiotic system intersecting the immune system and microbiota, understanding their connection has implications for immune system development and function as the gut microbiome is involved in protecting

against pathogens, influencing states of inflammation, and even affecting cancer patient outcomes [275,276].

The gut microbiome primes immune responses [277]. Alteration in the microbial composition can induce changes in T cells function in infectious disease, autoimmunity and cancer [278]. For example, mice treated with antibiotics which restrict or reduce the microbial environment exhibit impaired immune response because their T cells have altered TCR signaling and compromised intracellular Ca^{2+} mobilization in infectious disease and cystic fibrosis models [279-281]. In contrast, administering oral antibiotics to mice with EAE increases the frequency of $CD5^{+}$ B cell subpopulations in distal lymphoid sites and confers disease protection [282]. In cancer, the microbiome also influences patient response to immune checkpoint inhibitors such as CTLA-4 and PD-1 [283,284]. Mice and melanoma patients immunized or populated with *Bacteriodes fragilis* respond better to treatment with Ipilimumab, a monoclonal antibody against CTLA-4 [275]. Similarly, tumor-specific immunity improved when anti-PD-1/PD-L1 monoclonal antibodies where used in the presence of *Bifidobacterium* [285].

Though little is known about how CD5 influences T cell interaction with the microbiome, some tantalizing details are available. As specific bacterium promotes cancer regression during CTLA-4 and PD-1 checkpoint blockades, a CD5 blockade in conjunction with bacterial selection may also improve immune response. Such studies would lead to novel immunotherapeutic treatments for cancer and autoimmune diseases.

3.5 Conclusions

CD5, widely known an inhibitory co-receptor in the thymus, appears to modulate the signaling intensity of peripheral T cells by increasing Ca^{2+} signaling activity and efficacy of $CD5^{hi}$ T cells. CD5 expression levels in the periphery correlates with intracellular Ca^{2+}

mobilization, suggesting that CD5 promotes peripheral T cell activation and immune response. As such, CD5 may be a novel checkpoint therapy to regulate T cell activation and metabolism through altering Ca^{2+} mobilization, and could be used to affect neurological behavior, alter microbiome interactions, and treat cancer and autoinflammatory diseases. While this paper focuses on the role of co-receptor CD5 effects on calcium signaling and activation of T cells, CD5 itself may be regulated through post-translational modifications, such as N-glycosylation, which may affect Ca^{2+} mobilization, T cell metabolism, activation and function.

3.6 Acknowledgments

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CHAPTER 4: Naïve Helper T Cells with High CD5 Expression have Increased Calcium Signaling

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Abstract

The adaptive immune response is orchestrated by T helper cells and their function is dependent upon interactions between the T cell receptor (TCR), peptide MHC (pMHC) and co-receptors. TCR-pMHC interactions initiate calcium signaling cascades which determine T cell activation, survival, proliferation and differentiation. CD5 is a co-receptor that plays an important role in regulating T cell signaling and fate during thymocyte education. CD5 surface expression on mature single positive thymocytes correlates with the TCR signal strength for positive selecting self-ligands. CD5 also plays a role in T cell function after thymic development is complete. Peripheral T cells with higher CD5 expression respond better to foreign antigen than those with lower CD5 expression and CD5-high T cells are enriched in memory populations. In our study, we examined the role of CD5 expression and calcium signaling in the primary response of T cells using two Listeria monocytogenes specific T helper cells (LLO118 and LLO56). These T cells recognize the same immunodominant epitope ($LLO_{190-205}$) of L. monocytogenes and have divergent primary and secondary responses and different levels of CD5 expression. We found that each T cell has unique calcium mobilization in response to in vitro stimulation with $LLO_{190-205}$ and that CD5 expression levels in these cells changed over time

following stimulation. LLO56 naïve T helper cells, which expresses higher levels of CD5, have higher calcium mobilization than naïve LLO118 T cells. Three days after in vitro stimulation, LLO118 T cells had more robust calcium mobilization than LLO56 and there were no differences in calcium mobilization 8 days after in vitro stimulation. To further evaluate the role of CD5, we measured calcium signaling in CD5 knockout LLO118 and LLO56 T cells at these three time points and found that CD5 plays a significant role in promoting the calcium signaling of naïve CD5-high LLO56 T cells.

4.1 Introduction

Helper T cells play a critical role in adaptive immunity by orchestrating and regulating the immune response [286,287]. In large part, the binding properties of the T cell receptor (TCR) regulates the development, activation, and proliferative response of T lymphocytes [288,289]. In the thymus, T cells are selected according to their avidity for self-peptide/MHC complexes. The TCR must be able to recognize self-peptide/MHC complexes with enough affinity to transduce a signal during positive selection while not binding so tightly that they are negatively selected [289-291]. TCR avidity and signal strength plays a key role in T cell function (calcium signaling, cytokine production, T cell proliferation and differentiation) [292-294]. In addition to the TCR and its interaction with peptide MHC (pMHC), multiple receptors such as CD4, CD8, PD-1, and CTLA-4 play a key role in determining whether TCR:pMHC binding results in T cell activation or anergy. CD5 is known to be a negative regulator of TCR signaling in developing thymocytes and its expression level in naïve T cells is determined during thymic development. CD5 levels are set during positive selection according to the strength of the TCR-self-peptide/MHC interaction. Typically, the stronger the avidity for self-peptide/MHC the higher the CD5 surface expression [120,191,295,296]. After completing thymic development, T cells with higher CD5 expression respond

better to foreign antigen than those with lower CD5 expression and CD5-high T cells are enriched in memory populations [193,297]. Although there are studies examining the role of T cell CD5 expression during thymic development and CD5-high cells are enriched in memory cell populations, it is not clear how CD5 is involved in calcium signaling during a helper T cell primary response. To better understand the role of CD5 in a T cell primary response to foreign antigen, we examined the *in vitro* calcium responses of CD5-high and CD5-low T helper cells that respond to the same epitope of *Listeria monocytogenes.*

Calcium (Ca^{2+}) is a ubiquitous second messenger important for a wide range of cellular functions. Ca^{2+} signaling plays an important role in T cell activation, cytokine production, proliferation and cell fate and is determined by TCR interactions with the pMHC complex as well as additional co-receptors [207,294]. Ca^{2+} signaling has been well characterized in lymphocytes and the calcium signal for specific helper T cell subsets has been identified, suggesting a strong relationship between Ca^{2+} mobilization in T helper cells and their functional response [19,298]. TCR engagement with pMHC initiates signal transduction pathways that result in a dramatic increase of intracellular Ca^{2+} [5,134]. Increases in intracellular Ca^{2+} enables transcription factors to enter the nucleus and turn on genes that play a critical role in immune responses. For example, NFAT, NF-κB, AP-1, and the Oct family transcription factors initiate transcription of the interleukin-2 (IL-2) gene [299]. IL-2 production is important for T cell proliferation and survival and plays a key role in promoting effector and memory cell differentiation [300-302]. Thus, TCR-dependent Ca^{2+} signals are essential for robust T cell primary and secondary immune responses.

The TCR avidity for self-peptide/MHC complex during selection affects the function and maintenance of these cells in the periphery and how they respond to infection [42,43]. CD5 is a monomeric cell surface glycoprotein expressed on thymocytes, mature T cells, and a subset of B

cells. High TCR avidity for self-peptide/MHC results in high surface expression of CD5 on double positive and single positive thymocytes, whereas lower avidity is correlated with lower surface expression of CD5 [295]. CD5 has been shown to negatively regulate the TCR signal during thymic development [190]. CD5 expression and Ca^{2+} mobilization correlate with TCR signal strength and T cell fate [209,288,290]. However, mature naïve T cells with higher expression levels of CD5 appear to respond better to foreign ligands, suggesting that CD5 influences T cell responsiveness at the post-selection level [41,193,207]. Thus, it appears that the negative regulatory effect of CD5 in the thymus may not depend on the extracellular region of CD5 whereas the positive co-stimulatory effect of CD5 in the periphery is dependent on extracellular engagement of an endogenous ligand (CD5 or CD5L) [195,303,304]. While the exact function of these CD5 ligands is unclear, there is evidence that CD5L (CD72; a C-type ligand) binds to CD5 and that CD5 is homophillic and may bind to CD5 on other cells [305]. Thus, CD5 has a critical and divergent role in regulating T cell activation depending on the time and location of activation.

LLO56 and LLO118 are two T helper cells that recognize the same immunodominant epitope ($LLO_{190-205}$) of *L. monocytogenes* and have divergent primary and secondary responses. They differ by 15 amino acids in their TCR sequences and have unique responses to *L. monocytogenes* infection *in vivo*, LLO118 has a better primary response whereas LLO56 has a more robust secondary response [43]. Previous analysis of thymocytes and T cells revealed that LLO56 has higher levels of CD5 and a more robust IL-2 response in addition to a reduced primary response caused by increased cell death compared to LLO118 T cells [42,43]. In order to better understand how CD5 levels affect T cell activation in cells that have completed thymic development, we determined to evaluate calcium signaling in LLO56 (CD5-high) and LLO118 (CD5-low) T cells. We also measured calcium signaling in CD5 knockout LLO118 and LLO56

T cells to better elucidate the role of CD5 in calcium signaling after thymic development is finished. This was accomplished by measuring LLO118 and LLO56 calcium mobilization at three different time points during the T cell response: Naïve T helper cells, day 3 post-stimulation, and day 8 post-stimulation. We also measured calcium signaling in CD5 knockout LLO118 and LLO56 T cells at these time points and found CD5 plays a significant role in promoting the calcium signaling of naïve CD5-high T cells, but does not alter calcium mobilization levels at later time points.

4.2 Materials and Methods

4.2.1 Mice

LLO56 (B6 Thy-1.1^{+} Rag1$^{-/-}$), LLO118 (B6 Ly5.1^{+} Rag1$^{-/-}$) and CD5 knockout (KO) LLO56 and LLO118 were bred and housed in pathogen free conditions [42,43]. All mice used in these experiments were 5-12 weeks old. All use of laboratory animals was done with approval of the Animal Care and Use Committee (IACUC protocol #15-801) at Brigham Young University.

4.2.2 T cell Isolation and Activation

CD4^{+} T cells were isolated from the spleens of LLO56 and LLO118 TCR transgenic (Tg) mice [42]. Spleen single cell suspensions from LLO56 and LLO118 mice were purified using a negative selection CD4^{+} T cell isolation kit (Miltenyi Biotec) [298]. T cells were isolated from the spleen of LLO56, LLO118, LLO56-CD5KO, and LLO118-CD5KO mice. Spleens were homogenized and passed through a nylon mesh cell strainer. The single-cell suspension was resuspended in R10 medium containing RPMI 1640, 10% of FBS (HyClone), 1% Glutamax (Gibco by Life Technologies), and 0.5% Gentamicin (Life Technologies), then transferred to a 6-well plate (1x10^{6} cell/ml) and loaded with 1 μM of *Listeria monocytogenes* peptide LLO$_{190\text{-}205}$. For T cell isolations, mice were euthanized using CO_2 inhalation.

4.2.3 Antigen Presenting Cell isolation

Bone marrow derived macrophages (BMDM) were obtained from B6/C57 mouse femurs and tibias and were cultured at 37°C and 5% CO_2 and matured for 7 days in macrophage medium with DMEM (HyClone), 10% FBS (HyClone), 20% supernatant from L929 mouse fibroblast as a source of macrophage colony-stimulating factor (M-CSF), 5% heat inactivated horse serum (Sigma), 1 mM Na Pyruvate (Gibco by Life Technologies), 1.5 mM L-glutamine (Thermofisher), 1100X Penicillin/Sreptomycin (Gibco by Life Technologies). Harvested cells were plate in an 8-chamber cover glass where they were loaded with the *Listeria monocytogenes* peptide $LLO_{190-205}$ overnight. For bone marrow derived macrophage isolations, mice were euthanized using CO_2 inhalation.

4.2.4 Calcium Imaging

Naïve T cells were incubated with 1 μM of Fura-2AM (Invitrogen) for 30 minutes at 37°C and 5% CO_2 in Ringers imaging solution (150 mM NaCl, 10mM glucose, 5 mM of HEPES, 5 mM of KCl, 1mM $MgCl_2$, and 2 mM $CaCl_2$, pH 7.4), washed, and then incubated in Ringers solution for another 30 minutes at 37°C. 200,000 Fura-2AM loaded naïve $CD4^+$ T cells were pipetted onto 200,000 bone marrow derived macrophages that were previously incubated with 1 μM of *Listeria monocytogenes* peptide $LLO_{190-205}$ overnight. Imaging was performed in Nunc 8-chamber covered glass slides (155411, Thermo Scientific). For day 3 and day 8 stimulation time points, LLO118 or LLO56 splenocytes were incubated overnight with 1 μM of *Listeria monocytogenes* peptide $LLO_{190-205}$. Calcium imaging was performed at room temperature using an Olympus IX51 inverted microscope equipped with a xenon arc lamp. Fura-2AM loaded T cells were excited at 340 nm and 380 nm excitation filters and capture by a florescence microscope camera (Q Imaging Exi Blue) using a 20x objective (N.A. 0.75). Images

(340/380/transmitted) were recorded at 3 second intervals over 20 minutes. Each individual cell fluorescence was normalized with the first recorded value according to the equation (F-Fo)/Fo where F is the fluorescence at the specific time point, and Fo is the fluorescence value at time 0 [60].

4.2.5 Flow Cytometry

Calcium mobilization was also measured using flow cytometry and the high affinity calcium indicator Fluo-4 (ex:470–490 nm and em: 520–540 nm). Cells were surface stained with an anti-$CD4^+$-APC antibody (17-0041; eBioscience). T cells were loaded for 30 mins as previously published with pluronic acid and 1mM Fluo-4-acetoxymethyl ester (Invitrogen) in Ringer solution (150 mM NaCl, 10 mM glucose, 5 mM of HEPES, 5 mM of KCl, 1 mM $MgCl_2$, and 2 mM $CaCl_2$, pH 7.4) [306]. Intracellular calcium mobilization was initiated by adding 50 ng/ml of PMA (phorbol 12-myristate 13-acetate) and 1 μg/mml of ionomycin [307]. For further analysis done in FlowJo, the lymphocyte population was gated in a forward and side scatter gate and singlets. From this gate a second gate was created specific for $CD4^+$ T cells [308]. Intracellular calcium flux was measured in the $CD4^+$ T cell gate using the FlowJo kinetics tool.

For CD5 expression analysis, spleen single cell suspensions from naïve and stimulated (day 3 and day 8) were stained with anti-CD5-PE (12-0051; eBioscience), and anti-CD4-APC (17-0041; eBioscience) and analyzed on the flow cytometer (BD Accurri C6).

4.2.6 Data Analysis

Live cell calcium imaging data was analyzed using CellSens Software from Olympus and the 340:380 ratio calculations were performed on randomly selected cells. The standard deviation of the calcium levels from the regression line was determined using GraphPad Prism. For calcium flow cytometry measurements, FlowJo kinetics tool was used to determine the area

under the curve (AUC) [306,309,310]. All assays were performed at least three times in triplicate and significant values were determined using student T test in GraphPad Prism.

4.3 Results

4.3.1 LLO118 and LLO56 T helper cells have different responses to antigen and CD5 expression levels

To examine the role of CD5 in regulating calcium signaling in the primary response of helper T cells, we used LLO56 and LLO118 T cells which are specific for the same epitope of *Listeria monocytogenes* (listeriolysin O, $LLO_{190-205}$) [43]. These LLO118 and LLO56 T cells differ in their *in vivo* responses upon *L. monocytogenes* infection; LLO118 helper T cells have a better primary response and LLO56 helper T cells exhibit a better secondary response [43]. Additionally, naïve LLO56 T cells have higher levels of CD5 and produce more IL-2 upon stimulation compared to those from LLO118 T cells [42,43]. We hypothesized that these differences in CD5 levels and T cell function would allow us to better understand the role of CD5 in calcium signaling and T cell activation in a primary response (See Table 1).

Table 1. Summary of differences between LLO56 and LLO118 T cells

	LLO56	LLO118
Primary Response	+	+++
Secondary Response	+++	+
IL-2 Response	+++	++
CD5 Expression (naïve T cells)	+++	+

4.3.2 LLO56 naïve helper T cells have higher calcium mobilization in vitro

To determine how CD5 plays a role in the primary immune response of LLO56 and LLO118 T cells, we first analyzed the calcium signaling of naïve T cells isolated and purified from the spleens of LLO56 and LLO118 TCR Tg mice. Calcium mobilization was measured using live cell imaging after loading the T cells with Fura-2AM and adding them to 8-chamber slides containing antigen presenting cells loaded overnight with the *L. monocytogenes* peptide ($LLO_{190-205}$). A calcium profile was generated by combining measurements (40^{+} cells) from 4 different experiments taking readings every 3 seconds over a 20-minute time span (Fig 16A). Upon stimulation with $LLO_{190-205}$ peptide, LLO56 T helper cells have higher peak calcium influx levels compared to LLO118 T cells (Fig 16B). There are not any significant differences in the mean calcium levels and variability (standard deviation) of the calcium signal between LLO56 and LLO118 T cells (Fig 16C and 16D) [311]. Thus, naïve LLO56 (CD5-high) and naïve LLO118 (CD5-low) T cells have significantly different peak calcium mobilization profiles.

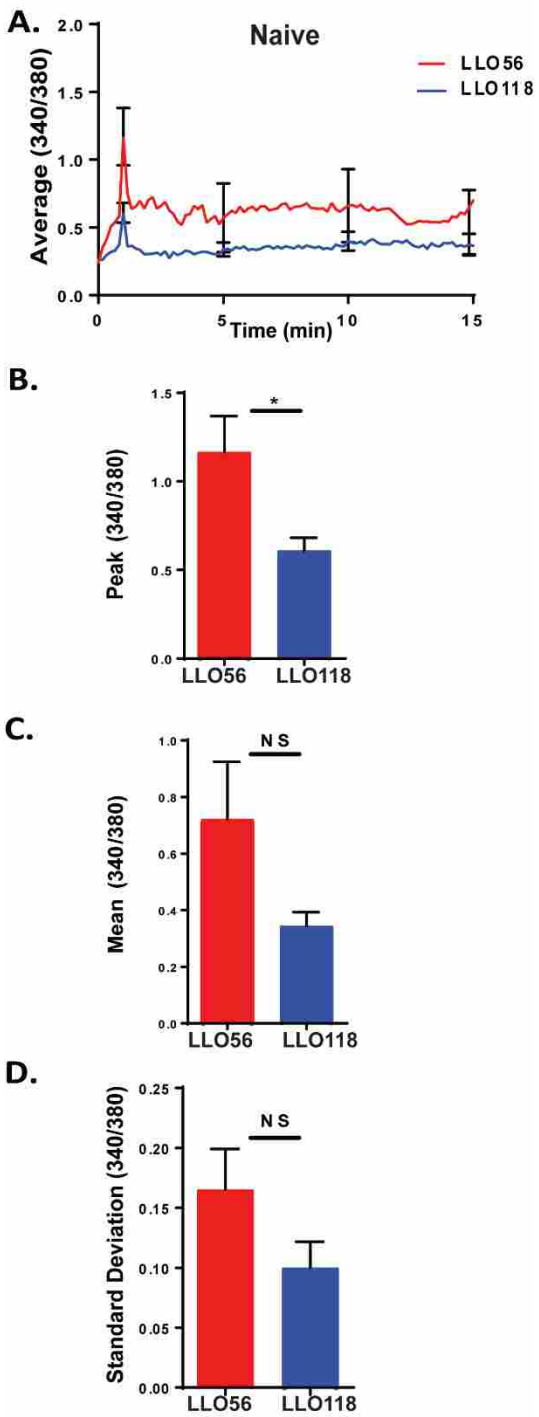

Figure 16. LLO56 naïve helper T cells have higher calcium mobilization *in vitro*.
Naïve T cells from LLO56 and LLO118 TCR transgenic mice were obtained from the spleen using negative selection and calcium levels were measured using live cell microscopy. T cells were added to antigen presenting cells (bone marrow derived macrophages) that were loaded overnight with the *L. monocytogenes* peptide ($LLO_{190-205}$). A. Average curves of intracellular Ca^{2+} mobilization from LLO56 and LLO118 naïve T cells (340/380 ratios) ($n=40^{+}$). Error bars show the SEM at the influx peak and every 5 minutes after the peak ($n=40^{+}$). B. Statistical analysis of peak calcium influx of stimulated LLO56 and LLO118 naïve T helper cells ($n=40^{+}$). C. Statistical analysis of the sustained intracellular Ca^{2+} levels

(Average 340/380 values between minutes 5 and 20) after initial stimulation response ($n=40^{+}$). D. Standard deviation was determined by linear regression analysis and shows variability in the calcium signal for each group ($n=40^{+}$). (* = $p<0.05$; NS = not significant).

4.3.3 LLO56 naïve T helper cells have higher levels of CD5 surface expression

Previous work has shown that naïve LLO56 T cells have higher expression of CD5 compared to naïve LLO118 T cells [43]. We wanted to know what happened to the levels of CD5 at the post-stimulation time points (day 3 and day 8) that we examined in this study. As previously reported, LLO56 naive T helper cells showed higher CD5 expression than naïve LLO118 T helper cells. However, upon stimulation, the CD5 expression differences between LLO56 and LLO118 T cells decrease over the course of 8 days of stimulation (Figs 17A and 17B). To further confirm CD5 expression a mean fluorescent intensity (MFI) profile was done, which confirmed significant CD5 expression levels differences between naïve LLO56 T cells with Day 3 and Day 8, however LLO56 did not have significant differences between Day 3 and Day 8 (Figs 17C and 171D).

4.3.4 LLO118 T cells have higher peak calcium influx on day 3 post-stimulation

To determine whether the Ca^{2+} influx difference seen in naïve T cells were maintained over the course of a primary response to infection, we measured calcium influx for LLO118 and LLO56 T helper cells 3 days post-stimulation with *L. monocytogenes* peptide ($LLO_{190\text{-}205}$) (Fig 18A). In contrast to naïve T cells, day 3 post-stimulated LLO118 T cells have significantly higher peak levels of calcium influx than LLO56 T helper cells (Fig 18B). While there were no differences in mean Ca^{2+} levels (Fig 18C), LLO118 did have significantly higher variability in

the calcium signal (standard deviation). Thus, on day 3 post-stimulation LLO56 T cells had significantly lower peak calcium influx and lower variability compared to LLO118 T cells.

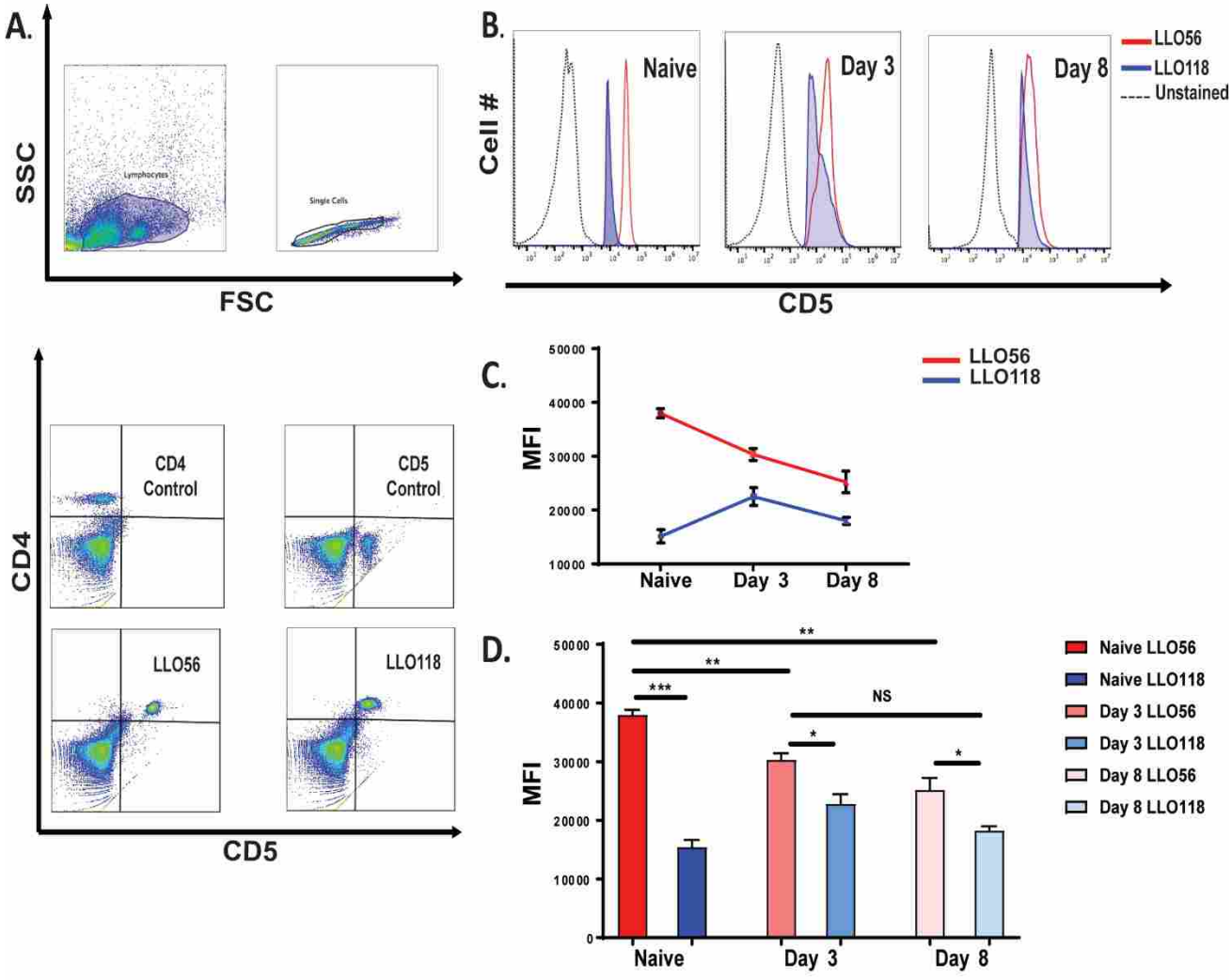

Figure 17. Naïve LLO56 T helper cells have higher levels of CD5
Flow cytometry analysis of CD5 expression of LLO56 and LLO118 T helper cells was done using LLO118 and LLO56 splenocytes at different time points after stimulation with the $LLO_{190-205}$ peptide from *L. monocytogene*s. A. Gating strategy for measuring CD4 and CD5 mean fluorescent intensity on LLO118 and LLO56 T cells. B. CD5 levels of naïve T cells, T cell stimulated for 3 days, and T cells stimulated for 8 days. T helper cells from LLO56 (red line) overlaid with the CD5 levels from LLO118 (shaded blue). Unstained cells are also included (black dots). C-D. Comparison of mean fluorescence intensity (MFI) profiles for LLO56 and LLO118 expression levels of CD5 at different time points were determined by flow cytometry quantitative analysis. (* = $p<0.05$; ** = $p<0.01$; *** =$p<0.001$; NS = not significant).

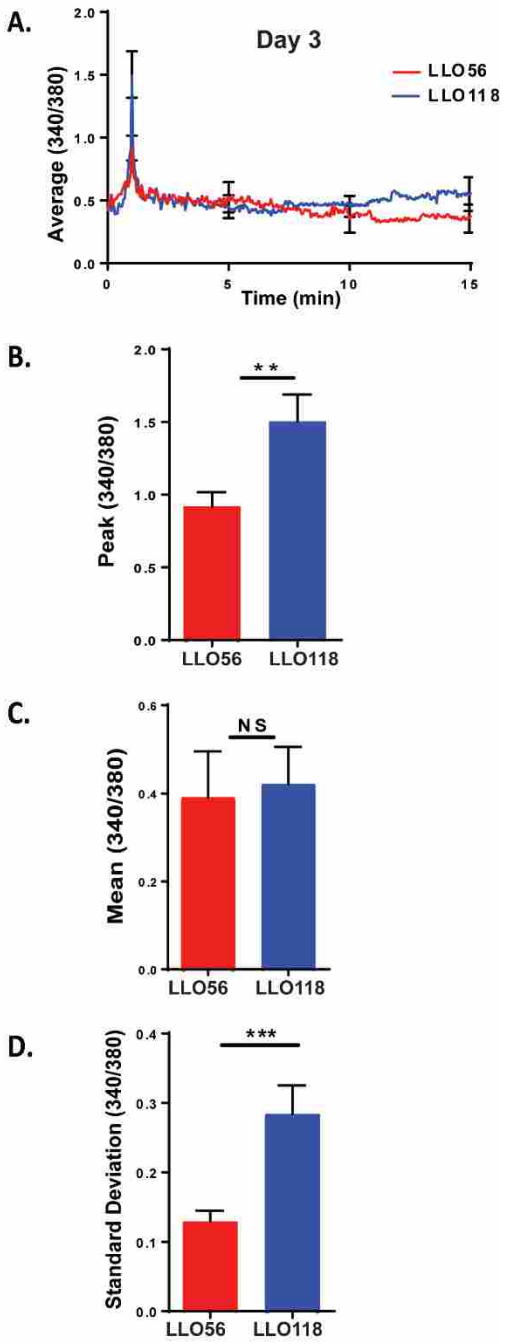

Figure 18. LLO118 helper T cells have higher calcium signaling on day 3 post stimulation. LLO56 and LLO118 splenocytes were isolated and cultured with the $LLO_{190\text{-}205}$ peptide from *L. monocytogenes* for 72 hours *in vitro*. 24 hours before live imaging, a second set of splenocytes were isolated and cultured in an 8-chamber slide loaded with $LLO_{190\text{-}205}$ peptide of *L. monocytogenes* for use as antigen presenting cells. T cells were stained with Fura-2AM, added to the antigen presenting cells and Ca^{2+} influx was measured. A. Average curves of intracellular Ca^{2+} mobilization from stimulated LLO56 and LLO118 splenocytes (340/380 ratios; n=30) on day 3 post stimulation. Error bars show the SEM at the influx peak. B. Statistical analysis of peak calcium influx of stimulated LLO56 and LLO118 naïve T helper cells ($n=30^{+}$). C. Statistical analysis of the sustained intracellular Ca^{2+} levels (Average 340/380 values between minutes 5 and 20) after initial stimulation response ($n=30^{+}$). D. Standard deviation was

determined by linear regression analysis and shows variability in the calcium signal for each group ($n=30^{+}$). (** = $p<0.01$; *** = $p<0.001$; NS = not significant).

4.3.5 LLO56 and LLO118 have similar in vitro calcium responses on day 8 post stimulation

To further characterize the LLO118 and LLO56 Ca^{2+} response, we isolated splenocytes and co-cultured them with the *L. monocytogenes* peptide for 8 days. On day 8 post-stimulation, the average Ca^{2+} profiles were similar to each other (Fig 19A). Upon evaluation, there were no significant differences between LLO118 and LLO56 T cells in calcium peak, mean, or standard deviation on day 8 post-stimulation (Fig 19B-D).

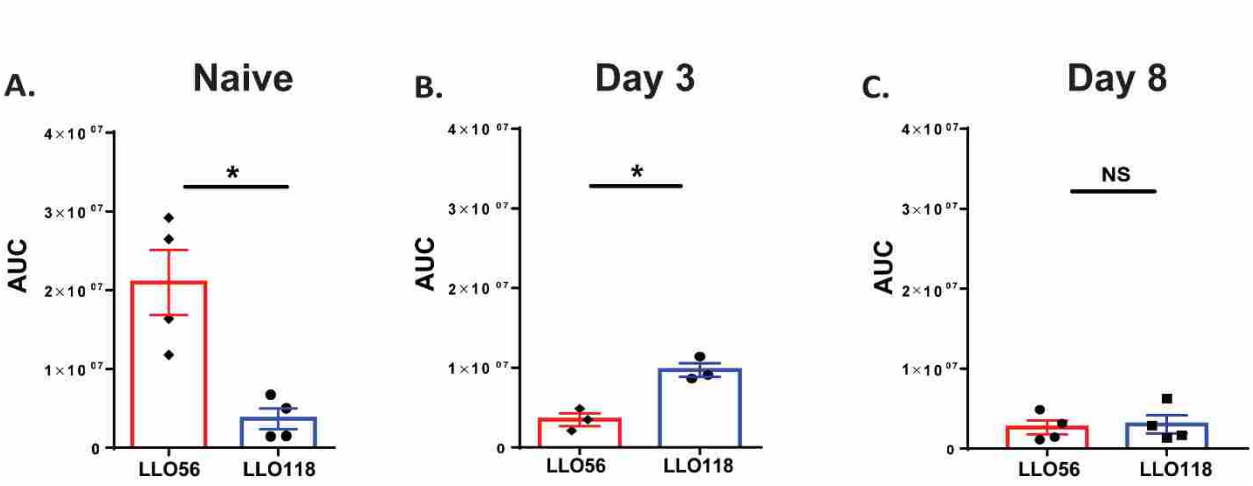

Figure 19. No calcium differences between LLO56 and LLO118 on day 8 post stimulation. LLO56 and LLO118 transgenic splenocytes were isolated and cultured with the $LLO_{190\text{-}205}$ peptide for a week. 24 hours before live imaging, a second set of splenocytes were isolated and cultured in an 8-chamber slide loaded with $LLO_{190\text{-}205}$ peptide of *L. monocytogenes* for use as antigen presenting cells. 8 days stimulated T cells were stained with Fura-2AM and Ca^{2+} influx was measured using live imaging microscopy. A. Average curves of intracellular Ca^{2+} mobilization from stimulated LLO56 and LLO118 splenocytes (340/380 ratios; n=30) on day 8 post stimulation. Error bars show the SEM at the influx peak. B. Statistical analysis of peak calcium influx of stimulated LLO56 and LLO118 naïve T helper cells ($n=30^{+}$). C. Statistical analysis of the sustained intracellular Ca^{2+} levels (Average 340/380 values between minutes 5 and 20) after initial stimulation response ($n=30^{+}$). D. Standard deviation was determined by linear regression analysis and shows variability in the calcium signal for each group ($n=30^{+}$). (NS = not significant).

4.3.6 Calcium Flow Cytometry data correlates with calcium microscopy data

To confirm the results obtained in live cell calcium microscopy and evaluate the role of TCR independent calcium signaling, we isolated LLO56 and LLO118 T cells and measured Ca^{2+} mobilization using flow cytometry. Cells were labeled with Flou-4AM and stimulated with PMA and ionomycin. Flou-4AM fluorescence was examined before and after stimulation. Measurements were collected for naïve T helper cells, day 3, and day 8 post-stimulated T cells. The data was consistent with our previous live cell imaging findings in which naïve LLO56 T helper cells and day 3 post-stimulated LLO118 T cells had higher Ca^{2+} mobilization compared to their counterparts while no calcium mobilization differences were seen at day 8 between LLO118 and LLO56 T cells (Fig 20A-C and Table 2). Collectively, these data show CD5 expression levels and calcium signaling changes over the course of a primary response in LLO118 and LLO56 T cells (Table 2, Fig. 17, and Fig. 19). Our live cell microscopy calcium imaging and flow cytometry calcium analysis differ in the parameters measured and the stimulation used (cells were stimulated in a TCR-dependent manner for live cell calcium imaging and in a TCR-independent manner for flow cytometry analysis). This calcium data is consistent with the TCR independent cytokine production differences between LLO118 and LLO56 identified by Persaud et al, in which they demonstrated that the LLO118 and LLO56 naive T cell response was set during thymic selection. Naive LLO56 T cells have higher expression of CD5, suggesting increased affinity for self-peptide, and produce higher levels of IL-2 even when stimulated in a TCR independent manner [42].

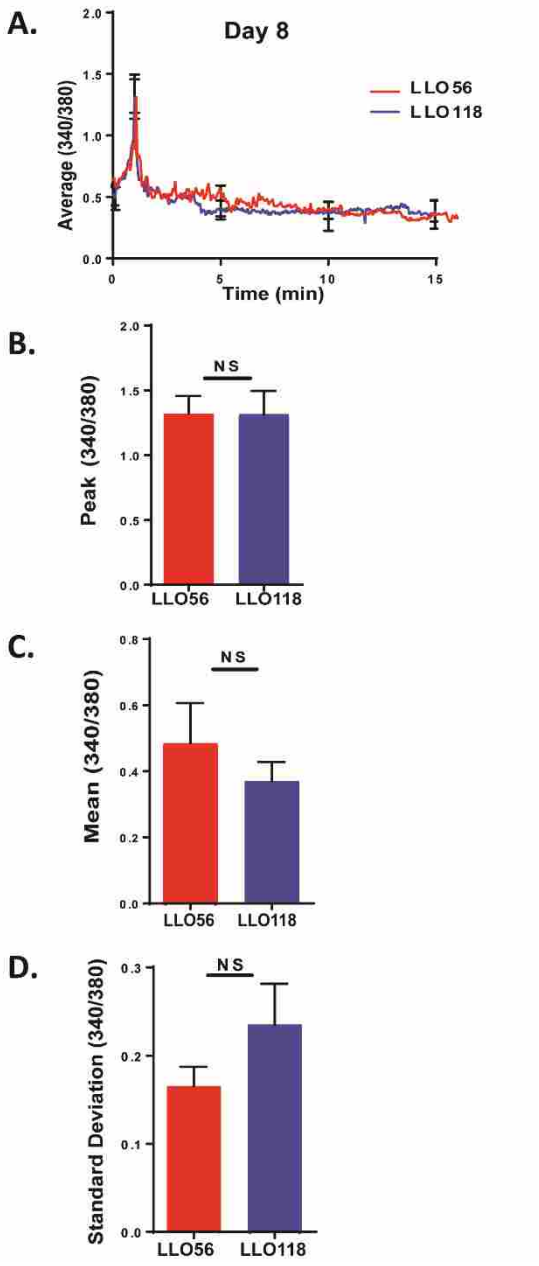

Figure 20. Flow cytometry calcium analysis confirms improved calcium mobilization for naïve LLO56 T cells and higher calcium mobilization for LLO118 T cells at day 3 post-stimulation. LLO56 and LLO118 splenocytes were isolated and cultured at different time points with the $LLO_{190\text{-}205}$ peptide. Calcium levels were quantified using the FlowJo kinetics tool to determine the area under the curve (AUC) for each sample. Calcium mobilization levels for LLO118 and LLO56 are quantified (mean ± SEM of the area under the curve). A. Statistical analysis of naïve LLO118 and LLO56 T helper cell calcium mobilization after activation with PMA and Ionomycin. B. Statistical analysis of day 3 post stimulated LLO118 and LLO56 calcium mobilization and C. Statistical analysis of day 8 post stimulated LLO118 and LLO56 calcium mobilization. (NS = not significant).

Table 2. Summary of CD5 and calcium findings for LLO56 and LLO118

	LLO56		LLO118	
	CD5 Expression	Ca^{2+} influx	CD5 Expression	Ca^{2+} mobilization
Naïve T cell	+++	+++	+	+
Day 3	++	++	+	+++
Day 8	++	++	+	++

4.3.7 CD5 expression in naïve LLO56 T helper cells is correlated with higher Ca^{2+} mobilization

To further investigate the role CD5 expression plays in Ca^{2+} mobilization, we measured the calcium signal in T cells from LLO118-CD5 knockout and LLO56-CD5 knockout mice. We found in the LLO118 T cells (CD5-low) that calcium mobilization was not significantly different from LLO118-CD5 knockout T cells at any of the three time points (Fig. 21A-C). Conversely, naïve LLO56-CD5 knockout T helper cells had significantly lower calcium levels compared to the naïve LLO56 T cells (CD5-high) (Fig. 21D). There was no calcium mobilization difference between LLO56 and LLO56-CD5 knockout T cells at day 3 or day 8 post-stimulation (Fig. 21E and 21F). Thus, in naïve LLO118 T cells (CD5-low), CD5 does not appear to play a strong role in regulating calcium mobilization at any of the time points. However, CD5 expression is important in regulating calcium mobilization in the naïve LLO56 T cells (CD5-high) during the initial response to antigen, but as CD5 levels decrease over time, its role in regulating calcium also decreases.

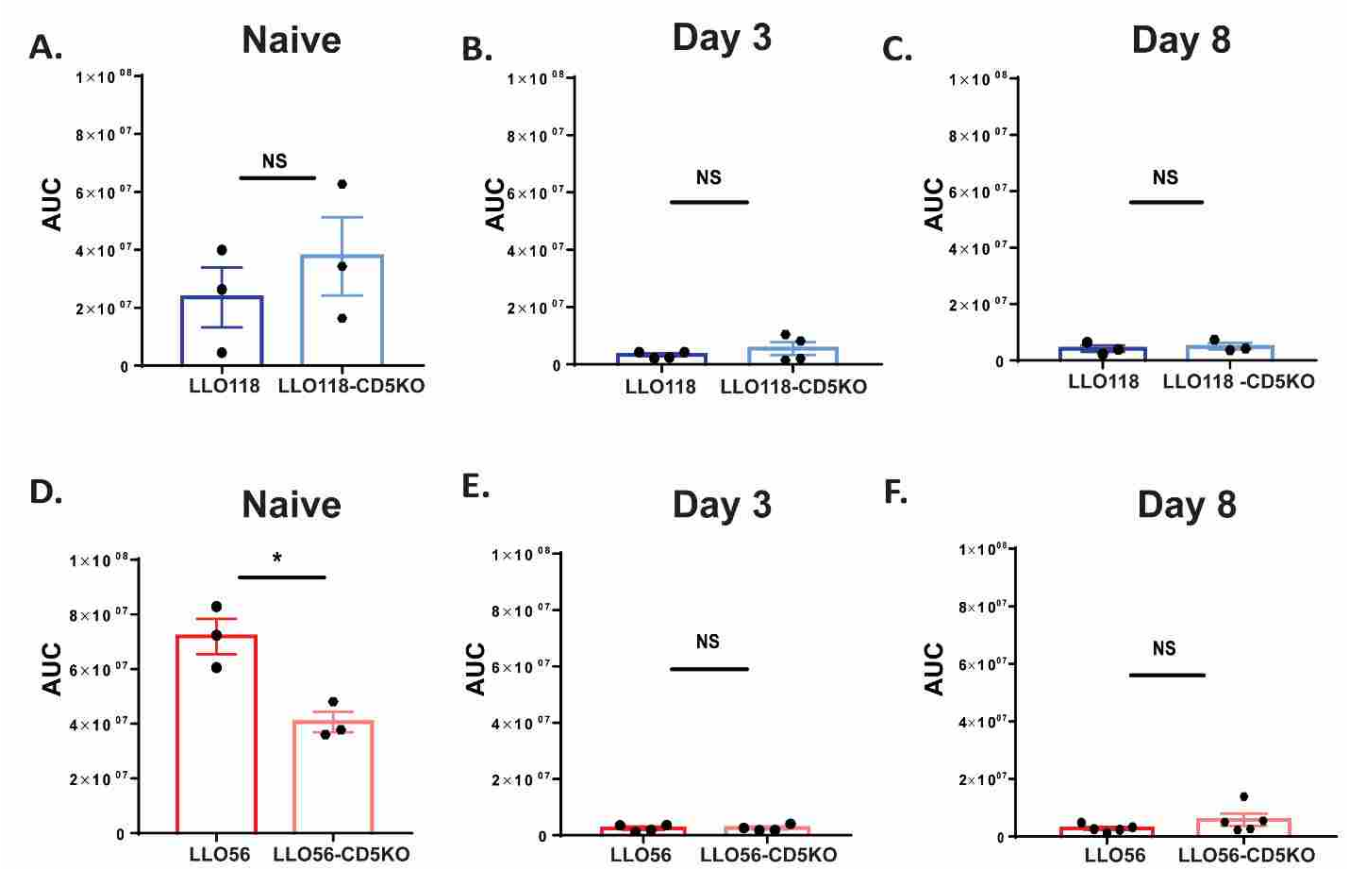

Figure 21. CD5 expression in naïve LLO56 T helper cells is correlated with higher Ca^{2+} mobilization
Flow cytometry analysis was performed to determine Ca^{2+} mobilization levels in LLO56, LLO118, LLO56-CD5 knockout and LLO118-CD5 knockout T cells stimulated with the *L. monocytogenes* peptide (naïve, day 3, and day 8 time points). Calcium levels were quantified using the FlowJo kinetics tool to determine the area under the curve (AUC) for each sample (mean ± SEM). A-C. Statistical analysis of calcium mobilization of LLO118 and LLO118-CD5 knockout T cells stimulated with PMA/Ionomycin. Data is shown for naive (A), day 3 post stimulation (B) and day 8 post stimulation (C). D-F. Statistical analysis of calcium mobilization of naïve LLO56 and LLO56-CD5 knockout T cells stimulated with PMA/Ionomycin. Data is shown for naive (D), day 3 post stimulation (E) and day 8 post stimulation (F). (* = $p<0.05$; NS = not significant).

4.4 Discussion

In this study, we examined the role of CD5 in regulating T cell activation during a primary response using two T helper cells, LLO56 and LLO118, which bind to the same *L. monocytogenes* epitope and have different levels of CD5 on the surface upon completion of thymic development [42,43]. Because of the described negative regulatory role of CD5 in the

thymus and the prevalence of CD5-high cells in memory cells, we wondered how CD5 influences T cell immune response at a post-thymic level. We found significantly different Ca^{2+} signaling levels between LLO56 and LLO118 T helper cells at the naïve and day 3 time points. The distinct Ca^{2+} mobilization patterns of LLO56 and LLO118 likely influence their particular responses to antigen, similar to observations made in B cells in which unique Ca^{2+} mobilization controls distinct B cell activation phenotypes [312,313]. Previous work has defined the important role of CD5 during T cell thymic development and that CD5-high cells are enriched in memory T cell populations, but how CD5 functions during the primary response stage of T helper cells has not been well defined. Here we characterized the role of CD5 expression and calcium mobilization in these CD5-high and CD5-low T cells over the course of 8 days. We found that naïve LLO56 T helper cells (CD5-high) have significantly higher calcium mobilization compared to the LLO56-CD5 knockout T cells, but at later time points the removal of CD5 did not significantly alter LLO56 calcium mobilization. Naïve LLO118 T helper cells (CD5-low) exhibit no differences in Ca^{2+} mobilization relative to their CD5 knockout counterpart. Thus, we found naive CD5-high T cells have improved calcium mobilization to an antigen they have never seen before.

T cell development shapes the T cell population by removing strongly self-reactive cells and helping determine future immune responses. T cells that are moderately self-reactive may be able to pass positive selection and evade negative selection and circulate in the periphery. These self-reactive cells, marked by high levels of CD5, appear to be primed to be the best responders to foreign antigens[42]. CD5 is a known negative regulator of TCR signaling during thymocyte development and its expression is correlated to the relationship of TCR avidity for self-pMHC [42,43,121,209]. Analysis in thymocytes showed that LLO56 and LLO118 CD5 knockout T cells had increased p-ERK and IL-2 production, providing additional evidence that CD5 has a

negative regulatory effect in developing thymocytes [42]. However, additional work has demonstrated that CD5-high and CD5-low T cells respond differently to self and foreign antigens, suggesting that CD5 has an important role in thymocyte selection and peripheral T cell function and fate [41,209].

Recent studies suggest that developing T cell CD5 levels affect naïve T cell responses to foreign antigens in the periphery [297,314]. While the negative regulatory function of CD5 in the thymus does not appear to be dependent upon engagement with a ligand, the positive co-stimulatory effect of CD5 in the periphery is likely due to CD5 engagement of a ligand (CD5 or CD5L) [195,303-305]. As previously reported, anti-CD5 antibodies enhance TCR-mediated activation and proliferation in peripheral T cells [41,201,315]. This calcium difference observed in naïve T cells is supportive of the previously published finding that LLO56 T cells have significantly higher phosphorylation levels of pERK and production of IL-2 before exposure to antigen, suggesting a role for self-peptide affinity in altering CD5 levels and naive T cell responses. CD5-high T cells respond strongly upon stimulation *in vitro* and have increased IL-2 secretion and greater Erk phosphorylation compared to CD5-low T cells [42]. Since CD5 expression is set by self-peptide reactivity in the thymus, our finding that naïve CD5-high LLO56 T cells have higher calcium influx is consistent with other studies that have shown that increased reactivity to self-peptides results in T cells with improved reactivity to foreign antigens [193,209].

We found that CD5 expression plays an important role in intracellular Ca^{2+} mobilization for naïve LLO56 helper T cells (CD5-high). CD5-high T cells have stronger avidity for self-peptide. It has been suggested that the enhanced activation response to foreign pathogens of CD5-high T cells could be due to their ability to more efficiently use self-peptide as a co-agonist peptide in the periphery [297]. Additionally, CD5-high cells have better basal TCR signaling and

improved functional characteristics which correlate with better response to foreign peptide [209]. Studies in naïve cytotoxic T cells suggest that the gene expression profile of CD5-high T cells transcriptionally engage into proliferative and effector functions faster than CD5-low T cells [35]. Furthermore, CD5 appears to help with CD5-high naïve T cell survival after antigen recognition [316]. In fact, T cells with high CD5 levels may maintain diversity within the memory population, which may outweigh the cost of increased self-reactivity [297]. Additionally, T cells with high CD5 expression are enriched in memory cell populations, suggesting that when designing vaccines, CD5 levels and self-peptide and foreign peptide interactions are an important consideration [193,297].

The data presented here helps to elucidate the role that CD5 plays in regulating calcium signaling in naïve cells early after cell activation during an *in vitro* primary response. We plan to further investigate whether the unique Ca^{2+} profiles of LLO56 and LLO118 T cells are consistent in an *in vivo* model and further quantify the role CD5 plays in effector and memory T cells. These future studies will help elucidate how CD5 influences naïve T cell responses and its potential role in memory T cell generation and maintenance.

4.5 Acknowledgements

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CHAPTER 5: The Role of CD5 Co-receptor in T cells Metabolism

The following chapter is a work in progress and will be submitted for scientific review upon completion. Current data and future directions for this project are presented here.

Abstract

During activation, T cells undergo metabolic reprogramming, which helps determine their distinct functional fates. CD5 is a co-receptor found on T cells and plays a significant role in regulating T cell thymic development, signaling, and cytokine production. Although CD5 is known best for its function as a regulatory coreceptor during selection in T cell development, it has been reported to play a regulatory role similar to PD-1 and CTLA-4 in mature T cells upon activation. We have previously discovered that CD5 levels influence Ca^{2+} mobilization and T cell activation. Differential calcium mobilization, calcineurin function, and nuclear factor of activated T cell (NFAT) activity are known to affect glycolysis and mitochondrial respiration. Previous studies have shown that CD5 knockout mice (CD5KO) have increased T cell activation, leading to elevated levels of cytokine production and T cell proliferation. These functional changes suggest that CD5 may be affecting T cell metabolic reprograming. We hypothesized that CD5 deficient T cells have different bioenergetic demands that alter metabolic pathways key to T cell activation and function. We evaluated the effects of the CD5 co-receptor on metabolism by measuring the metabolic profiles of CD5KO and wild type T cells. Our preliminary data suggests that CD5KO T cells have higher mitochondrial respiration than wild type T cells and we are currently examining other mechanisms that can affect T cell mitochondrial respiration. Thus, CD5 may play an important role in metabolic reprograming in T cells and could potentially be useful in modulating the T cell response in cancer immunotherapy treatments.

5.1 Introduction

Helper T cells are key regulators of the adaptive immune system and play an important role in protecting against foreign peptides and cancer. During an immune response, activated T cells undergo metabolic changes transitioning from mitochondrial respiration [oxidative phosphorylation (OXPHOS) and fatty acid oxidation (FAO)] to glycolysis. This significant metabolic shift affects T cell activation, proliferation and function[216,235,317]. Naïve T cells remain relatively quiescent and rely predominantly on OXPHOS to meet basal metabolic needs[219,318]. As T cells become activated, they have different energy demands and switch to aerobic glycolysis which supports effector T cells functions such as differentiation and cytokine synthesis[219,319]. T cell activation involves the interaction between the T cell receptor (TCR) and the peptide major histocompatibility complex (TCR-pMHC), the signal strength initiated by this interaction is modulated by co-stimulatory or co-inhibitory receptors. Thus, these co-receptors contribute to a metabolic switch according to the energy requirements of the cell[140,219,235,320]. CD5, is a T cell co-receptor that negatively regulates T cell activation during T cell development in the thymus. It belongs to the group B scavenger-receptor cysteine-rich (SRCR) superfamily, and is associated with the TCR/CD3 complex[121,203]. CD5 expression levels correlate with the strength of the signal between the TCR-self-pMHC, and help fine tune the TCR repertoire by altering the strength of the antigen receptor signal during the selection process in the thymus[196,321]. This regulation appears to be independent of SHP-1 and CD5 dependent signal transduction appears to be dependent on specific tyrosine and serine motifs that allow for regulation of T cell activation[132,304]. In mature cells, it appears CD5 may function in a scaffolding role for the ubiquitin ligases CBL and CBLB[322].

The main characterized co-receptors that negatively regulate T cell responses are PD-1 and CTLA-4; they mediate a defective transmission of the TCR-pMHC signal which correlates with decreased Ca^{2+} mobilization and proliferation[145,175]. Upon activation T cells undergo metabolic changes from OXPHOS to glycolysis[323]. Signals such as those from cytokines and the TCR signal strength determines the magnitude of the glycolytic switch[324]. CD5, like the PD-1 and CTLA-4 co-receptors, negatively regulates the T cell response by affecting the TCR signal strength and glucose metabolism[140,235]. CD5 also influences the immune response due to its ability to regulate the TCR signaling response and T cell functions[207]. Previous publications showed that T cells with different CD5 expression levels have different primary and secondary immune responses and different calcium (Ca^{2+}) mobilization responses[43,61]. It has also been reported that CD5 deficient T cells are hyperproliferative, have enhanced AICD, and produce higher levels of cytokines upon stimulation[321]. These reported inhibitory functions of CD5 suggest that like PD-1 and CTLA-4, the CD5 co-receptor might influence the T cell metabolic response[321].

To determine if the CD5 co-receptor does in fact alter T cell metabolism, we compared the metabolic profiles of T cells from CD5WT and CD5KO mice. Our preliminary data suggests that the mitochondrial respiration and spare respiratory capacity (SRC) of CD5KO naive T cells is significantly higher than CD5WT. We also observed that naïve CD5KO T cells have higher metabolite consumption. While this project remains a work in process, these initial findings suggest an important role of CD5 in T cell metabolism. If confirmed, then CD5 might potentially be useful in immunotherapy to modulate T cell metabolic responses in the tumor microenvironment.

5.2 Materials and Methods

5.2.1 T cell Isolation

$CD4^+$ naïve T cells were isolated from the spleens of CD5WT and CD5KO mice[42]. Splenic single cell suspensions from CD5WT and CD5KO mice were purified using a negative selection $CD4^+$ T cell isolation kit (Miltenyi Biotec)[298]. Spleens were homogenized and passed through a nylon mesh cell strainer. The single-cell suspension was resuspended in R10 medium containing RPMI 1640, 10% of FBS (HyClone), 1% Glutamax (Gibco by Life Technologies), and 0.5% Gentamicin (Life Technologies), then transferred to a 6-well plate ($1x10^6$ cell/ml). For T cell isolations, mice were euthanized using CO_2 inhalation.

5.2.2 T cell Activation and Culture

Isolated $CD4^+$ T cells were initially plated into 24-well plates and stimulated with 4.5 μm diameter dynabeads coated with anti-CD3 and anti-CD28 (11456, Thermo Fisher Scientific), in a bead to cell ratio of 1:1 as per manufacture instructions. The cells were cultured for 24 hours, 96 hours and 120 hours at 37 °C, 5% CO_2. For cultures longer than 24 hours, 30 U/mL of IL-2 was added after 72 hours (402-ML-100, R&D).

5.2.3 Metabolism Assays

The extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were measured using an Extracellular Flux Analyzer XFp (Agilent Technologies, Santa Clara, CA, USA). The culture medium was changed to Seahorse XF RPMI Base Medium (Cat#103336-100) before the analysis. Briefly, 150,000 T cells were seeded onto a Poly-d coated seahorse 8-well plate allowing the adhesion of T-cells and were pre-incubated at 37 °C for 60 min in the absence of CO_2. Mitochondrial stress and glycolytic parameters were measured *via* oxygen consumption rate (OCR) (pmoles/min) and extracellular acidification rate (ECAR) (mpH/min), respectively,

with use of real-time injections: oligomycin, fluorocarbonyl cyanide phenylhydrazone (FCCP), rotedone/antimycin, glucose, and 2-deoxy-glucose (2-DG).

For mitochondrial stress, cells were resuspended in XF assay media supplemented with 25 mM glucose, 2 mM L-glutamine and 1 mM sodium pyruvate under basal conditions, and in response to 1 μM oligomycin, 1.5 μM fluorocarbonyl cyanide phenylhydrazone (FCCP), and 0.5 μM rotenone/antimycin A (XFp mito stress test kit; cat#103010-100; Agilent Technologies).

For glycolysis, cells were resuspended in XF assay media. The ECAR were measured at the baseline and in response to 10 mM glucose, 1 μM oligomycin and 50 mM 2-DG (XFp glycolysis stress test kit; cat#103017-100; Agilent Technologies). All chemicals were purchased from Seahorse Bioscience (North Billerica, MA). Calculations for individual parameters represents the average of individual well calculations for each assay group. Error bars are calculated based on the individual well calculation for each parameter (Report Generator User guide, Agilent Seahorse).

5.2.4 Metabolite Analysis

Naïve T cells were isolated under the indicated culture conditions at 1×10^6cells / ml per well in six-well culture plates. After 1 hour of culture, 1×10^6 cells/ml cells were harvested separately, flash-frozen in liquid nitrogen and stored at −80 °C. A total of four replicate samples for CD5KO and CD5WT were generated and analyzed via Gas chromatography–mass spectrometry (GC/MS). Metabolomics analysis was performed at the Metabolomics Core Facility at the University of Utah which is supported by 1 S10 OD016232-01, 1 S10 OD021505-01 and 1 U54 DK110858-01 grants. Samples preparation and analysis was as described below:

5.2.5 Metabolite Extraction

To each sample was added cold 90% methanol (MeOH) solution containing the internal standard d4-succinic acid (Sigma 293075) to give a final concentration of 80% MeOH to each cell pellet. Samples were then briefly vortexed, sonicated for 5 min and incubated at -20 ˚C for 1 hour. After incubation the samples were centrifuged at 20,000 x g for 10 minutes at 4˚C. The supernatant was then transferred from each sample tube into a labeled, fresh micro centrifuge tube. Pooled quality control samples were made by removing a fraction of collected supernatant from each sample and process blanks were made using only extraction solvent and no cell culture. The samples were then dried *en vacuo*.

5.2.6 GC-MS Analysis

All GC-MS analyses were performed at the Metabolomics Core Facility at the University of Utah was done with an Agilent 7200 GC-QTOF and an Agilent 7693A automatic liquid sampler. Dried samples were suspended in 40 µL of a 40 mg/mL O-methoxylamine hydrochloride (MOX) (MP Bio #155405) in dry pyridine (EMD Millipore #PX2012-7) and incubated for one hour at 37°C in a sand bath. 13 µL of this solution was added to auto sampler vials. 60 µL of N-methyl-N-trimethylsilyltrifluoracetamide (MSTFA with 1%TMCS, Thermo #TS48913) was added automatically via the auto sampler and incubated for 30 minutes at 37 °C. After incubation, samples were vortexed and 1 µL of the prepared sample was injected into the gas chromatograph inlet in the split mode with the inlet temperature held at 250°C. A 5:1 split ratio was used for analysis for the majority of metabolites. For those metabolites that saturated the instrument's detector at the 5:1 split concentration, a split of 50:1 was used for analysis. The gas chromatograph had an initial temperature of 60°C for one minute followed by a 10°C/min ramp to 325°C and a hold time of 10 minutes. A 30-meter Agilent Zorbax DB-5MS with 10

meters Duraguard capillary column was employed for chromatographic separation. Helium was used as the carrier gas at a rate of 1 mL/min.

5.3 Preliminary Results

5.3.1 CD5KO naïve T cells have higher amounts of metabolites involved in glycolysis and mitochondrial respiration compared to CD5WT naïve T cells

Naïve T cells are quiescent cells and their energy demands are supplied by the OXPHOS of intracellular metabolites generated from the tricarboxylic acid (TCA) cycle and β-oxidation using fatty acids to help in their homeostatic survival[217,325]. The metabolic state of the quiescent cells is also regulated by genes and/or proteins with specific roles such as the co-receptors in T cells[216]. CD5 is an important co-receptor during T cell development and negative regulates TCR-self peptide MHC interaction. Differences in CD5 expression levels [CD5 high ($CD5^{hi}$) and CD5 low ($CD5^{lo}$)] in naïve T cells are known to affect gene expression related with cytokine interaction[326]. To examine the role of CD5 in naïve T cells metabolism we isolated $CD4^+$ T cells from CD5WT and CD5KO mice and compared metabolites within these cells. We observed in our preliminary data that the intracellular metabolites levels from the glycolytic pathway: glucose-6-P, fructose-6-P and D-glucose (Fig. 22A), the OXPHOS pathway in specifically the tricarboxylic acid cycle (TCA): citric acid, succinic acid and fumaric acid (Fig. 22B) and amino acids from the amino acid metabolic pathway: tyrosine, tryptophan, alanine, glutamic acid and glycine (Fig. 22C), from CD5KO T cells were higher compared to those of the CD5WT T cells, suggesting that CD5 influences the metabolic response in naïve T cells.

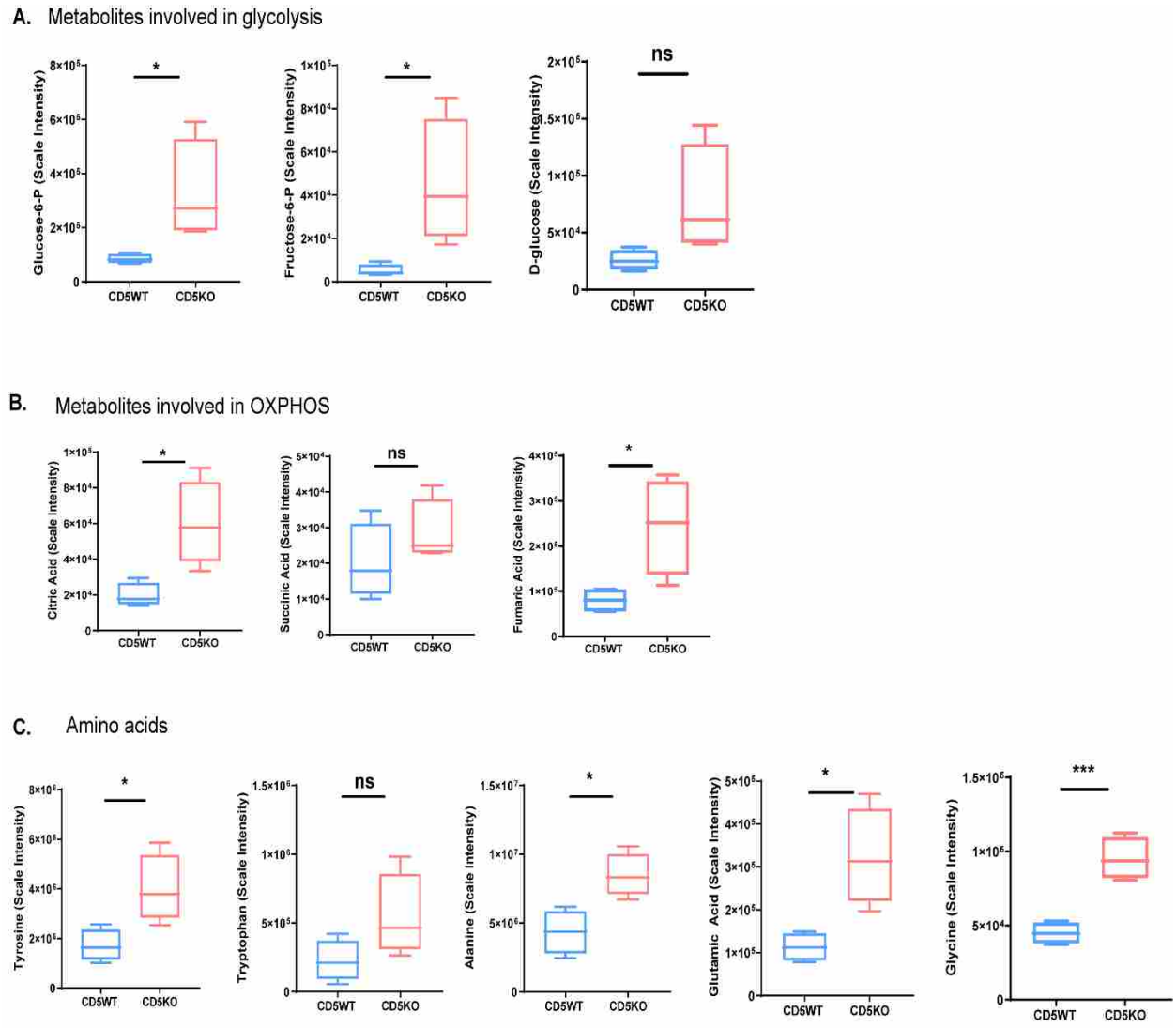

Figure 22. CD5KO T cells have higher intracellular levels of metabolites.
Analysis of metabolites of naïve $CD4^+$ T cells isolated from the spleen of CD5WT and CD5KO mice. A. Metabolites involved in glycolysis: glucose-6-P, fructose-6-P and D-glucose. B. Metabolites involved in OXPHOS/mitochondrial respiration: citric acid, succinic acid and fumaric acid. C. Metabolites involve in amino acid metabolism: tyrosine, tryptophan, alanine, glutamic acid and glycine. The amounts of the indicated metabolites in naïve T cells were plotted in whisker boxes. n=4 (* = $p<0.05$; *** = $p<0.001$; ns = not significant).

5.3.2 CD5KO naïve T cells have increased glycolytic profile

To examine whether CD5 inhibits glycolysis in T cells, we isolated naïve $CD4^+$ T cells isolated from CD5WT and CD5KO mice and measured their glycolytic profile. We observed that naïve CD5KO T cells have a higher ECAR rate in contrast of the CD5WT naïve T cells which is

associated with increased glycolysis, reflected in the extracellular acidification response in the cells upon glucose addition (Fig. 23A-B). We wondered if upon stimulation, the same glycolytic phenotype would remain. $CD4^+$ T cells were stimulated using dynabeads coated with anti-CD3 and anti-CD28 at different time points (24 hours, 96 hours and 120 hours). We observed that in contrast to naïve T cells, upon stimulation the CD5WT T cells ECAR levels (glycolytic profile) was either the same after 24 hours, or higher as seen at the 96 hour and 120 hour time points (Fig. 23B-H), suggesting that CD5 influences the glycolytic function of naïve and effector T cells in a divergent manner over time.

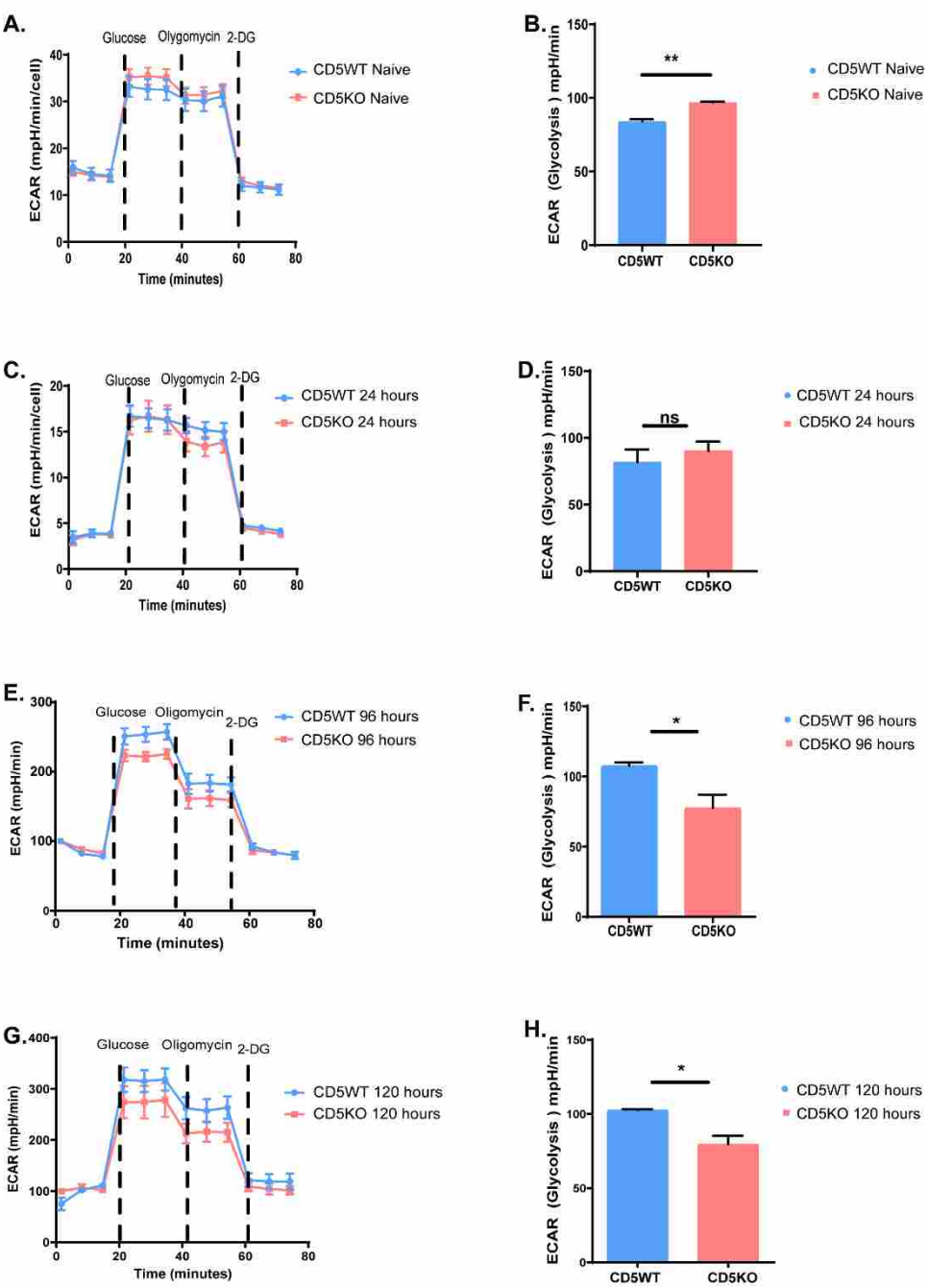

Figure 23. CD5KO naive T cells have increased levels of ECAR (higher glycolytic profile) and CD5KO effector cells have equivalent or decreased ECAR levels (decreased glycolytic profile). Seahorse extracellular acidification rate (ECAR) was measured in $CD4^+$ isolated T cells from naïve and activated T cells using the glycolytic stress test from Agilent. Activated T cells were stimulated with dynabeads coated with anti-CD3/CD28 at different time points (24 hours, 96 hours and 120 hours). A, C, E and G Show the ECAR at different time points. B, D, F and H show the differences in glycolysis response between CD5WT and CD5KO upon glucose stimulation. (* = $p<0.05$; ** = $p<0.01$, ns = not significant).

5.3.3 CD5KO naïve and effector T cells have increased mitochondrial respiration and SRC

To investigate how mitochondrial respiration of $CD4^+$ naive T cells and activated T cells is regulated by the CD5 co-receptor via TCR/CD3 and CD28 stimulation we measured the oxygen consumption rate (OCR), an indicator of OXPHOS, and spare respiratory capacity (SRC) which is the difference between the maximum respiratory capacity and basal respiratory capacity[327]. In other words, when energy demand exceeds supply, like in increased work or stress, the reserve mitochondrial capacity or SRC has the potential to provide that energy supply[327,328]. While unstimulated naïve T cells are metabolic quiescent [they rely on mitochondrial respiration (OXPHOS) for their bioenergetic demands], we observed that CD5KO naïve T cells OCR and SRC was increased in comparison to CD5WT naïve T cells (Fig. 24 A-B). This suggests that naïve CD5KO $CD4^+$ T cells have a greater reserve of energy before activation. To examine if the OCR and SRC changed after activation, we stimulated T cells using dynabeads coated with anti-CD3 and anti-CD28 at different time points (24 hours, 96 hours and 120 hours), and we observed that CD5KO T cells had equivalent or higher OCR and SRC than CD5WT after 24 hours, and significantly different at 96 hours (Fig. 24 E and F). These data suggested that CD5 plays an important role in the bioenergetic properties of T cells.

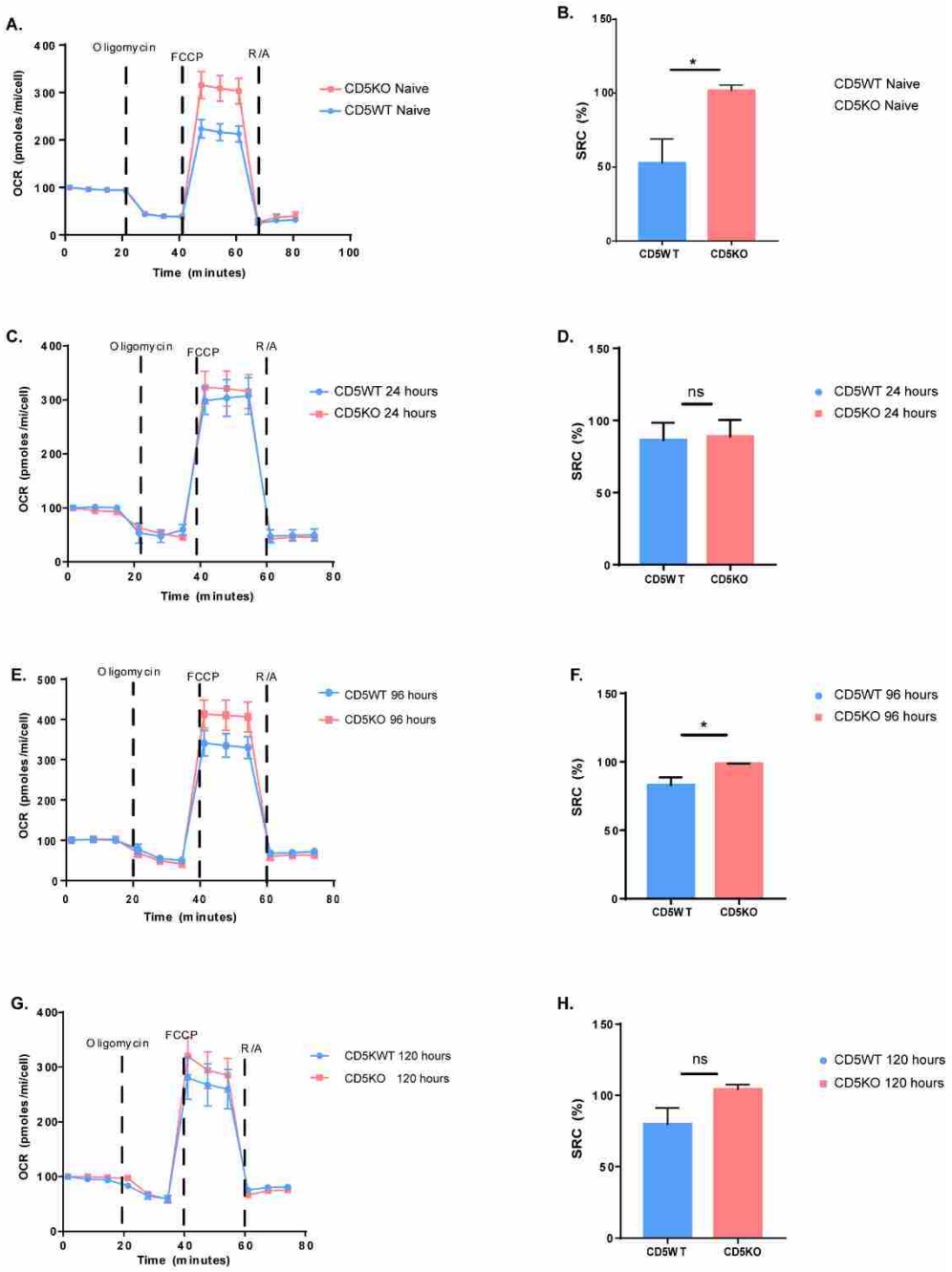

Figure 24. CD5KO naive T cells have higher mitochondrial respiration profile
Seahorse oxygen respiratory capacity (OCR) and spare respiratory capacity (SRC) was measured in $CD4^+$ isolated T cells from naïve and activated T cells. Activated T cells were stimulated with dynabeads coated with anti-CD3/CD28 at different time points (24 hours, 96 hours and 120 hours). A, C, E and G Show the OCR at different time points. B, D, F and H show the differences in SRC. (* = $p<0.05$; ns = not significant).

5.4 Conclusions and Future Direction

Upon activation, T cell metabolism shifts from mitochondrial respiration to a rapid increase in aerobic glycolysis[216]. In fact, naïve T cells rely on glycolysis for their activation, even in the presence of oxygen, to help with the proliferation, effector, and differentiation functions[219,329]. T cells from PD-1 deficient mice, a co-receptor with similar regulatory functions as CD5, have an increase in lymphocyte activation[330]. This increase in lymphocyte sustained activation induces metabolic changes, and affects T cell function[179]. During an immune response, T cells change their metabolic programing upon activation when energy demands increase[331].

Ca^{2+} signaling pathways are involved in T cell metabolic homeostasis[101]. CD5KO thymocytes and T cells are hyperresponsive to TCR stimulation, have increased proliferation, and their signaling pathways lead to an increase in intracellular Ca^{2+}[190,191]. We previously reported that CD5 expression levels in T cells alter Ca^{2+} mobilization responses[61], and since Ca^{2+} levels are known to influence the T cell metabolic switch[332], we wondered about the metabolic role of CD5 in T cells (Fig 25). Here we described work examining the impact of the CD5 co-receptor in T cell metabolism by comparing the bioenergy demands of naïve and activated $CD4^{+}$ T cells from CD5KO and CD5WT mice. CD5 is a T cell co-receptor that plays a critical role during T cell early development by negative regulating the signal strength interaction between the TCR and self-pMHC complex, and participating in the positive selection process. Evidence of CD5 importance in T cells signaling is evident in CD5KO thymocytes which are hyperresponsive to TCR stimulation, and in CD5KO T cells that produce higher cytokines levels[321 333]. Therefore, we hypothesized that CD5 regulatory functions alter T cells metabolism.

CD5 regulatory role in metabolic function is supported by our preliminary data, which suggests that CD5KO and CD5WT T cells have significantly different metabolic demands. We observed that while CD5KO naïve T cell have a higher ECAR, OCR and SRC in comparison to CD5WT T naïve cells, the ECAR from activated CD5KO T cells was equivalent or lower than CD5WT activated T cells. T cell co-receptors are important during the switch of energy as costimulatory and inhibiting signals are the ones regulating proliferation, differentiation and cytokine production[334,335]. Without costimulatory signals, T cells metabolic mechanisms will be affected, and T cells will not be fully activated or will be anergic.

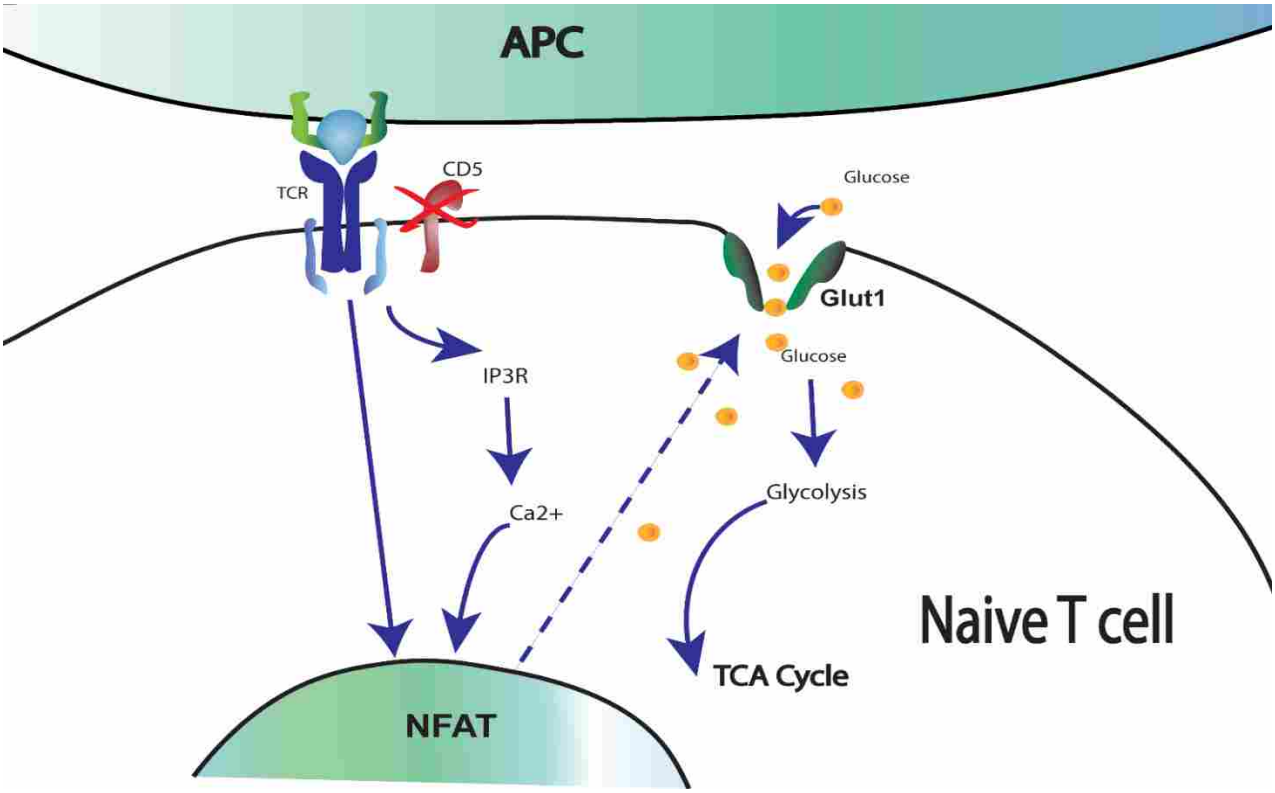

Figure 25. Summary of how CD5 influences metabolic function.
CD5KO naive T cells have higher mitochondrial respiration and glycolytic profiles. Representation of CD5KO naïve T cells upon activation and the effect on T cells metabolism.

On the other hand, when co-inhibitory signals are not present, there is no control of the activation upon stimulation of the T cell leading to hyperactivation of metabolic pathways such as glycolysis, mitochondrial respiration (OXPHOS) and lipid biosynthesis[336,337].

Co-inhibitory signals such as CD5 affect naïve T cells activation responses by negatively modulating TCR signaling[338]. Inadequate inhibitory signals result in altered T cell activation and immune tolerance[339]. Altered T cells in autoimmune diseases show low levels of metabolites involved in glycolysis, mitochondrial respiration, and amino acid metabolism in serum, implying a higher use of these metabolites by the T cells[235,340]. We observed in our preliminary data that naïve CD5KO T cells had higher concentrations of intracellular metabolites involved in glycolysis, mitochondrial respiration (OXPHOS) and in amino acids synthesis, compared to CD5WT naïve T cells. Previous studies have shown that CD5KO thymocytes are hyperesponsive[190]. We propose that such effects in activation are also maintained in the periphery in CD5KO naïve T cells, as suggested by the concentration of intracellular metabolites in these cells[133]. This observation suggests a relevant role of CD5 in T cell metabolic immune homeostasis not only during T cell development, but also peripheral naïve T cell maintenance.

In addition, we observed that CD5KO naïve T cells have a higher mitochondrial respiration response (OCR) and a higher SRC than CD5WT naïve T cells. Previous work done in T cells from mice deficient in mitochondrial proteins (mitofusin 1 and optic atrophy1) demonstrate abnormalities in their mitochondrial mass, elevate mitochondrial membrane potential (generate by proton pumps in the ETC), and ATP depletion[341]. Interestingly, they also show a sustained mitochondrial respiration (OXPHOS) similar to that observed in memory T cells[342]. Furthermore, elevated SRC (a measurement of the reserve mitochondrial capacity available to the cell for energy production in response to increased work or metabolic stress)

allows memory T cells to maintain ATP levels and improve survival[230,343]. Increased SRC promotes mitochondrial biogenesis and the expression of CPT1 (carnitine palmitotransferase 1a)[344]. CPT1, is a transmembrane protein of the mitochondrial outer membrane that converts long-chain acyl-CoA such as palmitoyl to acyl carnitine, and enters the mitochondrial matrix where it undergoes fatty acid oxidation[345]. To better understand the increased SRC in CD5KO naive T cells, it would be useful to measure CPT1 expression levels since CPT1 impairment affects SRC in T cells[328]. While it is not clear what the influencing factors are for the higher OCR and SRC in CD5KO naïve T cells, we propose that mitochondria function in these cells plays an important role. Thus, measurement of mitochondrial mass should be performed as increased mitochondrial mass is connected with fatty acid use in OXPHOS[328,343]. Mitochondrial mass has also been connected to increase SRC and a reduction of mitochondrial mass increases dependency on glycolysis[328]. In our preliminary data we observed that CD5KO T cells seemed to have an equivalent or decreased glycolytic profile after activation in comparison to CD5WT. This could support previous claims in which CD5 modulatory signals also influence T cell immune responses to foreign peptide, meaning CD5 influences the T cell metabolic response not only in development, but also in peripheral T cells upon activation[207,346].

Finally, besides fatty acids metabolism, mitochondrial respiration (OXPHOS) is also involved in amino acid metabolism[235,347,348]. Amino acid synthesis rates increase during T cell activation and it is important for proliferation and cell growth[317,348,349]. Since, amino acid availability is important for cell proliferation and is related to OXPHOS, amino acid availability might also affect SRC of the cell. One way to measure CD5KO and CD5WT T cell ability to utilize amino acids is by targeting glutamine. Glutamine is essential for T cell function as it helps in nucleotide synthesis[350]. SNAT1 and SNAT2 are glutamine transporters, and their expression is

upregulated during T cell activation as increased levels of glutamine are needed to support proliferation[350]. Measurement of these glutamine transporters would provide additional insight in the role of CD5 in amino acid biosynthesis and support the important role of CD5 in T cell metabolism.

CHAPTER 6: CD5 Affects Cognitive Behavior and the Microbiome

The following chapter is a work in progress and will be submitted for scientific review upon completion. Current data and future directions for this project are presented here.

Abstract

The immune system helps in the maintenance of central nervous system (CNS) homeostasis and the CNS is influenced by immune cells and cytokines. T cells are key regulators of the adaptive immune response, and they respond to CNS injuries and can affect learning and behavior. While our understanding of immune-CNS interactions remains incomplete, the role of T cells in the CNS is known to be critically important. Previous work has shown that the absence or hyperactivation of T cells affects cognitive behavior. Additionally, there is evidence that T cell hyperactivation leads to changes in the diversity of the microbiome population. CD5, a co-receptor found on T cells, modulates T cell signaling, activation and proliferation. We hypothesized that because CD5KO T cells have altered activation and proliferation profiles, they would also alter cognitive behavior and microbiome diversity in mice. To evaluate the influence of CD5 influence on behavior and the microbiome, we used CD5KO and CD5WT mice. We found that CD5KO mice had significant behavioral differences when compared to CD5WT, in marble burying rates and open field activity. We also found that CD5KO mice have significantly different microbiome diversity, compared to CD5WT mice. These results suggest that CD5 deficient mice have altered cognitive function (higher levels of fear and anxiety-like behavior) and altered bacterial populations in the gut microbiota.

6.1 Introduction

The immune system, the central nervous system (CNS) and the gut microbiota, are not only individual entities, but are in fact, systems that often work in synergy. Systemic immune activation is believed to be affected by the gut microbiota and to have behavioral effects[351,352]. T cells, as part of the adaptive immune response, are important in cognition and behavior[238,239,353]. Autoreactive T cell hyperactivation, proliferation, and cytokine production (IFNγ) have been shown to affect neuronal plasticity and alter behavior[354,355]. Similarly, depletion of T cells from meningeal spaces results in impairment of learning and memory, which can be reversed by injection of wild-type T cells[356]. Hence, T cells can have protective or harmful effects as they infiltrate the CNS in several autoimmune and infectious diseases settings and affect cognitive behavior[357,358].

Microbial colonization starts during delivery as the neonate moves from what is believed to be a "sterile" location and is exposed to the mother's microbes in the birth canal[359]. This exposure affects neonate's metabolism and maturation of the immune system[352,360,361]. The microbiome, consisting of hundreds of bacterial species, has been implicated in the regulation of inflammatory, infectious and metabolic diseases[362]. Interactions between the gut microbiota and the immune system are numerous, with multiple effects on T cells responses[363-365]. While the microbiota can influence immune cell function, studies performed in T cell deficient mice have found significantly altered microbiota populations, suggesting that T cells also influence microbiome composition[366,367]. Gut microbiota also contributes to the development and maturation of the brain, affecting locomotive and cognitive functions[368]. In fact, microbiota acquired during birth often forms a "core microbiome" during the first 2-3 years old of life, contribute to nervous system development, and have a direct impact on cognition and behavior

later in life[369,370]. Furthermore, germ-free mice have been shown to have impaired brain development and function, as well as impaired immune responses and alterations in their $CD4^+/CD8^+$ T cell populations[363,371,372].

PD-1, is an inhibitory co-receptor found on the surface of activated T cells and regulates T cell activation and promotes self-tolerance[181]. Studies performed in PD-1 deficient mice showed T cell dysregulation resulting in hyperactivation, increased production of IFNγ, and upregulated proliferation that leaded to impairment of the gut barrier, increased inflammation, and defects in IgA regulation, all of which impacted gut microbial composition[373,374]. In addition, increased T cell responses in mice that lack the inhibitory receptor PD-1 were associated with depletion of amino acids necessary for the synthesis of neurotransmitters such as serotonin and dopamine, that may be responsible for altered cognitive functions and an increase in fear based behavior[179]. Thus, an alteration in a T cell co-receptor can significantly influence multiple systems in the organism.

Similar to PD-1, CD5 is a T cell co-receptor that plays an important regulatory function in T cell activation and immune responses. It is known to play an important role during T cell development, immune homeostasis, regulation of activation induced cells death (AICD) and tolerance[121]. T cells from CD5KO mice are hyper-responsive, have higher rates of proliferation and increased calcium mobilization[124]. Because alterations in T cell activation have been associated with altered cognitive behavior and dysbiosis in the gut microbiota, we endeavored to examine the role of CD5 in cognitive behavior and microbiome diversity[179,374]. To do this, we compared CD5 knock out (CD5KO) to CD5 wild type (CD5WT) mice. Our preliminary data suggests that there is an impairment in cognitive behavior of CD5KO mice resulting in an increase in the anxiety-like response in the marble burying test and elevated plus maze. We also

observed gut microbiota differences in CD5KO from CD5WT mice, suggesting that CD5 also influences the diversity of the gut microbiota.

6.2 Materials and Methods

6.2.1 Mice

CD5 wild type (CD5WT) and CD5 knockout (KO) mice (C57BL/6 genetic background) were bred and housed in pathogen free conditions and fed with standard chow[42,43]. All mice used in these experiments were 9-12 weeks old. All use of laboratory animals was done with approval of the Animal Care and Use Committee (IACUC protocols #18-0303, 18-0707, and 18-0708) at Brigham Young University. For the behavioral protocols, mice were maintained on a 12 hour light/dark cycle.

6.2.2 Behavior assessments

6.2.2.1 Marble burying Test

The marble burying test measures anxiety-like behavior[375]. Mice were individually housed and placed in a clear plastic cage filled with approximately 4-cm-deep bedding of "Celu-Nest bedding" (by Shepherd Specialty Paper Company) lightly pressed to give a flat surface[375]. Twenty glass marbles were placed on the surface in five rows of four marbles each. The number of buried marbles (to 2/3 their depth) were recorded during the 10-minutes test, and later quantified by 8 -10 individuals unaware of the groups.

6.2.2.2 Open field activity test

The open field activity test measures locomotor activity and anxiety-like behavior[376]. The open field consisted of a white PVC arena (50 cm × 50 cm). Mice were brought into the experimental room 30 minutes before testing. Each mouse was placed in one of the corner squares facing the wall, observed and recorded for 10 minutes. The total distance traveled,

movement duration and time spent in the center area and outer area over the 10 minutes were recorded and quantified using the Noldus EthoVision XT software. After each test, the arena was cleaned with 70 % ethanol to attenuate and homogenize olfactory traces.

6.2.2.3 Elevated-plus maze test

The elevated-plus maze test measures fear-based and anxiety-like behavior[377]. The elevated-plus maze test was conducted as previously described[377]. The maze (O'Hara & Co) consists of two open arms (30 cm × 5 cm) and two closed arms of the same size, with dark walls (15 cm). The arms and center square (5 cm × 5 cm) are made of white plastic plates elevated to a height of 30 cm above the floor. 3-mm-high plastic ledges decrease open arm falls. Individual mice were placed in the center square facing a closed arm, and activity was recorded or 10 minutes and quantified using the Noldus EthoVision XT software.

6.2.2.4 Morris Water Maze (MWM)

The Morris Water Maze (MWM) measures cognitive function[238,240]. Mouse cognitive function was examined using the MWM for 7 days (n = 17). Mice were given three, 2-minutes trials a day for 7 consecutive days for training to locate a 12 cm diameter circular platform, which was placed 1 cm below water level in an open round pool. The water and room temperature were kept constant between 26.5°C and 27.5° C. An initial day of *training* was given where the mice were given 2 minute trials to locate the platform which was revealed at the end of the trial.

During the acquisition phase of the task, each mouse was allowed a maximum of 2 minutes to locate and climb onto the platform. Once the mouse had located the platform, it was given 5 seconds to remain on the platform, then briefly washed in warm (40°C) water before returning to its home cage. Mice that failed to locate the platform, within 2 minutes were gently

guided to the platform and allowed to acclimatize for 5 seconds before returning to the home cage.

During days 1 – 4, mice were placed in the Southeast quadrant with the platform hidden in the opposing Northwest quadrant. On the fifth day, a probe trial was performed with the platform removed to test reference memory. Each mouse was given only one trial and maximum of 2 minutes in the MWM. On days 6 and 7, the platform was placed in the quadrant opposite the original training quadrant (Northwest), and the mouse was retrained for 3 sessions/trials each day.

Data were recorded using the Noldus EthoVision XT software using a Basler ace acA1300-60gm GigE camera in an isolated environment with only four visual references in the MWM to facilitate the testing subject's learning and memory: A red star on the north end, green triangle on south end, blue square on east end, and yellow circle on west end. 12 ounces of white non-toxic Art-Time Tempera Paint (Sargent Art) was used to make the MWM opaque.

Statistical Analysis of behavioral experiments

Groups were tested the same day, and done in successive training days as in the case of MWM. For MWM Two-way measures ANOVA was used for statistical analyses, with a Bonferroni post hoc test used for individual time point comparisons. Statistical analyses for the other behavioral test (marble burying, open field, and elevated plus maze) were performed using unpaired Student *t* test. All testing was performed between 9am and 1pm, (during the first 4 hours of the lights-off phase). All groups were between the ages of 9 and 12 weeks during the behavior tests. $p<0.05$; *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

6.2.3 Metagenomics analysis of gut microbiota

6.2.3.1 Bacterial DNA extraction, isolation, and purification protocol

Bacterial DNA was isolated and purified from fecal pellets stored at -80°C using a previously published extraction protocol[378], with the following changes: samples were homogenized in the Next Advance Bullet Blender Storm (Next Advance, Averill Park, NY) using 3.2 mm stainless steel beads (SSB32; Next Advance, Averill Park, NY). Cells were then disrupted with 0.1 mm glass beads (GB01; Next Advance, Averill Park, NY). After isolation, purified DNA was suspended in 10mM Tris (pH 8.5) and stored at -20°C.

6.2.3.2 Purity and concentration checks

DNA concentration was measured by absorbance at 260 nm (A260) and purity was estimated by measuring the A260/A280 ratio with a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington DE). Integrity of purified DNA was checked using 0.8% agarose gel electrophoresis with ethidium bromide staining.

6.2.3.3 Bacterial DNA library preparation and sequencing

16S rDNA gene libraries were prepared using the protocol[379] from the John Chaston Lab (Brigham Young University). Amplicon primer sequences (Table 2) were used to amplify the V4 region of the 16S rDNA using the protocol found in Table 4. Integrity of PCR product was checked on 1.2% agarose gels. Following PCR, SequalPrep normalization plates (Invitrogen, Frederick, MD) were used for DNA clean-up and normalization of all samples. Paired-end sequencing was performed on the Illumina Hi-Seq 2500 platform in the BYU DNA Sequencing Center.

Table 3. Amplicon primer sequences

Forward	
Primer	Sequence
v4.SB501	AATGATACGGCGACCACCGAGATCTACACCTACTATATATGGTAATTG TGTGCCAGCMGCCGCGGTAA
v4.SB502	AATGATACGGCGACCACCGAGATCTACACCGTTACTATATGGTAATTG TGTGCCAGCMGCCGCGGTAA
v4.SB503	AATGATACGGCGACCACCGAGATCTACACAGAGTCACTATGGTAATT GTGTGCCAGCMGCCGCGGTAA
v4.SB504	AATGATACGGCGACCACCGAGATCTACACTACGAGACTATGGTAATT GTGTGCCAGCMGCCGCGGTAA
v4.SB505	AATGATACGGCGACCACCGAGATCTACACACGTCTCGTATGGTAATTG TGTGCCAGCMGCCGCGGTAA
v4.SB506	AATGATACGGCGACCACCGAGATCTACACTCGACGAGTATGGTAATT GTGTGCCAGCMGCCGCGGTAA
v4.SB507	AATGATACGGCGACCACCGAGATCTACACGATCGTGTTATGGTAATTG TGTGCCAGCMGCCGCGGTAA
Reverse	
Primer	Sequence
v4.SA701	CAAGCAGAAGACGGCATACGAGATAACTCTCGAGTCAGTCAGCCGGA CTACHVGGGTWTCTAAT
v4.SA702	CAAGCAGAAGACGGCATACGAGATACTATGTCAGTCAGTCAGCCGGA CTACHVGGGTWTCTAAT
v4.SA703	CAAGCAGAAGACGGCATACGAGATAGTAGCGTAGTCAGTCAGCCGGA CTACHVGGGTWTCTAAT
v4.SA704	CAAGCAGAAGACGGCATACGAGATCAGTGAGTAGTCAGTCAGCCGGA CTACHVGGGTWTCTAAT
v4.SA705	CAAGCAGAAGACGGCATACGAGATCGTACTCAAGTCAGTCAGCCGGA CTACHVGGGTWTCTAAT
v4.SA706	CAAGCAGAAGACGGCATACGAGATCTACGCAGAGTCAGTCAGCCGGA CTACHVGGGTWTCTAAT
v4.SA708	CAAGCAGAAGACGGCATACGAGATGGAGACTAAGTCAGTCAGCCGG ACTACHVGGGTWTCTAAT
v4.SA709	CAAGCAGAAGACGGCATACGAGATGTCGCTCGAGTCAGTCAGCCGGA CTACHVGGGTWTCTAAT
v4.SA710	CAAGCAGAAGACGGCATACGAGATTAGCAGACAGTCAGTCAGCCGGA CTACHVGGGTWTCTAAT
v4.SA711	CAAGCAGAAGACGGCATACGAGATTCATAGACAGTCAGTCAGCCGGA CTACHVGGGTWTCTAAT
v4.SA712	CAAGCAGAAGACGGCATACGAGATTCGCTATAAGTCAGTCAGCCGGA CTACHVGGGTWTCTAAT

Table 4. Protocol used to amply the V4 region of the 16s rDNA

Amplicon PCR		
Baseline Sample	Reagents	Your # of Samples ↓
1 Sample		35
13.2	dd H_20	462
4	10x Buffer	140
0.5	50mM $MgSO_4$	17.5
3	2.5 mM dNTP's	105
0.3	Platinum PFX	10.5
2	Template (7ng/ul)	
23	Master mix (2ul) DNA/well	

6.2.3.4 Sequence analysis

16S rDNA sequences were analyzed using the QIIME2/2018.6. Software package[380]. Read joining, denoising, demultiplexing, and feature assignments were accomplished using the Dada2 plug-in[381]. Forward and reverse reads were not trimmed. Reverse reads were truncated at 230 base pairs to insure overlap of reads. Samples from the created BIOM table[382] were then filtered to remove features that appear in less than 2 total samples (singletons) and features not assigned to at least phyla level. Phylogenetic distances were computed using q2-feature-classifier[383] with naïve-bayes fit[384]. Alpha (how many different species could be detected within microbial ecosystem) and beta (how different is the microbial composition between CD5WT and CD5KO) diversity were calculated using core metrics rarefied to a sampling depth of 5000. Principle coordinate analysis (PCoA) visualizations were created using EMPeror[385,386]. Permutation Multivariate Analysis of Variance (PERMANOVA)[387] was used to compare differences in beta diversity between groups. Alpha diversity was calculated using Faith's

Phylogenetic Diversity (PD) and Kruskal-Wallis one-way analysis of variance[388,389]. Taxonomy was assigned using q2-feature-classifier plug-in using Greengenes13_8 99% classifier[390,391].

6.2.3.5 Statistical analysis of microbiome

Alpha diversity (microbial diversity) of microbiota data was analyzed using Kruskal-Wallis one-way ANOVA [388]. Permutation Multivariate Analysis of Variance (PERMANOVA) and Bray– Curtis distance metric was used to compare differences in beta diversity between groups. [387]. Significance levels were assigned as $p<0.05$; *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

6.3 Preliminary Results

6.3.1 CD5 does not influence learning and memory

Previous studies have reported the importance of T cells in learning and memory, as well as how altered immune responses could impair such behavior[392,393] . CD5 modulates T cell activation, and CD5KO T cells are known for their hyperactivation upon peptide stimulation[124]. Because CD5KO T cells have increased proliferation and cytokine production, we wondered if these T cell alterations would influence the learning and memory in CD5KO mice. In order to answer this question, CD5KO and CD5WT mice were trained on the Morris water maze test (MWM). We observed that both strains recorded similar swimming distances and took similar amount of time to locate the platform during the first 4 days, ruling out deficiencies in simple learning/training behavior (Fig. 26A). We next tested the probe trial performance (Day 5; platform removed from water maze), in which the time spent in the original platform quadrant of the pool is measured and which is part of the memory assessment of the MWM[238]. We found that there were not significant differences in the frequency that the mice entered the quadrant where the probe had been, suggesting that CD5 does not affected memory (Fig. 26B). On days 6 and 7,

we examined complex or reversal learning by placing the platform in a different (reversal) quadrant. On day 6 CD5KO mice had significantly improved abilities to find the platform than CD5WT mice, however, at day 7 there were no significant differences between CD5KO and CD5WT mice in finding the reversed probe (Fig. 26C). In summary, in contrast to previous behavioral findings with SCID, nude, Rag1$^{-/-}$ and Rag2$^{-/-}$mice, we did not observe significant learning and memory differences between CD5KO and CD5WT mice in the Morris water maze test (Fig. 26).

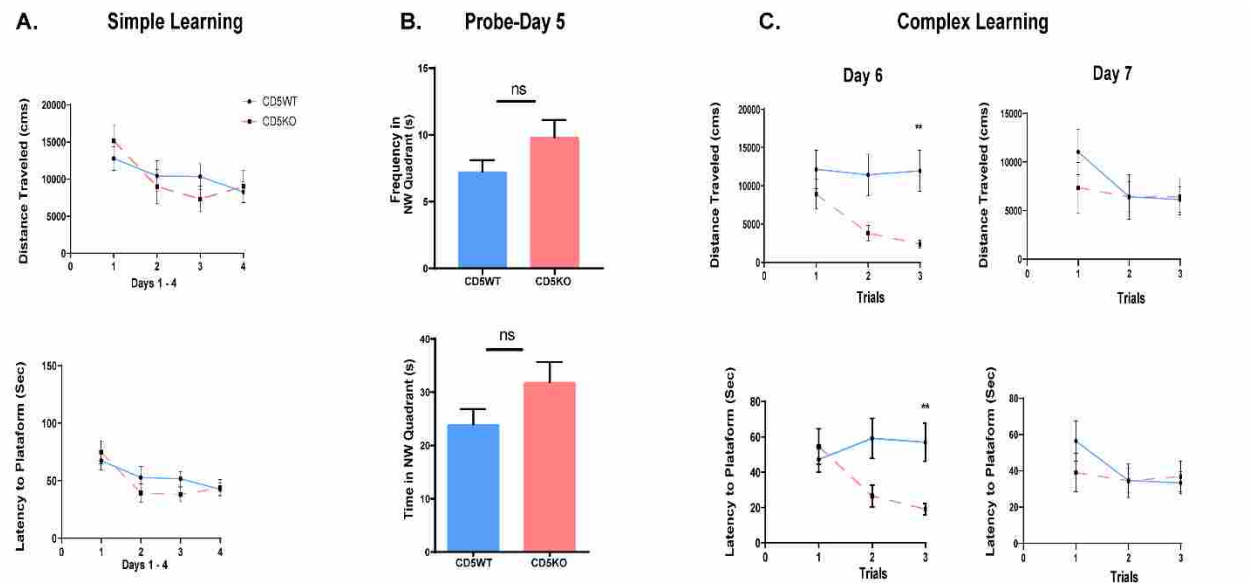

Figure 26. CD5 deletion in mice is not associated with abrogated spatial learning.
A. As a control for cognitive differences during simple learning measurements (days 1-4), mice were tested on the distance they swam. All groups traveled similar distances during the acquisition phase of the task. B. On day 5 a no-platform trial was used to test memory. Frequency and time spent in the NW quadrant (location of the platform on days 1-4) was measured using the student-t test. C. Complex learning or reversal assessment was measured on days 6 and 7 by placing the platform in the quadrant opposite the original training quadrant. Two-way measures ANOVA was used for statistical analyses, with a Bonferroni post hoc test used for individual time point comparisons (n = 17 mice per group;**$p<0.01$). Results are representative of three independent experiments.

6.3.2 CD5KO mice did not exhibit increased anxiety-like or fear-based behavior in the elevated plus maze test

While CD5 did not show to affect memory or learning behavior, we wanted to address if CD5KO mice showed elevated fear-based behavior using a plus maze test. This test measured responses to unconditioned anxiety-related behavior, based on an approach-avoidance conflict, forcing the mouse to decide between the desire to explore a novel environment and the fear that comes from being in an elevated open area[394]. Mice were placed in the center of the maze facing closed arms, and were allowed to freely explore for 10 minutes. There were no significant differences in the time spent in the open arms or frequency in open arms (Fig. 27A-B), the time spent in the closed arms (Fig. 27C), the frequency of visits to the closed arms (Fig. 27D), and the total distance traveled between CD5KO and CD5WT mice (Fig. 27E).

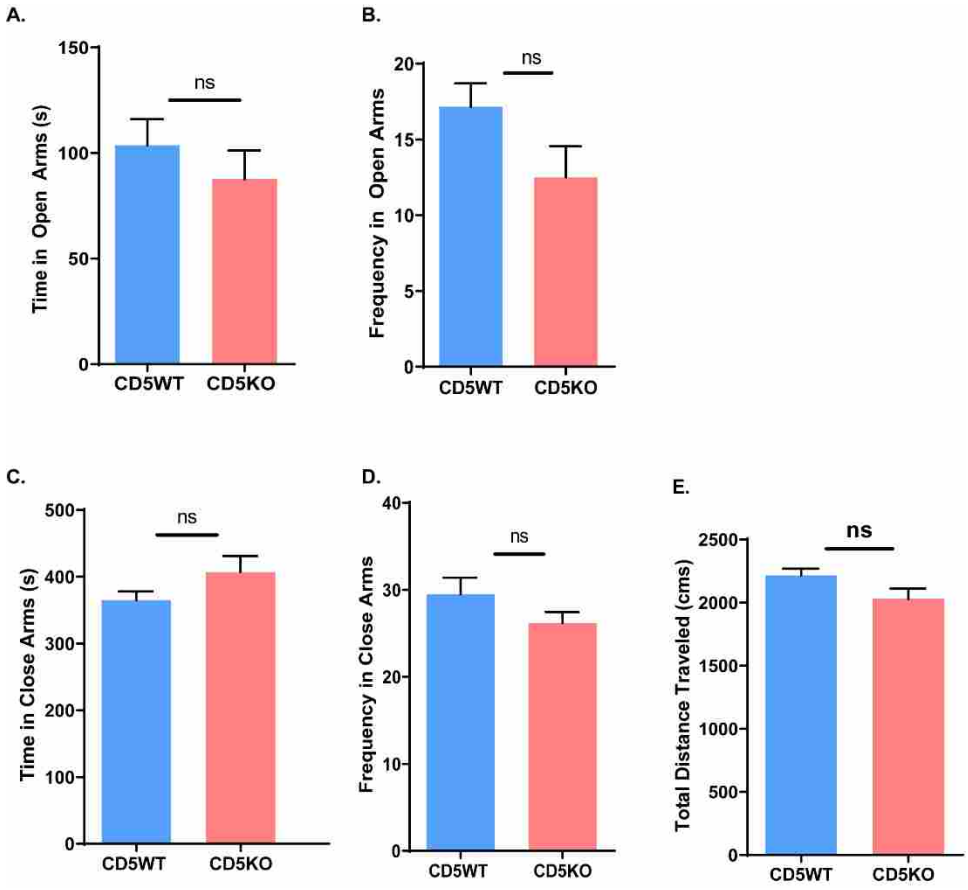

Figure 27. Elevated-plus maze results for CD5WT and CD5KO mice.
A. Total time nice spent in the open arms. B. The frequency of times mice entered the open arms. C. Total time spend in the close arms. D. The frequency of times mice entered the closed arms. E. The total distance traveled by the mice (n = 12 for all figures) ns = no significant differences (two-tailed unpaired *t*-test)

6.3.3 CD5KO mice have increased marble-burying behavior

We determined to examine the anxiety-like behavior in the CD5WT and CD5KO mice using marble-burying tests and found that CD5KO mice buried a significantly higher number of marbles compared with CD5WT mice (Fig. 28). We also observed in the CD5KO cage an extensive scattering of marbles from their initial locations, and disturbed bedding which suggested a higher burying and even digging activity by the CD5KO mice in comparison to the CD5WT mice.

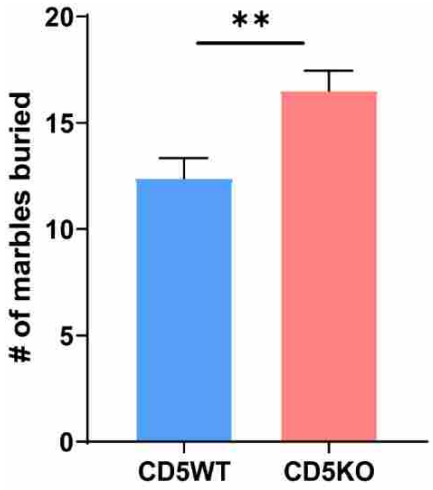

Figure 28. CD5KO mice have increased marble-burying behavior.
The bar graphs show the total number of buried marbles measured during the 10 min marble-burying test. **p<0.01 two-tailed unpaired *t*-test, (*n* = 13 per group)

6.3.4 CD5KO mice have decreased locomotion levels compare to CD5WT mice in the open field test

To further corroborate the anxiety-like behavior phenotype we used the open field activity test[179,395]. Distance traveled on the box area in the 10 minutes was how locomotor activity was measured. There were no significant differences in the time spent in the outer area (Fig. 29A) or the center area (Fig. 29B) compared with the CD5WT mice. However, CD5KO mice did travel significantly less distance in the 10 minutes than the CD5WT mice (Fig. 29C).

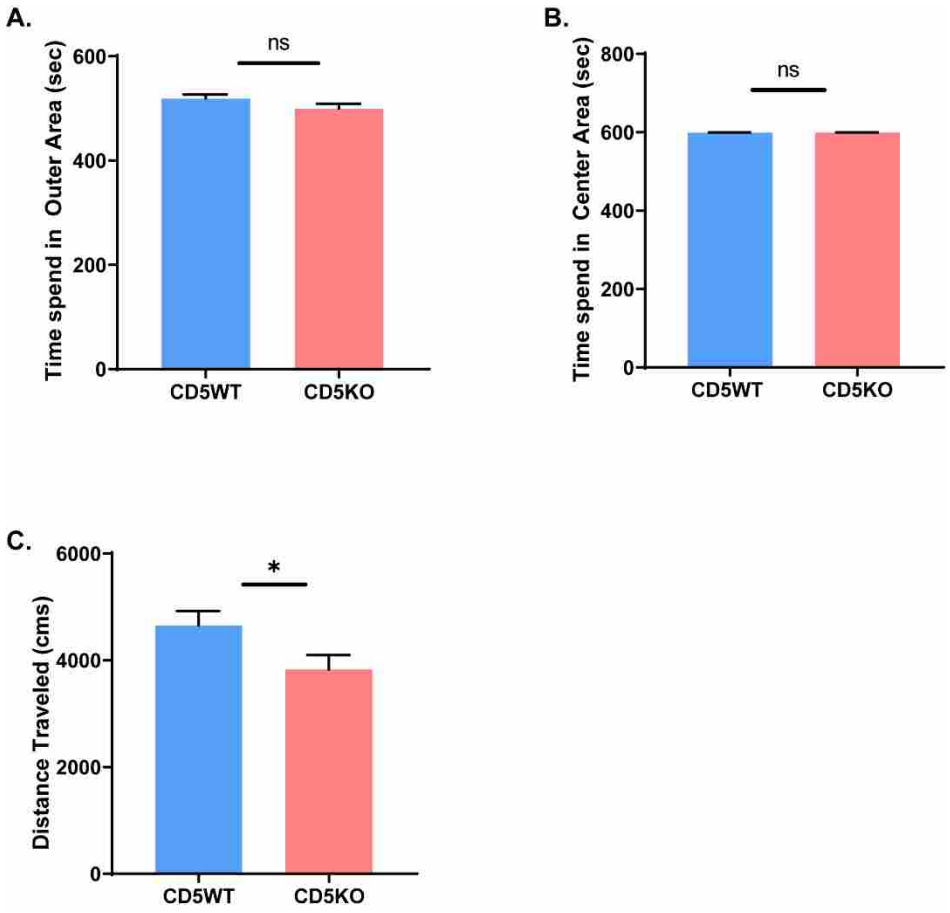

Figure 29. Open field test measuring locomotion activity of CD5KO and CD5WT mice.
Open-field test results for CD5KO and CD5WT mice. A. Time spent by CD5KO and CD5WT mice in the outer area. B. Time spent by CD5KO and CD5WT mice in the center area. C. Total distance traveled by the CD5KO and CD5WT mice in 10 minutes. (n = 13/group) *P < 0.05, (two-tailed unpaired t-test).

6.3.5 CD5KO have significantly different microbiome composition than CD5WT mice

There is increased evidence of the influence of the gut microbiota in behavior and the immune responses[179,396,397]. In fact, changes in the microbiome and immune systems have been shown to significantly affect behavior and are suggested to play a role in autism spectrum disorders and anxiety[398]. It appears that the gut microbiota influences the homeostasis of the immune response by inducing the polarization of specific T cells, such as Th17 and Th1[399,400]. Although, alterations in the microbiota can trigger inflammatory diseases, studies done in Rag 1-

/- mice (fail to perform V (D) J recombination and do not have mature B or T cells) have found that these mice have altered microbiota, suggesting a mutual relationship between gut microbiota and the immune cells[401-403]. Because it is known that CD5 deficiency dramatically alters T cell function, we decided to test if CD5KO and CD5WT mice differed in their microbiota. We examined the bacterial composition found in the fecal samples from CD5WT and CD5KO mice that were fed with normal chow by metagenomics analysis (Fig. 30A). Our data from the unweighted unifrac PCoA plot suggested that there is a significant separation in the microbial communities between CD5WT and CD5KO (Fig. 30B). Furthermore, we observed when comparing CD5KO to CD5WT samples, Phylum Firmicutes is more abundant, phylum Bacteroidetes is reduced, and in CD5KO samples there was no detectable levels of the phylum Verrucomicrobia compared while there were detectable levels in the CD5WT samples CD5WT (Fig. 30C). *Akkermansia muciniphila* is a mucin-degrading bacterium with anti-inflammatory function, is the sole member of the Verrucomicrobia phylum, and is known to be more abundant in the gut of healthy individuals[404]. It has also been suggested that the presence of *A. muciniphila* could be involved in the microbiota-immunity symbiotic loop, since it seems to influence the transcriptional modulation of several immune genes[405]. Thus, these data suggest that there is dysbiosis found in the gut microbiota composition of CD5KO mice, and that this alteration could be influenced by the immune system.

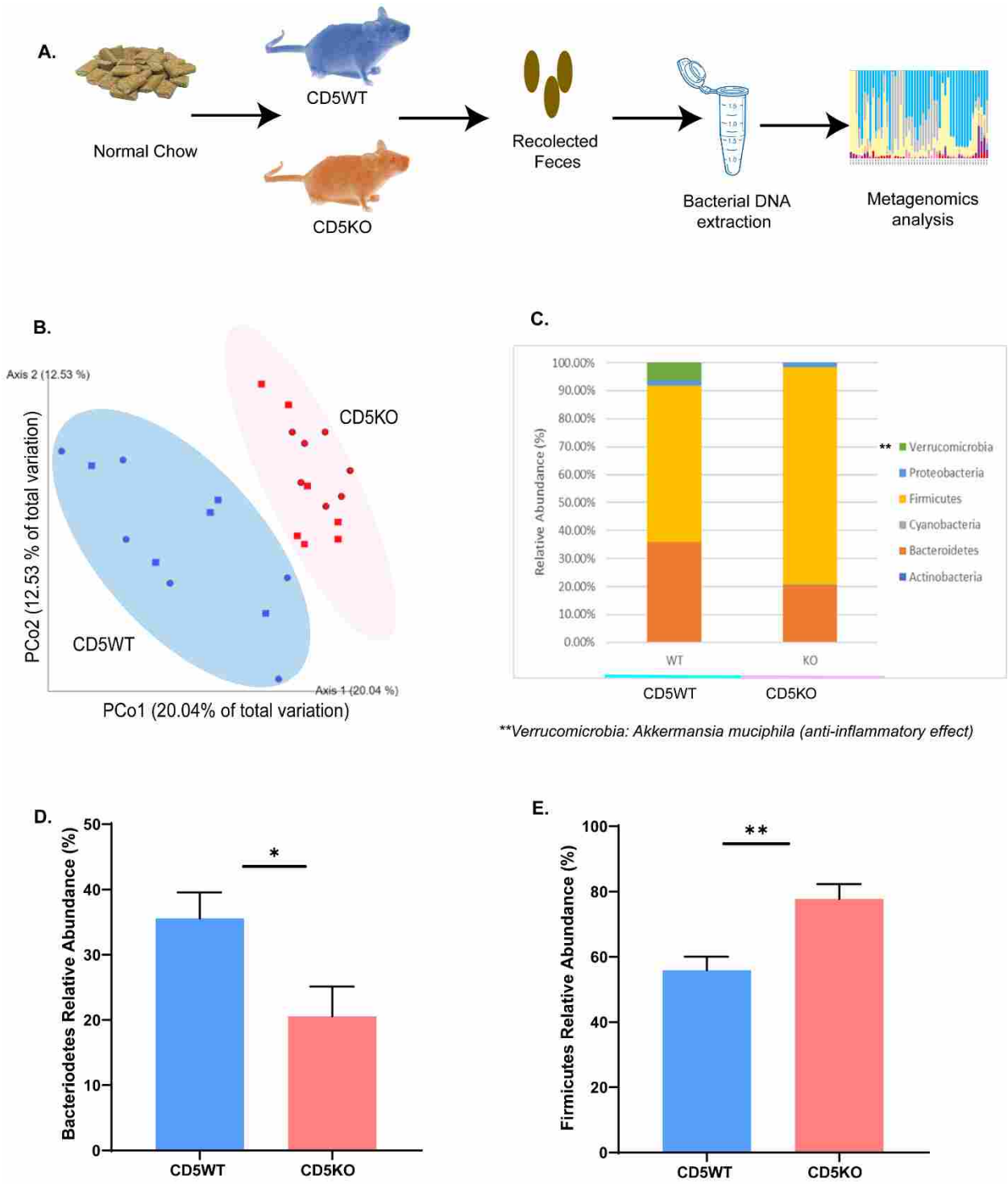

Figure 30. Microbiome analysis of CD5KO and CD5WT mice.
A. Experimental outline for the metagenomics analysis (Figure adapted from "Altered Gut Microbiota Composition in Rag1-deficient Mice Contributes to Modulating Homeostasis of Hematopoietic Stem and Progenitor Cells" by Kwon, O., et al, 2015)[402]. B. Principal coordinate analysis plot (PCoA) based upon unweighted unifrac metrics of fecal microbiota between CD5WT and CD5KO. The axes explain that there is a 12.52% and a 20.04% of data variation in the gut microbiota respectively, further analysis with

the Bray-curtis test shows a high compositional dissimilarity based in their genotypes with a p-value of $p<0.001$. Clustering shows this separation of groups (Marked by the ovals: blue for CD5WT and pink for CD5KO)(n = 29). C. Bacterial phyla distribution on CD5WT and CD5KO mice. *Verrucomicrobia Phylum* was present in CD5WT but not in CD5KO mice. D. There was significant more *Bacteroidetes* in CD5WT than in CD5KO mice. E. There was a significant amount of *Firmicutes* species in CD5KO than in CD5WT mice $*P < 0.05$, $**p<0.01$ two-tailed unpaired *t*-test. (n = 29).

6.4 Conclusions and Future directions

Studies done in the RAG-$1^{-/-}$ mice model that lacks of mature lymphocytes, support the theory that altered immune system function influences cognitive behavior and gut microbiota[406-408]. In fact, RAG-$1^{-/-}$ mice have a significant increase in anxiety-like behavior in behavioral tests such as the open field and the marble-burying tests[409].

PD-1 is a co-receptor that negatively regulates T cell immune responses, suppressing proliferation and cytokine production in activated T cells[338]. Deletion of PD-1 in T cell leads to hyperactivation of T cells which depletes amino acids necessary for neurotransmitter synthesis, resulting in altered cognitive function and behavior[179]. The CD5 co-receptor has a similar co-inhibiting function in T cells as PD-1. The modulatory role of CD5 impacts T cells function during development and in the periphery and has an important role in T cell immune homeostasis and tolerance[132]. CD5KO T cells have increased levels of cytokine production and proliferation, affecting the role of T cells in immune responses, auto reactivity, and activation induced cell death (AICD)[321,410]. T cells are known to influence cognitive function[238]. T cells producing higher amounts of inflammatory cytokines such as TNF-α have been associated with increased depression in humans and laboratory animals under chronic stress[411,412]. Because of the CD5 costimulatory function on T cells, we sought to examine if there is a direct connection between CD5 deficiency and mice cognitive function and behavior.

Work in other models systems with altered T cell function [SCID (Rag1/2 $^{-/-}$) and nude (deficient in mature T cells)] used the Morris water maze test (MWM) to evaluate the effect of

lymphocytes in memory and learning, and found that an absence of functional T cells leaded to cognitive dysfunction[239]. Here we used the CD5KO mouse model in which the T cell have increased rates of cytokine production and proliferation[321]. We did not see a significant impairment in the learning or memory response of CD5KO mice in the MWM test as was seen in studies in mice with a T cell deficiency (SCID and nude mice). Thus, a deficiency of functional T cells appears to initiate impaired cognitive function in the MWM test. While T cells from CD5KO mice have altered function, they do still have functional T cells which could explain why no learning and memory differences were observed in MWM.

We next decided to perform different tests that could evaluate anxiety-like behavior in these mice. First, we use the elevated plus maze test which measures the fear response to open spaces, our results showed not significant differences. In the open field test we found that the CD5KO mice traveled a significantly shorter distance than the CD5WT mice. We also performed the marble-burying test and observed that CD5KO mice had significantly increased the marble burying activity compared to their counterpart the CD5WT mice, suggesting that the CD5KO mice have increased anxiety-like behavior. The idea of altered T cell function influencing cognitive function and behavior has been seen before. Behavioral research performed on experimental autoimmune encephalomyelitis (EAE) mice, suggest that T cells alterations influence mice anxiety-like behavior and can influence serotonin levels[413,414]. In addition, PD-1 deficiency in T cells has been associated with depletion of essential amino acids precursors of neurotransmitters such as serotonin and dopamine in the brain, affecting cognitive behavior, again supporting the important role a T cell co-receptor plays in cognitive function and behavior[179].

Additional studies have also demonstrated that gut microbiota can affect behavior and also the influence immune cells function[415,416]. For example, dysfunction in gut microbiota can affect the balance of Tregs and Th17cells altering the immune response which leads to chronic inflammation and contributes to neurological disorders such as depression[415,416]. RAG-1$^{-/-}$ mice have altered gut microbiota including decreased representation of bacterial species such as Lactobacillales and Enterobacteriales in neonate mice, and increased amount of Verrucomicrobiales (*A. muciniphila* at the species level) in both neonatal and adult mice, suggesting that the adaptive immune system can alter the microbiome population as well[408].

Studies have found that altered T cell function from PD-1 deficiency causes hyperactivation, increased production of IFNγ and impairment of the gut barrier[373].These alterations in immune function also lead to an increase in inflammation, and defects in IgA regulation that impacted gut microbial composition[373,374]. Our CD5 microbiome data analysis revealed that while the microbial composition of CD5WT and CD5KO mice share specific phylum such as Bacteroidetes, Firmicutes, and Proteobacteria, they are found in significantly different quantities (Fig. 32C). One dramatic difference was the Verrucomicrobia; in specific *Akkermansia muciniphilia* which is known for its protective anti-inflammatory effects was not found in CD5KO mice but was found in CD5WT mice[417]. *A. muciniphila* is a gram-negative anaerobic bacterium and the only isolated representative of the Verrucomicrobia phylum. It can degrade mucin and *A. muciniphila* altered expression of mucus genes related to altered immune response and homeostasis[418]. Furthermore, presence of *A. muciniphila* has been shown to improved anti-PD-1 blockage therapy, underlining even more the important role that this bacteria has in the immune response[419]. The myriad of interactions between the innate and adaptive immune cells and gut microbiota, demonstrated the importance of the immune cells in the

development of gut microbiota. There is not only a cause-effect relationship, but also a cooperative connection among these two different systems[420].

Our data indicate that CD5WT and CD5KO differ significantly in gut microbiota diversity and that deficiency of CD5 might play a role in gut microbiota dysbiosis. At the current stage of our study we cannot prove that there is a direct connection between the CD5 co-receptor and the anxiety-like behavior or the microbiome observed in CD5KO mice. We do see a correlation suggesting CD5 is influencing T cells function resulting in alteration in cognitive function, behavior and gut microbiota diversity. A number of previous studies have linked altered T cell responses in inflammation and infection and the cause of neurological disorders as in bacterial dysbiosis[402,421-423]. Our understanding of how immune dysfunction influences cognitive function, behavior and gut microbiome is still in early development. This research aims to contribute to this work, by showing how the CD5 co-receptor plays a role in the immune-microbiota-brain axis synergy.

Further studies are needed to evaluate the role of T cells in behavior and gut microbiota of CD5KO mice (Fig. 31). The following question have to be addressed: Is it the microbiome or the T cells responsible for altered behavior in CD5KO mice? We could address this question by cohousing CD5KO and CD5WT mice (this will allow for them to have similar gut microbiota), or T cell adoptive transfer (Transfer of CD5WT T cells to CD5KO mice) and upon behavioral testing provide or discard microbial or T cell influence[392,424]. Such answer will direct the functional association between CD5 co-receptor in T cells and altered emotional behavior and gut microbiota.

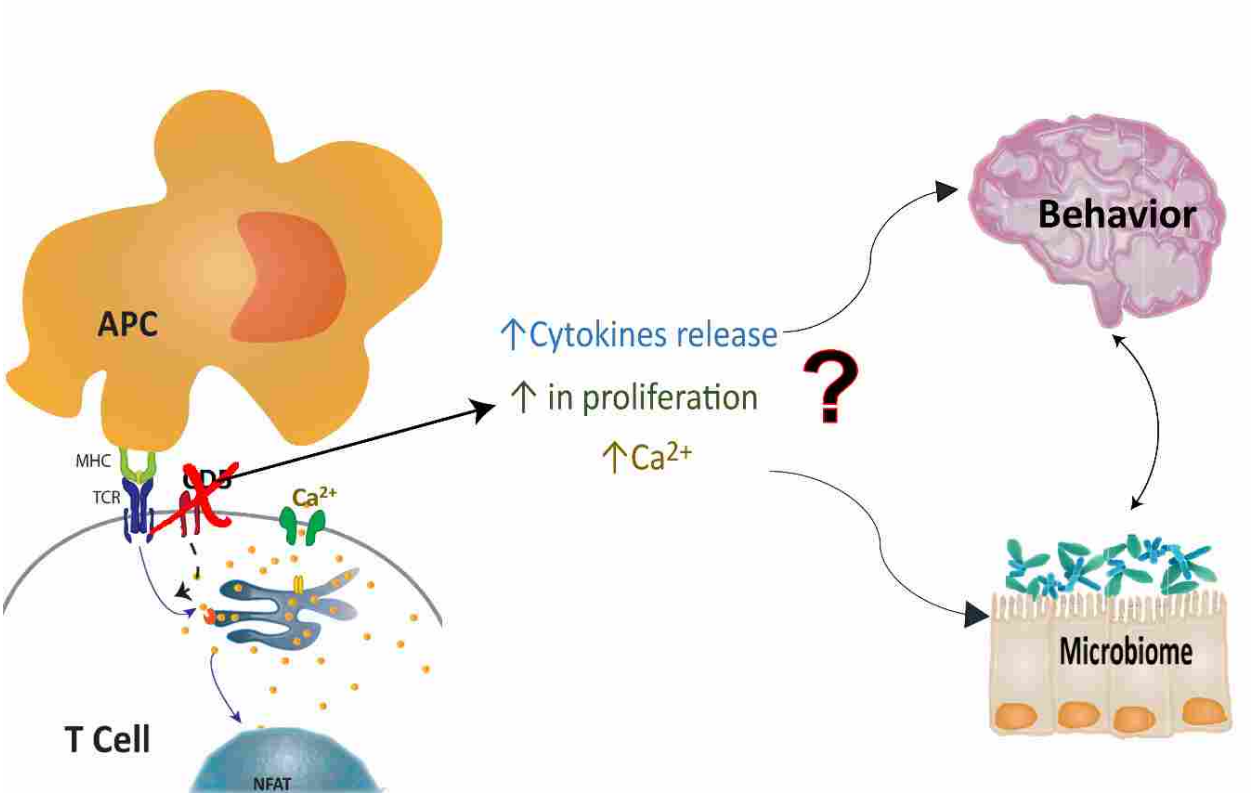

Figure 31. Summary of the potential effect of CD5 in microbiome and behavior.
CD5KO T cells are hyper activation increases cytokine release, proliferation and intracellular Ca^{2+} concentration, which could potentially influence cognitive behavior and gut microbiota.

6.5 Acknowledgments

We want to thank and recognize the significant role that the Bridgewater Lab played in the work reported in this chapter. In special we want to recognize Jeralyn Jones Franson who helped with the training for the behavioral tests, the microbiome experiment and the metagenomics analysis which was essential for us to collect and analyze our preliminary data.

CHAPTER 7: Concluding Remarks and Future Directions

The work presented in this dissertation summarizes research performed to understand the function of two main proteins: 1) nBMP2, a nuclear variant of the BMP2 and 2) CD5, a T cell co-receptor. Both of these proteins influence cellular Ca^{2+} mobilization responses. Concluding remarks and future directions for each of these two proteins will be addressed in individual sections.

7.1 nBMP2 role in immune cells

The Bone morphogenetic protein 2 (Bmp2) is a secreted growth factor important in embryonic development[425]. The nBMP2 is a nuclear variant of the BMP2, translated from an alternative start codon downstream of the signal peptide sequence, which allows a bipartite nuclear localization signal (NLS) to direct translocation to the nucleus[31]. To better understand the role of the nBMP2, studies in muscle, hippocampal neurons and behavior were done in six month old mice (age when phenotype was most pronounced) and the results suggested an impairment in intracellular Ca^{2+} transport[30-32]. Additional work examining the role of nBMP2 on immune cells function found that nBMP2 mutant mice have an impaired secondary response. Here we described work testing the hypothesis that this impaired secondary immune response is due to altered intracellular Ca^{2+} mobilization[32,50]. The impaired immune response in nBMP2 mutant mice was to a systemic bacterial challenge and it appeared these mice had reduced phagocytic activity by macrophages in the spleen. We determined to examine the role that nBMP2 played in macrophages activation and phagocytosis. Our findings revealed a Ca^{2+} mobilization impairment in splenic macrophages from nBmp2NLS^{tm} mutant mice after

secondary infection along with a decreased phagocytic response, supporting the hypothesis that intracellular Ca^{2+} response played a role[426].

Macrophages are phagocytic cells activated by cytokines such as IFN-γ that are produced by innate and adaptive immune cells including natural killer cells, cytotoxic T cells, and helper T cells[427]. Altered Ca^{2+} regulation in these cells affects their metabolism, proliferation, cytokine production and cytotoxicity as well[428]. Future work should examine if the nBMP2 dependent Ca^{2+} impairment observed in macrophages is also present in these other innate and adaptive immune cells after secondary infection.

In addition, it would be important to address if the nBMP2 plays a role in the SERCA activity of macrophages and other immune cells. Sarco/endoplasmic reticulum Ca^{2+}-ATPases (SERCAs) pump Ca^{2+} into the endoplasmic reticulum. It was observed that the SERCA activity in skeletal muscle from nBmp2NLS^{tm} mutant mice was impaired[31]. In fact, previous studies have suggested that the mechanism for reduced intracellular Ca^{2+} mobilization upon activation is due to increased sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA)-mediated reuptake rather than changes in Ca^{2+} influx capacity, meaning that the alterations in Ca^{2+} mobilization response could be due to an altered SERCA function[429]. To accomplish this, test such as the Ca^{2+} pumping activity (which measure Ca^{2+}-ATPase pumps activity and Ca^{2+} transport) could be used[430]. For this purpose it is relevant to inhibit the plasma membrane Ca^{2+} pumps (PMCA) activity to determine if the defect observed in Ca^{2+} mobilization from nBmp2NLS^{tm} mutant mice is dependent of SERCA activity in the cell[431]. In addition, the SERCA pump is encoded by a family of 3 genes (SERCA1 (*ATP2A1*), -2 (*ATP2A2*), and -3 (*ATP2A3*)), and defects in any of them will affect SERCA activity[432,433]. Expression

measurement of these genes will help to elucidate specific components of the SERCA pump where the nBMP2 protein is playing a relevant role.

7.2 CD5 Modulatory Response

CD5 functions as an inhibitory co-receptor in the thymus, and has a modulatory role in the signaling intensity of peripheral T cells by controlling Ca^{2+} signaling activity according to the CD5 expression levels in the T cells. CD5 promotes peripheral T cell activation and immune response by increasing Ca^{2+} signaling in $CD5^{hi}$ T cells as they come in contact to a foreign peptide. Thus, CD5 may be a novel checkpoint to regulate T cell activation and metabolism by altering Ca^{2+} mobilization (chapter 5). CD5 also appears to affect cognitive function and behavior and alter microbiome interactions (Chapter 6). Detailed molecular characterization of the mechanisms of CD5 function could potentially lead to novels immunotherapies.

7.2.1 CD5 role in T cell metabolism

Co-receptors are signaling domains that can influence the T cell activation and metabolic responses[434]. CD5 is a co-receptor with negative regulatory role during positive selection in the thymus and modulatory function in naïve and mature T cell activation as T cells with distinctive CD5 expression levels respond differently to foreign peptide. Previous studies suggest that differences in proliferation and survival in T cells with different CD5 expression levels are due to differences in the TCR signaling strength which will affect their metabolic state[230,294]. Additional work suggested that CD5 levels influence T cell differentiation and effector cytokine production by demonstrating that *ex vivo* activated murine $CD5^{lo}$ $CD4^{+}$ T cells produce relatively greater amounts of the Th1 cytokine IFNγ compared to their $CD5^{hi}$ counterparts[435]. Our work has shown that CD5 expression levels in T

cells affects their activation response which we measured looking at Ca^{2+} mobilization upon activation[61,320]. Ca^{2+} plays an important role in T cell metabolism, as it directs the metabolic reprogramming of naive T cells by regulating the expression of glucose transporters, glycolytic enzymes, and metabolic regulators through the activation of nuclear factor of activated T cells (NFAT) and the PI3K-AKT kinase-mTOR nutrient-sensing pathway[8].

In order to address if CD5 influenced T cell metabolic response, we worked with CD5KO mice T cells, and used as a control CD5WT mice (C57/BL6). Our preliminary data suggests that CD5KO naïve T cells have a significantly higher mitochondrial respiration profile, increased SRC, higher usage of metabolites involved in glycolysis, and increased mitochondrial respiration and amino acids metabolism (See Fig 22 and Fig. 24A-B). To better understand this preliminary data, future research should look into the possible causes that lead to an increase SRC in T cells. One possible cause includes a higher presence of mitochondrial mass in CD5KO T cells. As T cells undergo metabolic changes they also change in mitochondrial content which provide them with a substantial SRC to produce energy in response to increase stress[328]. This can be tested by using MitoTracker, a fluorescent mitochondrial stain used to quantify mitochondrial density, and the mitochondrial membrane potential which is a major component of the proton motive force that increases during ATP synthesis[436,437].

Another hypothesis related to the SRC increase in CD5KO T cells is related with a possible enhancement in the fatty acid metabolism during increase mitochondrial biogenesis (growth and division of mitochondria)[328,438]. SRC promotes mitochondrial biogenesis and the carnitine palmitoyl transferase (CPT1a) expression[328]. CPT1a is a metabolic enzyme that controls fatty acid oxidation (FAO)[328,439]. Measurement of differences of the mRNA of CPT1a

expression between CD5KO and CD5WT T cells would help to elucidate if FAO metabolism plays a role in the higher SRC levels of the CD5KO T cells. Another way to examine if the enhanced SRC in CD5KO is FAO dependent is to run a Mito stress test by adding an extra injection of an CPT1 inhibitor (Etomoxir). CD5KO and CD5WT naïve T cells will be exposed to the etomoxir to block mitochondrial FAO before or after FCCP injection, if SCR in CD5KO T cells is FAO dependent we should expect that addition of etomoxir will decrease the SRC observed in CD5KO T cells[328]. It would also be valuable to examine metabolic function in the CD5KO and CD5WT T cells by measuring levels of key amino acids such as glutamine and the glutamine transporters from the SNAT family SNAT1 (SLC38A1) and SNAT2 (SLC38A2) that are important in T-cell activation[235]. Finally, it would be important to obtain measurements of the GLUT1 (glucose transporters) and glucose uptake rates since glucose fuels mitochondrial FAO and OXPHOS[440]. All this information together can help to elucidate the importance of CD5 in T cell metabolism.

7.2.2 CD5 role in cognitive behavior

Studies done in PD-1 deficient mice showed a connection between T cell hyperactivation and behavior, suggesting that T cell hyperactivation affected systemic metabolism by decreasing free amino acids important for neurotransmitter synthesis which resulted in the increased fear response[179]. Because CD5KO T cells also have a hyperactive phenotype, we determined to see if the cognitive function in CD5 mice was altered. We performed behavioral analysis to characterize the phenotype of CD5KO mice using the Morris water maze (MWM), the elevated plus maze, the open field test and the marble burying test to observe any learning and memory impairment and fear-based or anxiety-like behavior. There were no differences in the learning and memory in the MWM although it was observed that the CD5KO mice appeared to be

swimming faster than CD5WT mice (empirical observation not measured). In future research it would be important to measure swim speed in the MWM. The elevate plus maze test did not show any significant differences between CD5WT and CD5KO mice. In the marble burying test the CD5KO mice had buried a significantly higher number in marbles than the CD5WT mice, suggesting increased anxiety-like behavior in the CD5KO mice. Thus, our results indicate that CD5KO mice have impaired cognitive behavioral function reflected in elevated fear-based and anxiety-like behavior.

Future work will need to elucidate the connection between the CD5 co-receptor in T cells and its influence in cognitive behavior by first performing adoptive T cell transfers from CD5WT T cells to CD5KO mice and evaluate if this recues the fear-based and anxiety-like behavioral responses. Adoptive T cell transfers have restored cognitive function and behavior in SCID, nude and Rag1 -/-mice, providing a means to determine if CD5 deficiency is in fact connected to cognitive function and behavior[392]. It would be important to measure free amino acids levels in serum before and after the T cell adoptive transfer. In the PD-1 study the decreased amino acids levels of tryptophan and tyrosine affected synthesis of monoamine neurotransmitters which were the mechanism of the altered behavioral response.

If amino acids levels are low in the serum of CD5KO mice another approach would be to measure the total amount of tryptophan in tissue (brain and liver) and plasma[441]. Tryptophan is an essential amino acid, and an important precursor of serotonin[442]. When tryptophan is limited in mice it has been shown to result in a defensive behavioral response, suggesting that tryptophan restriction alters the emotional response to stress[443]. It is also possible to address whether the CD5KO phenotype is due to altered amino acid levels is to feed CD5KO mice a tryptophan

supplemented chow (or oral administration) and then measure behavior to see if fear and anxiety-like behavior decreased in CD5KO mice[444,445] .

7.2.3 CD5 role in gut microbiota

There is increased evidence that host-microbe interactions play a key role in maintaining homeostasis and alterations in gut microbial composition is associated with marked behavioral changes[446]. Dysfunction of the microbiome-brain-gut axis has been implicated in stress-related disorders such as depression, anxiety and irritable bowel syndrome and neurodevelopmental disorders such as autism[447]. Studies done in $Rag^{-/-}$ mice showed that they have a distinct microbiota, suggesting that lymphocytes play an important role in the microbiome composition[406]. Since we have documented altered behavior in our CD5KO mice, we hypothesized that an additional component that could affect behavior is microbiome dysbiosis. Our data analysis showed significant differences between the microbial populations composition from CD5WT and CD5KO mice (Fig. 32B). Further characterization of these differences revealed that the phylum *Verrucomicrobia* (*Akkermansia muciniphila* is the only identified member of this phylum) was not present in the CD5KO gut microbiota (Fig. 32C). This is an important finding considering that *Akkermansia muciniphila* is considered a hallmark of healthy gut due to its anti-inflammatory and immune-stimulant properties and its ability to improve gut barrier function[448].

Of note, hyperactivation of the immune cells increases production of pro-inflammatory cytokines which then increase the permeability of the gut-blood barrier and affect the diversity and composition of gut microbiota[449]. In fact, PD-1 deficient T cells hyperactivity has influenced the selection of IgA plasma cells and leads to gut microbiota dysbiosis[373,374]. CD5 has similar function than PD-1 in terms of regulating T cell activation and it would be of great interest to see

if CD5 deficiency also impacts IgA levels. This can be done by determining any differences in the number of IgA plasma cells in the lamina propia, total levels of IgA, or differences in IgA bacterial binding capacity between CD5WT and CD5KO[373,374].

To further establish the role of CD5KO microbiota on mouse behavior, it would be useful to do a fecal transplant. This can be done in mice by cohousing CD5KO and CD5WT mice. Co-housing allows for normalization of the composition of the gut microbiota to see if it will alleviate the fear and anxiety-like behavior in CD5KO mice[450].

Future work should also address if there are changes in gut microbiota after adoptive T cell transfer from CD5WT to CD5KO mice. In addition, it could be ideal to have a group of mice that will be co-housed following adoptive transfer[451]. In this way, it is possible to better elucidate the associations and mechanism of action between the CD5 coreceptor in T cells, the microbiome, and behavior.

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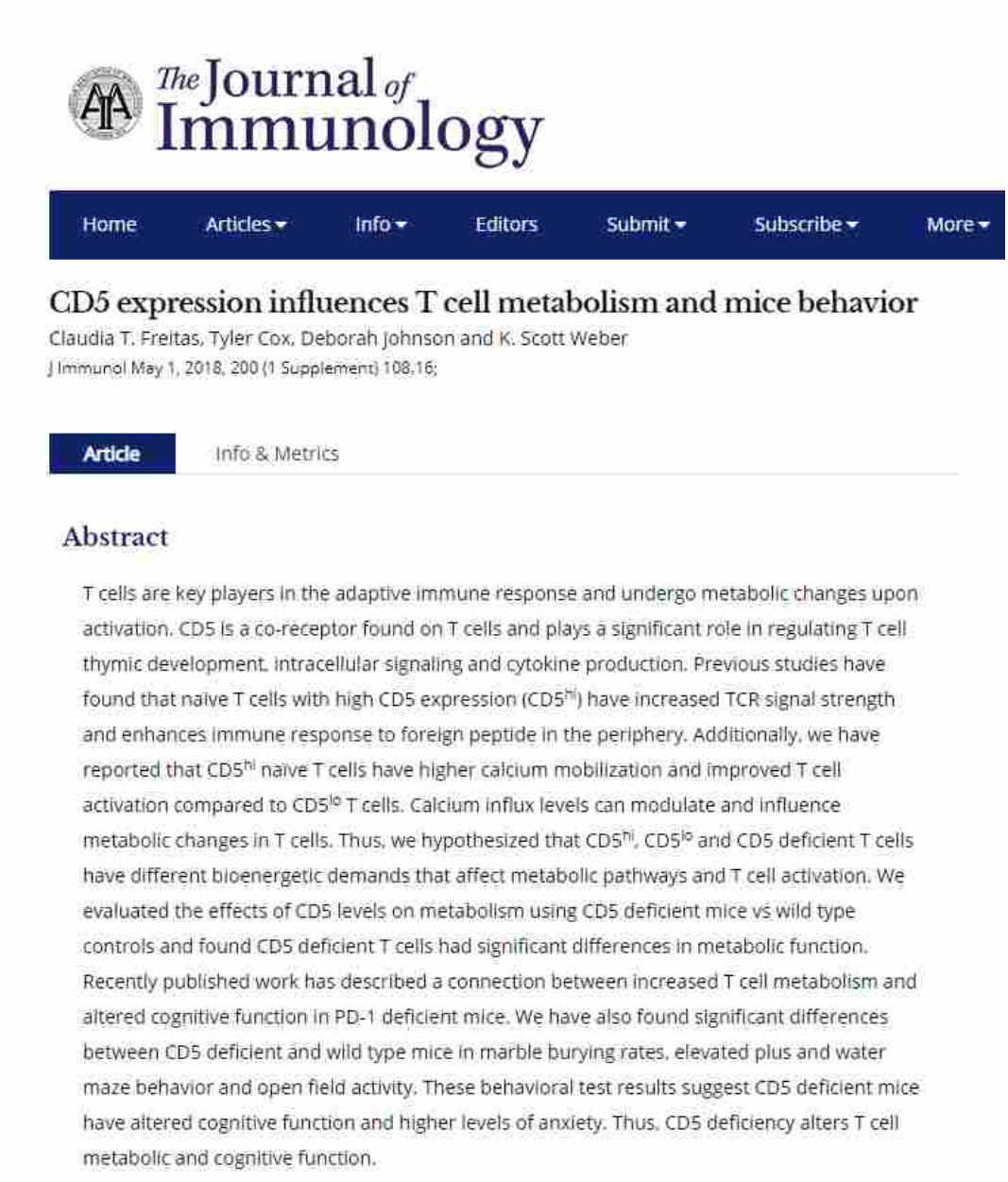
APPENDIX I: CD5 Expression Influences T cell Metabolism and Mice Behavior

The following appendix contains a published abstract in the journal of Immunology Freitas, C. T., Cox, T., Johnson, D. & Weber, K. S. CD5 expression influences T cell metabolism and mice behavior. *The Journal of Immunology* 200, 108.116-108.116 (2018). The data for this abstract was presented at the American Association of Immunologist (AAI) Annual Meeting in Austin, TX, 2018 .

Abstract

T cells are key players in the adaptive immune response and undergo metabolic changes upon activation. CD5 is a co-receptor found on T cells and plays a significant role in regulating T cell thymic development, intracellular signaling and cytokine production. Previous studies have found that naïve T cells with high CD5 expression ($CD5^{hi}$) have increased TCR signal strength and enhances immune response to foreign peptide in the periphery. Additionally, we have reported that $CD5^{hi}$ naïve T cells have higher calcium mobilization and improved T cell activation compared to $CD5^{lo}$ T cells. Calcium influx levels can modulate and influence metabolic changes in T cells. Thus, we hypothesized that $CD5^{hi}$, $CD5^{lo}$ and CD5 deficient T cells have different bioenergetic demands that affect metabolic pathways and T cell activation. We evaluated the effects of CD5 levels on metabolism using CD5 deficient mice vs wild type controls and found CD5 deficient T cells had significant differences in metabolic function. Recently published work has described a connection between increased T cell metabolism and altered cognitive function in PD-1 deficient mice. We have also found significant differences between CD5 deficient and wild type mice in marble burying rates, elevated plus and water maze behavior and open field activity.

These behavioral test results suggest CD5 deficient mice have altered cognitive function and higher levels of anxiety. Thus, CD5 deficiency alters T cell metabolic and cognitive function.



The Journal of Immunology

CD5 expression influences T cell metabolism and mice behavior

Claudia T. Freitas, Tyler Cox, Deborah Johnson and K. Scott Weber

J Immunol May 1, 2018, 200 (1 Supplement) 108.16;

Abstract

T cells are key players in the adaptive immune response and undergo metabolic changes upon activation. CD5 is a co-receptor found on T cells and plays a significant role in regulating T cell thymic development, intracellular signaling and cytokine production. Previous studies have found that naive T cells with high CD5 expression ($CD5^{hi}$) have increased TCR signal strength and enhances immune response to foreign peptide in the periphery. Additionally, we have reported that $CD5^{hi}$ naive T cells have higher calcium mobilization and improved T cell activation compared to $CD5^{lo}$ T cells. Calcium influx levels can modulate and influence metabolic changes in T cells. Thus, we hypothesized that $CD5^{hi}$, $CD5^{lo}$ and CD5 deficient T cells have different bioenergetic demands that affect metabolic pathways and T cell activation. We evaluated the effects of CD5 levels on metabolism using CD5 deficient mice vs wild type controls and found CD5 deficient T cells had significant differences in metabolic function. Recently published work has described a connection between increased T cell metabolism and altered cognitive function in PD-1 deficient mice. We have also found significant differences between CD5 deficient and wild type mice in marble burying rates, elevated plus and water maze behavior and open field activity. These behavioral test results suggest CD5 deficient mice have altered cognitive function and higher levels of anxiety. Thus, CD5 deficiency alters T cell metabolic and cognitive function.

APPENDIX II: Presentations

1 **Freitas CMT**, Cox TD, Franson JJ , Bridgewater LC, and Weber KS. CD5 co-receptor influences T cell metabolism, gut microbiota and cognitive behavior. Tri-Branch American Society of Microbiology Meeting. April 13, 2019. Provo, UT

2 **Freitas CMT**, Cox T, Dunne A, and Weber KS. CD5 co-receptor plays a role in T cell metabolism. Midwinter Immunology Conference. Jan 26-29, 2019. Asilomar CA

3 Townsend MH, Bitter EK, Larsen D, **Freitas CMT**, Piccolo SR, Weber KS, Robison RA, O'Neill KL. HPRT impact on immune regulation influences the tumor microenvironment. Midwinter Immunology Conference. Jan 26-29, 2019. Asilomar CA

4 Townsend MH, Ewell ZD, **Freitas CMT**, Larsen DJ, Lawrence EL, Bennion KB, Piccolo SR, Weber KS, Robison RA, and O'Neill KL. HPRT Overexpression May Contribute to the Immunosuppressive Tumor Microenvironment. American Association for Cancer Research Annual Meeting. March 29- April 3 Atlanta, GA

5 **Freitas CMT**, Cox TD, Johnson DK, and Weber KS. CD5 expression influences T cell metabolism and mice behavior. The American Association of Immunologists Annual Meeting. May 4-8, 2018. Austin, TX (Poster and Oral presentations)

6 Cox TD, **Freitas CMT**, Yorgason JT, Franson JJ , Bridgewater LC, Steffensen SC, and Weber KS. CD5 deficient mice exhibit altered cognitive function in behavioral studies. TriBranch American Society of Microbiology Meeting. April 7, 2018. Durango, CO

7 Johnson DK, **Freitas CMT**, Hancock JC, Tueller JA, Myers SJ, Hamblin GJ and Weber KS. CD5 expression influences helper T cell metabolic state. Tri-Branch American Society of Microbiology Meeting. April 7, 2018. Durango, CO

8 **Freitas CMT**, Cox TD, Johnson DK, Franson JJ , Bridgewater LC, and Weber KS. Role of CD5 expression on T cell metabolism. Tri-Branch American Society of Microbiology Meeting. April 7, 2018. Durango, CO

9 Cox TD, **Freitas CMT**, Yorgason JT, Franson JJ , Bridgewater LC, Steffensen SC, and Weber KS. CD5 deficient mice exhibit altered cognitive function in behavioral studies. BYU College of Life Science Poster Competition. March 29, 2018. Provo, UT

10 Garland KS, Kener K, Hancock J, **Freitas CMT**, Bickman B, Hancock C, Weber KS, and Tessem J. The effects of Nr4a1 full-body knockout in mice. Utah Conference on Undergraduate Research. February 9, 2018. Cedar City, UT

11 **Freitas CMT** and Weber KS. nBMP2 Mutant Macrophages have impair calcium mobilization. BYU Microbiology and Molecular Biology Graduate Retreat. August, 2017. Provo, UT

12 Cox TD, **Freitas CMT**, Yorgason JT, Franson JJ, Bridgewater LC, Steffensen SC, and Weber KS. CD5 deficient mice exhibit altered cognitive function in behavioral studies. Autumn Immunology Conference 46th Annual Meeting. November 17-20, 2017. Chicago, IL

13 Johnson DK, **Freitas CMT**, Hancock JC, Tueller JA, Myers SJ, Hamblin GJ and Weber KS. CD5 expression influences helper T cell metabolic state. Autumn Immunology Conference 46th Annual Meeting. November 17-20, 2017. Chicago, IL

14 **Freitas CMT**, Hamblin GJ, Larsen CM, Weber KS. Naïve Helper T Cells with high CD5 expression have increased calcium signaling. American Society for Microbiology Intermountain Branch Meeting. April 15, 2017. Ogden, UT

15 **Freitas CMT**, Hamblin GJ, Larsen CM, Weber KS. Naïve Helper T Cells with high CD5 expression have increased calcium signaling. Midwinter Immunology Conference. Jan 28-31, 2017. Asilomar, CA

16 **Freitas CMT** and Weber KS. CD5 expression in T cells affect calcium mobilization. BYU Microbiology and Molecular Biology Graduate Retreat. August, 2016. Provo, UT

17 Hamblin GJ, **Freitas CMT**, Steadman N, Williams K, and Weber KS. Calcium Signaling in Primary and Secondary Responses of Listeria specific T helper cells. 10th Annual Utah Conference on Undergraduate Research. February 19, 2016. Salt Lake City, UT

18 Hamblin GJ, **Freitas CMT**, Steadman N, Williams K, and Weber KS. Calcium Signaling in Primary and Secondary Responses of Listeria specific T helper cells. Autumn Immunology Conference 44th Annual Meeting. November 20-23, 2015. Chicago, IL

19 **Freitas CMT** and Weber KS. The importance of Calcium signaling in T cells and macrophages. BYU Microbiology and Molecular Biology Graduate Retreat. August, 2015. Provo, UT

20 **Freitas CMT**, Williams KR, and Weber KS. Calcium Signaling in T helper cell Primary and Secondary Responses. Midwinter Conference of Immunologists. January 24-27, 2015. Asilomar, CA

21 **Freitas CMT** and Weber KS. Calcium signaling in macrophage and T cell activation. BYU Microbiology and Molecular Biology Graduate Retreat. August, 2014. Provo, UT

22 **Freitas CMT**, Williams KR, Weagel EG, O'Neill KL, and Weber KS. Macrophage polarization by necrotic and apoptotic cancer cells. American Society for Microbiology Intermountain Branch Meeting. March 8, 2014. Provo, UT

APPENDIX III: Compiled Publications

The following are the compiled published work of first author and co-author papers. (See next pages)

PLOS ONE

RESEARCH ARTICLE

Naïve helper T cells with high CD5 expression have increased calcium signaling

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Abstract

The adaptive immune response is orchestrated by T helper cells and their function is dependent upon interactions between the T cell receptor (TCR), peptide MHC (pMHC) and co-receptors. TCR-pMHC interactions initiate calcium signaling cascades which determine T cell activation, survival, proliferation and differentiation. CD5 is a co-receptor that plays an important role in regulating T cell signaling and fate during thymocyte education. CD5 surface expression on mature single positive thymocytes correlates with the TCR signal strength for positive selecting self-ligands. CD5 also plays a role in T cell function after thymic development is complete. Peripheral T cells with higher CD5 expression respond better to foreign antigen than those with lower CD5 expression and CD5-high T cells are enriched in memory populations. In our study, we examined the role of CD5 expression and calcium signaling in the primary response of T cells using two *Listeria monocytogenes* specific T helper cells (LLO118 and LLO56). These T cells recognize the same immunodominant epitope ($LLO_{190-205}$) of *L. monocytogenes* and have divergent primary and secondary responses and different levels of CD5 expression. We found that each T cell has unique calcium mobilization in response to *in vitro* stimulation with $LLO_{190-205}$ and that CD5 expression levels in these cells changed over time following stimulation. LLO56 naïve T helper cells, which expresses higher levels of CD5, have higher calcium mobilization than naïve LLO118 T cells. Three days after *in vitro* stimulation, LLO118 T cells had more robust calcium mobilization than LLO56 and there were no differences in calcium mobilization 8 days after *in vitro* stimulation. To further evaluate the role of CD5, we measured calcium signaling in CD5 knockout LLO118 and LLO56 T cells at these three time points and found that CD5 plays a significant role in promoting the calcium signaling of naïve CD5-high LLO56 T cells.

Citation: Freitas CMT, Hamblin GJ, Raymond CM, Weber KS (2017) Naïve helper T cells with high CD5 expression have increased calcium signaling. PLoS ONE 12(5): e0178799. https://doi.org/10.1371/journal.pone.0178799

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Introduction

Helper T cells play a critical role in adaptive immunity by orchestrating and regulating the immune response [1, 2]. In large part, the binding properties of the T cell receptor (TCR) regulates the development, activation, and proliferative response of T lymphocytes [3, 4]. In the thymus, T cells are selected according to their avidity for self-peptide/MHC complexes. The

TCR must be able to recognize self-peptide/MHC complexes with enough affinity to transduce a signal during positive selection while not binding so tightly that they are negatively selected [4–6]. TCR avidity and signal strength plays a key role in T cell function (calcium signaling, cytokine production, T cell proliferation and differentiation) [7–9]. In addition to the TCR and its interaction with peptide MHC (pMHC), multiple receptors such as CD4, CD8, PD-1, and CTLA-4 play a key role in determining whether TCR:pMHC binding results in T cell activation or anergy. CD5 is known to be a negative regulator of TCR signaling in developing thymocytes and its expression level in naïve T cells is determined during thymic development. CD5 levels are set during positive selection according to the strength of the TCR-self-peptide/MHC interaction. Typically, the stronger the avidity for self-peptide/MHC the higher the CD5 surface expression [10–13]. After completing thymic development, T cells with higher CD5 expression respond better to foreign antigen than those with lower CD5 expression and CD5-high T cells are enriched in memory populations [14, 15]. Although there are studies examining the role of T cell CD5 expression during thymic development and CD5-high cells are enriched in memory cell populations, it is not clear how CD5 is involved in calcium signaling during a helper T cell primary response. To better understand the role of CD5 in a T cell primary response to foreign antigen, we examined the *in vitro* calcium responses of CD5-high and CD5-low T helper cells that respond to the same epitope of *Listeria monocytogenes*.

Calcium (Ca^{2+}) is a ubiquitous second messenger important for a wide range of cellular functions. Ca^{2+} signaling plays an important role in T cell activation, cytokine production, proliferation and cell fate and is determined by TCR interactions with the pMHC complex as well as additional co-receptors [9, 16]. Ca^{2+} signaling has been well characterized in lymphocytes and the calcium signal for specific helper T cell subsets has been identified, suggesting a strong relationship between Ca^{2+} mobilization in T helper cells and their functional response [17, 18]. TCR engagement with pMHC initiates signal transduction pathways that result in a dramatic increase of intracellular Ca^{2+} [19, 20]. Increases in intracellular Ca^{2+} enables transcription factors to enter the nucleus and turn on genes that play a critical role in immune responses. For example, NFAT, NF-κB, AP-1, and the Oct family transcription factors initiate transcription of the interleukin-2 (IL-2) gene [21]. IL-2 production is important for T cell proliferation and survival and plays a key role in promoting effector and memory cell differentiation [22–24]. Thus, TCR-dependent Ca^{2+} signals are essential for robust T cell primary and secondary immune responses.

The TCR avidity for self-peptide/MHC complex during selection affects the function and maintenance of these cells in the periphery and how they respond to infection [25, 26]. CD5 is a monomeric cell surface glycoprotein expressed on thymocytes, mature T cells, and a subset of B cells. High TCR avidity for self-peptide/MHC results in high surface expression of CD5 on double positive and single positive thymocytes, whereas lower avidity is correlated with lower surface expression of CD5 [10]. CD5 has been shown to negatively regulate the TCR signal during thymic development [27]. CD5 expression and Ca^{2+} mobilization correlate with TCR signal strength and T cell fate [3, 5, 28]. However, mature naïve T cells with higher expression levels of CD5 appear to respond better to foreign ligands, suggesting that CD5 influences T cell responsiveness at the post-selection level [14, 16, 29]. Thus, it appears that the negative regulatory effect of CD5 in the thymus may not depend on the extracellular region of CD5 whereas the positive co-stimulatory effect of CD5 in the periphery is dependent on extracellular engagement of an endogenous ligand (CD5 or CD5L) [30–32]. While the exact function of these CD5 ligands is unclear, there is evidence that CD5L (CD72; a C-type ligand) binds to CD5 and that CD5 is homophillic and may bind to CD5 on other cells [33]. Thus, CD5 has a critical and divergent role in regulating T cell activation depending on the time and location of activation.

LLO56 and LLO118 are two T helper cells that recognize the same immunodominant epitope ($LLO_{190-205}$) of *L. monocytogenes* and have divergent primary and secondary responses. They differ by 15 amino acids in their TCR sequences and have unique responses to *L. monocytogenes* infection *in vivo*, LLO118 has a better primary response whereas LLO56 has a more robust secondary response [25]. Previous analysis of thymocytes and T cells revealed that LLO56 has higher levels of CD5 and a more robust IL-2 response in addition to a reduced primary response caused by increased cell death compared to LLO118 T cells [25, 26]. In order to better understand how CD5 levels affect T cell activation in cells that have completed thymic development, we determined to evaluate calcium signaling in LLO56 (CD5-high) and LLO118 (CD5-low) T cells. We also measured calcium signaling in CD5 knockout LLO118 and LLO56 T cells to better elucidate the role of CD5 in calcium signaling after thymic development is finished. This was accomplished by measuring LLO118 and LLO56 calcium mobilization at three different time points during the T cell response: Naïve T helper cells, day 3 post-stimulation, and day 8 post-stimulation. We also measured calcium signaling in CD5 knockout LLO118 and LLO56 T cells at these time points and found CD5 plays a significant role in promoting the calcium signaling of naïve CD5-high T cells, but does not alter calcium mobilization levels at later time points.

Materials and methods

Mice

LLO56 (B6 Thy-1.1$^+$ Rag1$^{-/-}$), LLO118 (B6 Ly5.1$^+$ Rag1$^{-/-}$) and CD5 knockout (KO) LLO56 and LLO118 were bred and housed in pathogen free conditions [25, 26]. All mice used in these experiments were 5–12 weeks old. All use of laboratory animals was done with approval of the Animal Care and Use Committee (IACUC protocol #15–801) at Brigham Young University.

T cell isolation and activation

$CD4^+$ T cells were isolated from the spleens of LLO56 and LLO118 TCR transgenic (Tg) mice [26]. Spleen single cell suspensions from LLO56 and LLO118 mice were purified using a negative selection $CD4^+$ T cell isolation kit (Miltenyi Biotec) [17]. T cells were isolated from the spleen of LLO56, LLO118, LLO56-CD5KO, and LLO118-CD5KO mice. Spleens were homogenized and passed through a nylon mesh cell strainer. The single-cell suspension was resuspended in R10 medium containing RPMI 1640, 10% of FBS (HyClone), 1% Glutamax (Gibco by Life Technologies), and 0.5% Gentamicin (Life Technologies), then transferred to a 6-well plate ($1x10^6$ cell/ml) and loaded with 1 μM of *Listeria monocytogenes* peptide $LLO_{190-205}$. For T cell isolations, mice were euthanized using CO_2 inhalation.

Antigen presenting cell isolation

Bone marrow derived macrophages (BMDM) were obtained from B6/C57 mouse femurs and tibias and were cultured at 37˚C and 5% CO_2 and matured for 7 days in macrophage medium with DMEM (HyClone), 10% FBS (HyClone), 20% supernatant from L929 mouse fibroblast as a source of macrophage colony-stimulating factor (M-CSF), 5% heat inactivated horse serum (Sigma), 1 mM Na Pyruvate (Gibco by Life Technologies), 1.5 mM L-glutamine (Thermofisher), 1100X Penicillin/Sreptomycin (Gibco by Life Technologies). Harvested cells were plate in an 8-chamber cover glass where they were loaded with the *Listeria monocytogenes* peptide $LLO_{190-205}$ overnight. For bone marrow derived macrophage isolations, mice were euthanized using CO_2 inhalation.

Calcium imaging

Naïve T cells were incubated with 1 μM of Fura-2AM (Invitrogen) for 30 minutes at 37˚C and 5% CO_2 in Ringers imaging solution (150 mM NaCl, 10mM glucose, 5 mM of HEPES, 5 mM of KCl, 1mM $MgCl_2$, and 2 mM $CaCl_2$, pH 7.4), washed, and then incubated in Ringers solution for another 30 minutes at 37˚C. 200,000 Fura-2AM loaded naïve $CD4^+$ T cells were pipetted onto 200,000 bone marrow derived macrophages that were previously incubated with 1 μM of *Listeria monocytogenes* peptide $LLO_{190\text{-}205}$ overnight. Imaging was performed in Nunc 8-chamber covered glass slides (155411, Thermo Scientific). For day 3 and day 8 stimulation time points, LLO118 or LLO56 splenocytes were incubated overnight with 1 μM of *Listeria monocytogenes* peptide $LLO_{190\text{-}205}$. Calcium imaging was performed at room temperature using an Olympus IX51 inverted microscope equipped with a xenon arc lamp. Fura-2AM loaded T cells were excited at 340 nm and 380 nm excitation filters and capture by a flores-cence microscope camera (Q Imaging Exi Blue) using a 20x objective (N.A. 0.75). Images (340/380/transmitted) were recorded at 3 second intervals over 20 minutes. Each individual cell fluorescence was normalized with the first recorded value according to the equation (F-Fo)/Fo where F is the fluorescence at the specific time point, and Fo is the fluorescence value at time 0 [34].

Flow cytometry

Calcium mobilization was also measured using flow cytometry and the high affinity calcium indicator Fluo-4 (ex:470–490 nm and em: 520–540 nm). Cells were surface stained with an anti-$CD4^+$-APC antibody (17–0041; eBioscience). T cells were loaded for 30 mins as previously published with pluronic acid and 1mM Fluo-4-acetoxymethyl ester (Invitrogen) in Ringer solution (150 mM NaCl, 10 mM glucose, 5 mM of HEPES, 5 mM of KCl, 1 mM $MgCl_2$, and 2 mM $CaCl_2$, pH 7.4) [35]. Intracellular calcium mobilization was initiated by adding 50 ng/ml of PMA (phorbol 12-myristate 13-acetate) and 1 μg/mml of ionomycin [36]. For further analysis done in FlowJo, the lymphocyte population was gated in a forward and side scatter gate and singlets. From this gate a second gate was created specific for $CD4^+$ T cells [37]. Intracellular calcium flux was measured in the $CD4^+$ T cell gate using the FlowJo kinetics tool.

For CD5 expression analysis, spleen single cell suspensions from naïve and stimulated (day 3 and day 8) were stained with anti-CD5-PE (12–0051; eBioscience), and anti-CD4-APC (17–0041; eBioscience) and analyzed on the flow cytometer (BD Accurri C6).

Data analysis

Live cell calcium imaging data was analyzed using CellSens Software from Olympus and the 340:380 ratio calculations were performed on randomly selected cells. The standard deviation of the calcium levels from the regression line was determined using GraphPad Prism. For calcium flow cytometry measurements, FlowJo kinetics tool was used to determine the area under the curve (AUC) [35, 38, 39]. All assays were performed at least three times in triplicate and significant values were determined using student T test in GraphPad Prism.

Results

LLO118 and LLO56 T helper cells have different responses to antigen and CD5 expression levels

To examine the role of CD5 in regulating calcium signaling in the primary response of helper T cells, we used LLO56 and LLO118 T cells which are specific for the same epitope of *Listeria monocytogenes* (listeriolysin O, $LLO_{190\text{-}205}$) [25]. These LLO118 and LLO56 T cells differ in

their *in vivo* responses upon *L. monocytogenes* infection; LLO118 helper T cells have a better primary response and LLO56 helper T cells exhibit a better secondary response [25]. Additionally, naïve LLO56 T cells have higher levels of CD5 and produce more IL-2 upon stimulation compared to those from LLO118 T cells [25, 26]. We hypothesized that these differences in CD5 levels and T cell function would allow us to better understand the role of CD5 in calcium signaling and T cell activation in a primary response (See Table 1).

LLO56 naïve helper T cells have higher calcium mobilization in vitro

To determine how CD5 plays a role in the primary immune response of LLO56 and LLO118 T cells, we first analyzed the calcium signaling of naïve T cells isolated and purified from the spleens of LLO56 and LLO118 TCR Tg mice. Calcium mobilization was measured using live cell imaging after loading the T cells with Fura-2AM and adding them to 8-chamber slides containing antigen presenting cells loaded overnight with the *L. monocytogenes* peptide ($LLO_{190\text{-}205}$). A calcium profile was generated by combining measurements (40^{+} cells) from 4 different experiments taking readings every 3 seconds over a 20-minute time span (Fig 1A). Upon stimulation with $LLO_{190\text{-}205}$ peptide, LLO56 T helper cells have higher peak calcium influx levels compared to LLO118 T cells (Fig 1B). There are not any significant differences in the mean calcium levels and variability (standard deviation) of the calcium signal between LLO56 and LLO118 T cells (Fig 1C and 1D) [40]. Thus, naïve LLO56 (CD5-high) and naïve LLO118 (CD5-low) T cells have significantly different peak calcium mobilization profiles.

LLO56 naïve T helper cells have higher levels of CD5 surface expression

Previous work has shown that naïve LLO56 T cells have higher expression of CD5 compared to naïve LLO118 T cells [25]. We wanted to know what happened to the levels of CD5 at the post-stimulation time points (day 3 and day 8) that we examined in this study. As previously reported, LLO56 naive T helper cells showed higher CD5 expression than naïve LLO118 T helper cells. However, upon stimulation, the CD5 expression differences between LLO56 and LLO118 T cells decrease over the course of 8 days of stimulation (Fig 2A and 2B). To further confirm CD5 expression a mean fluorescent intensity (MFI) profile was done, which confirmed significant CD5 expression levels differences between naïve LLO56 T cells with Day 3 and Day 8, however LLO56 did not have significant differences between Day 3 and Day 8 (Fig 2C and 2D).

LLO118 T cells have higher peak calcium influx on day 3 post-stimulation

To determine whether the Ca^{2+} influx difference seen in naïve T cells were maintained over the course of a primary response to infection, we measured calcium influx for LLO118 and LLO56 T helper cells 3 days post-stimulation with *L. monocytogenes* peptide ($LLO_{190\text{-}205}$) (Fig 3A). In contrast to naïve T cells, day 3 post-stimulated LLO118 T cells have significantly

Table 1. Summary of differences between LLO56 and LLO118 T cells.

	LLO56	LLO118
Primary Response	+	+++
Secondary Response	+++	+
IL-2 Response	+++	++
CD5 Expression (naïve T cells)	+++	+

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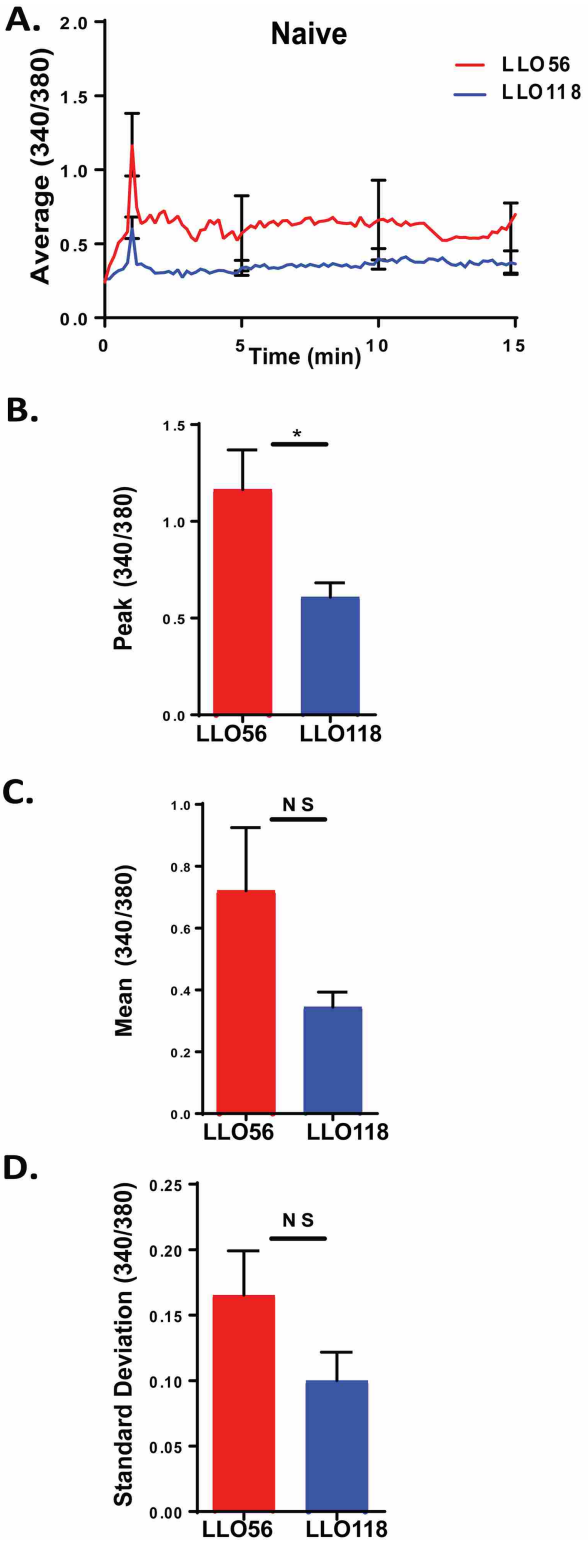

Fig 1. LLO56 naïve helper T cells have higher calcium mobilization *in vitro*. Naïve T cells from LLO56 and LLO118 TCR transgenic mice were obtained from the spleen using negative selection and calcium levels were measured using live cell microscopy. T cells were added to antigen presenting cells (bone marrow derived macrophages) that were loaded overnight with the *L. monocytogenes* peptide ($LLO_{190-205}$). **A.** Average curves of intracellular Ca^{2+} mobilization from LLO56 and LLO118 naïve T cells (340/380 ratios)

(n = 40^{+}). Error bars show the SEM at the influx peak and every 5 minutes after the peak (n = 40^{+}). **B.** Statistical analysis of peak calcium influx of stimulated LLO56 and LLO118 naïve T helper cells (n = 40^{+}). **C.** Statistical analysis of the sustained intracellular Ca^{2+} levels (Average 340/380 values between minutes 5 and 20) after initial stimulation response (n = 40^{+}). **D.** Standard deviation was determined by linear regression analysis and shows variability in the calcium signal for each group (n = 40^{+}). (* = p < .05; NS = not significant).

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higher peak levels of calcium influx than LLO56 T helper cells (Fig 3B). While there were no differences in mean Ca^{2+} levels (Fig 3C), LLO118 did have significantly higher variability in the calcium signal (standard deviation). Thus, on day 3 post-stimulation LLO56 T cells had significantly lower peak calcium influx and lower variability compared to LLO118 T cells.

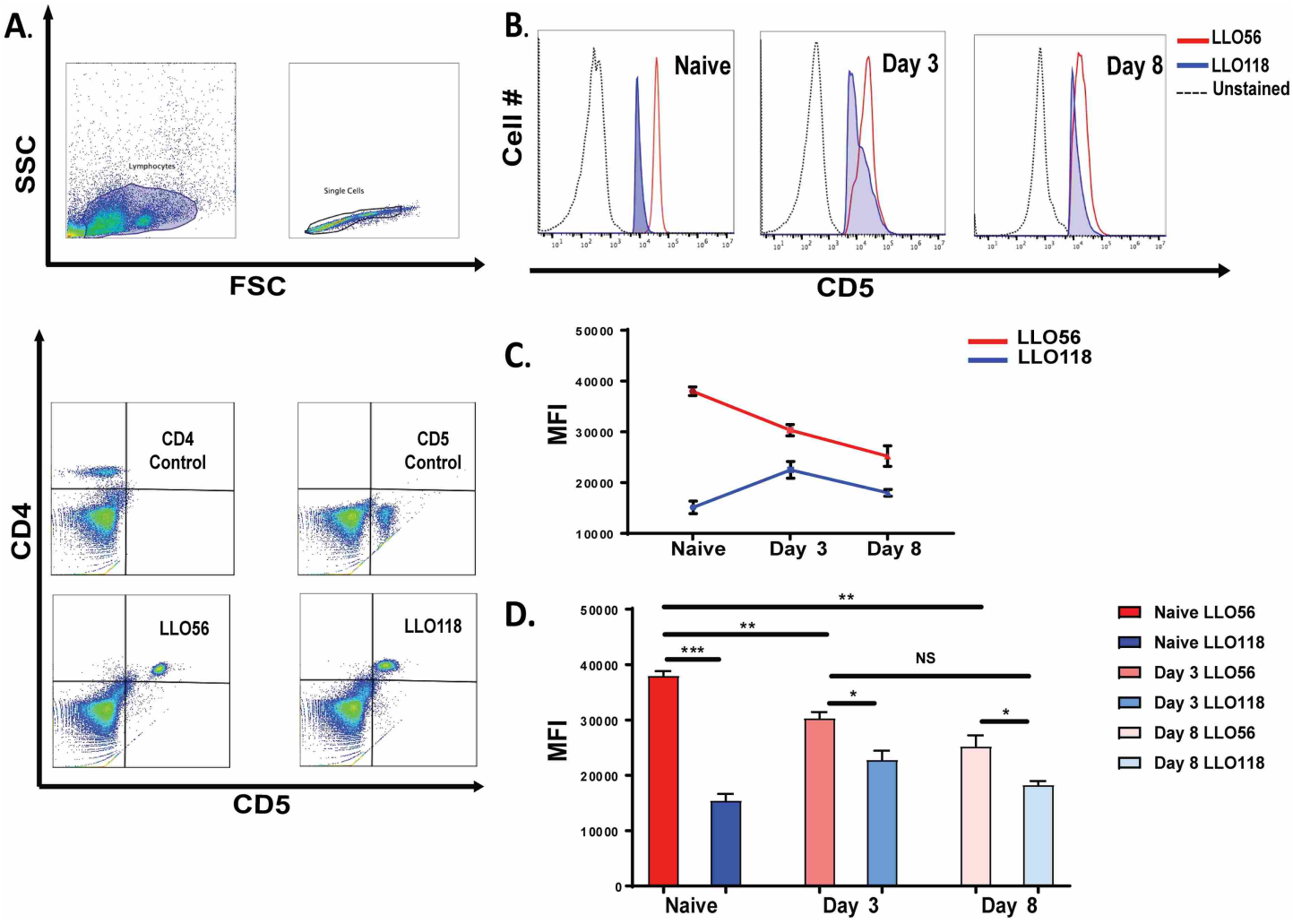

Fig 2. Naïve LLO56 T helper cells have higher levels of CD5. Flow cytometry analysis of CD5 expression of LLO56 and LLO118 T helper cells was done using LLO118 and LLO56 splenocytes at different time points after stimulation with the $LLO_{190\text{-}205}$ peptide from *L. monocytogenes*. **A.** Gating strategy for measuring CD4 and CD5 mean fluorescent intensity on LLO118 and LLO56 T cells. **B.** CD5 levels of naïve T cells, T cell stimulated for 3 days, and T cells stimulated for 8 days. T helper cells from LLO56 (red line) overlaid with the CD5 levels from LLO118 (shaded blue). Unstained cells are also included (black dots). **C-D.** Comparison of mean fluorescence intensity (MFI) profiles for LLO56 and LLO118 expression levels of CD5 at different time points were determined by flow cytometry quantitative analysis. (* = p < .05; ** = p < .01; *** = p < .001; NS = not significant).

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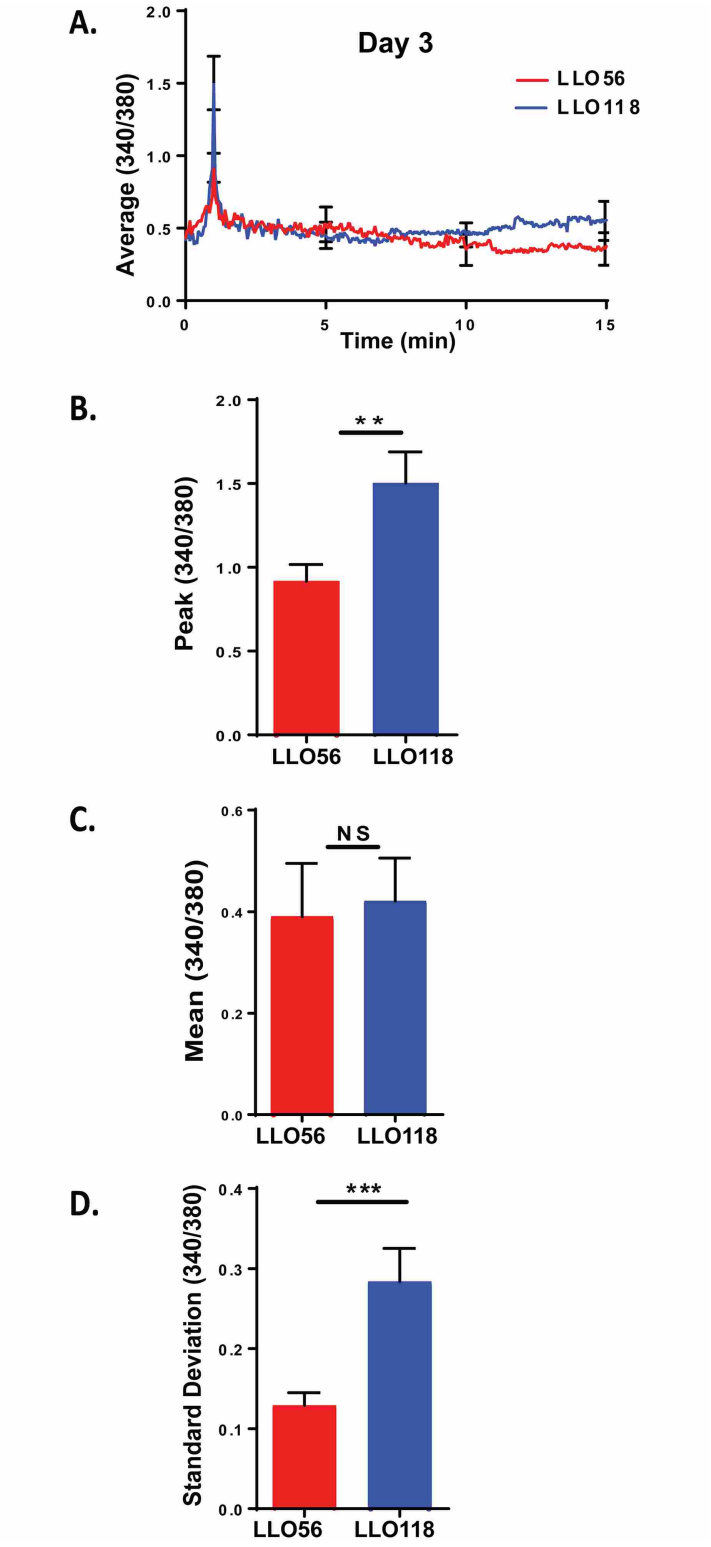

Fig 3. LLO118 helper T cells have higher calcium signaling on day 3 post stimulation. LLO56 and LLO118 splenocytes were isolated and cultured with the $LLO_{190\text{-}205}$ peptide from *L. monocytogenes* for 72 hours *in vitro*. 24 hours before live imaging, a second set of splenocytes were isolated and cultured in an 8-chamber slide loaded with $LLO_{190\text{-}205}$ peptide of *L. monocytogenes* for use as antigen presenting cells. T cells were stained with Fura-2AM, added to the antigen presenting cells and Ca^{2+} influx was measured. **A.**

Average curves of intracellular Ca^{2+} mobilization from stimulated LLO56 and LLO118 splenocytes (340/380 ratios; n = 30) on day 3 post stimulation. Error bars show the SEM at the influx peak. **B.** Statistical analysis of peak calcium influx of stimulated LLO56 and LLO118 naïve T helper cells (n = 30^{+}). **C.** Statistical analysis of the sustained intracellular Ca^{2+} levels (Average 340/380 values between minutes 5 and 20) after initial stimulation response (n = 30^{+}). **D.** Standard deviation was determined by linear regression analysis and shows variability in the calcium signal for each group (n = 30^{+}). (** = p < .01; *** = p < .001; NS = not significant).

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LLO56 and LLO118 have similar in vitro calcium responses on day 8 post stimulation

To further characterize the LLO118 and LLO56 Ca^{2+} response, we isolated splenocytes and co-cultured them with the *L. monocytogenes* peptide for 8 days. On day 8 post-stimulation, the average Ca^{2+} profiles were similar to each other (Fig 4A). Upon evaluation, there were no significant differences between LLO118 and LLO56 T cells in calcium peak, mean, or standard deviation on day 8 post-stimulation (Fig 4B–4D).

Calcium flow cytometry data correlates with calcium microscopy data

To confirm the results obtained in live cell calcium microscopy and evaluate the role of TCR independent calcium signaling, we isolated LLO56 and LLO118 T cells and measured Ca^{2+} mobilization using flow cytometry. Cells were labeled with Flou-4AM and stimulated with PMA and ionomycin. Flou-4AM fluorescence was examined before and after stimulation. Measurements were collected for naïve T helper cells, day 3, and day 8 post-stimulated T cells. The data was consistent with our previous live cell imaging findings in which naïve LLO56 T helper cells and day 3 post-stimulated LLO118 T cells had higher Ca^{2+} mobilization compared to their counterparts while no calcium mobilization differences were seen at day 8 between LLO118 and LLO56 T cells (Fig 5A–5C and Table 2). Collectively, these data show CD5 expression levels and calcium signaling changes over the course of a primary response in LLO118 and LLO56 T cells (Table 2, Figs 2 and 5). Our live cell microscopy calcium imaging and flow cytometry calcium analysis differ in the parameters measured and the stimulation used (cells were stimulated in a TCR-dependent manner for live cell calcium imaging and in a TCR-independent manner for flow cytometry analysis). This calcium data is consistent with the TCR independent cytokine production differences between LLO118 and LLO56 identified by Persaud et al, in which they demonstrated that the LLO118 and LLO56 naive T cell response was set during thymic selection. Naive LLO56 T cells have higher expression of CD5, suggesting increased affinity for self-peptide, and produce higher levels of IL-2 even when stimulated in a TCR independent manner [26].

CD5 expression in naïve LLO56 T helper cells is correlated with higher Ca^{2+} mobilization

To further investigate the role CD5 expression plays in Ca^{2+} mobilization, we measured the calcium signal in T cells from LLO118-CD5 knockout and LLO56-CD5 knockout mice. We found in the LLO118 T cells (CD5-low) that calcium mobilization was not significantly different from LLO118-CD5 knockout T cells at any of the three time points (Fig 6A–6C). Conversely, naïve LLO56-CD5 knockout T helper cells had significantly lower calcium levels compared to the naïve LLO56 T cells (CD5-high) (Fig 6D). There was no calcium mobilization difference between LLO56 and LLO56-CD5 knockout T cells at day 3 or day 8 post-stimulation (Fig 6E and 6F). Thus, in naïve LLO118 T cells (CD5-low), CD5 does not appear to play a strong role in regulating calcium mobilization at any of the time points. However, CD5

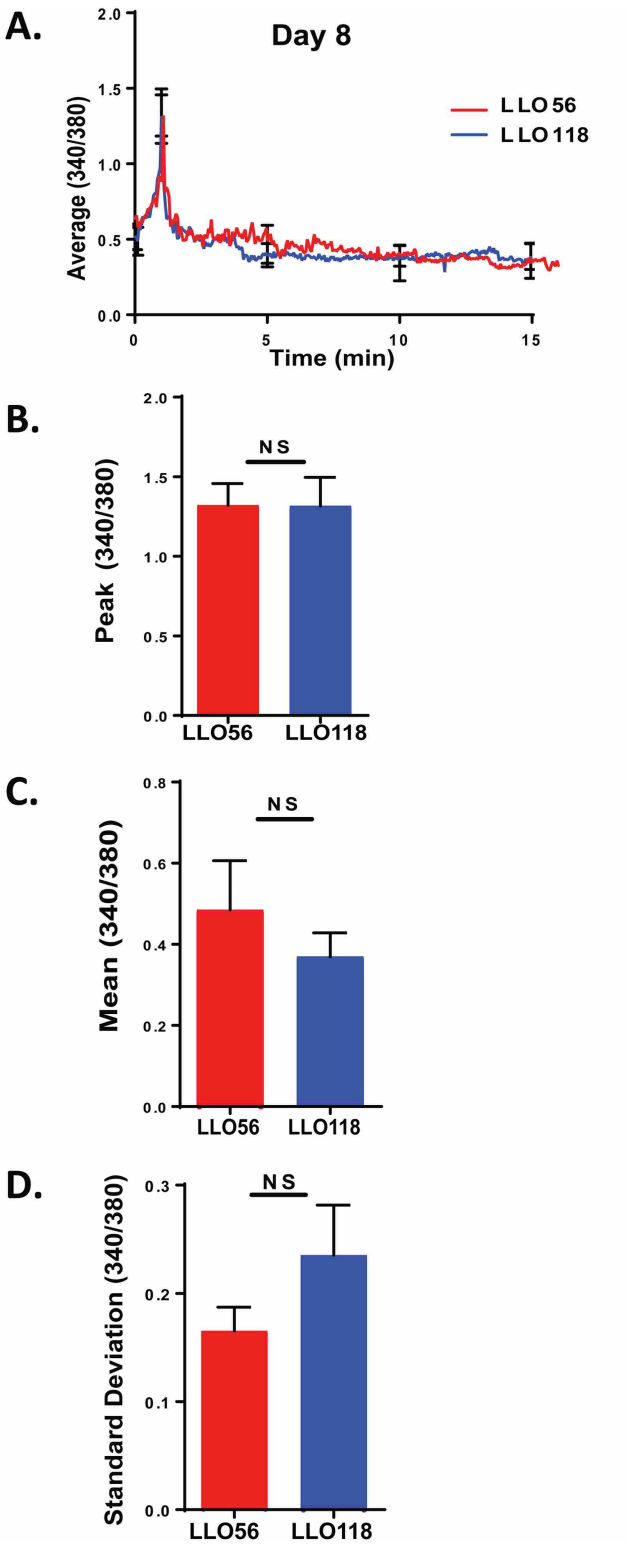

Fig 4. No calcium differences between LLO56 and LLO118 on day 8 post stimulation. LLO56 and LLO118 transgenic splenocytes were isolated and cultured with the $LLO_{190\text{-}205}$ peptide for a week. 24 hours before live imaging, a second set of splenocytes were isolated and cultured in an 8-chamber slide loaded with $LLO_{190\text{-}205}$ peptide of *L. monocytogenes* for use as antigen presenting cells. 8 days stimulated T cells were stained with Fura-2AM and Ca^{2+} influx was measured using live imaging microscopy. **A.** Average curves of

intracellular Ca^{2+} mobilization from stimulated LLO56 and LLO118 splenocytes (340/380 ratios; n = 30) on day 8 post stimulation. Error bars show the SEM at the influx peak. **B.** Statistical analysis of peak calcium influx of stimulated LLO56 and LLO118 naïve T helper cells (n = 30^{+}). **C.** Statistical analysis of the sustained intracellular Ca^{2+} levels (Average 340/380 values between minutes 5 and 20) after initial stimulation response (n = 30^{+}). **D.** Standard deviation was determined by linear regression analysis and shows variability in the calcium signal for each group (n = 30^{+}). (NS = not significant).

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expression is important in regulating calcium mobilization in the naïve LLO56 T cells (CD5-high) during the initial response to antigen, but as CD5 levels decrease over time, its role in regulating calcium also decreases.

Discussion

In this study, we examined the role of CD5 in regulating T cell activation during a primary response using two T helper cells, LLO56 and LLO118, which bind to the same *L. monocytogenes* epitope and have different levels of CD5 on the surface upon completion of thymic development [25, 26]. Because of the described negative regulatory role of CD5 in the thymus and the prevalence of CD5-high cells in memory cells, we wondered how CD5 influences T cell immune response at a post-thymic level. We found significantly different Ca^{2+} signaling levels between LLO56 and LLO118 T helper cells at the naïve and day 3 time points. The distinct Ca^{2+} mobilization patterns of LLO56 and LLO118 likely influence their particular responses to antigen, similar to observations made in B cells in which unique Ca^{2+} mobilization controls distinct B cell activation phenotypes [20, 41]. Previous work has defined the important role of CD5 during T cell thymic development and that CD5-high cells are enriched in memory T cell populations, but how CD5 functions during the primary response stage of T helper cells has not been well defined. Here we characterized the role of CD5 expression and calcium mobilization in these CD5-high and CD5-low T cells over the course of 8 days. We found that naïve LLO56 T helper cells (CD5-high) have significantly higher calcium mobilization compared to the LLO56-CD5 knockout T cells, but at later time points the removal of CD5 did not

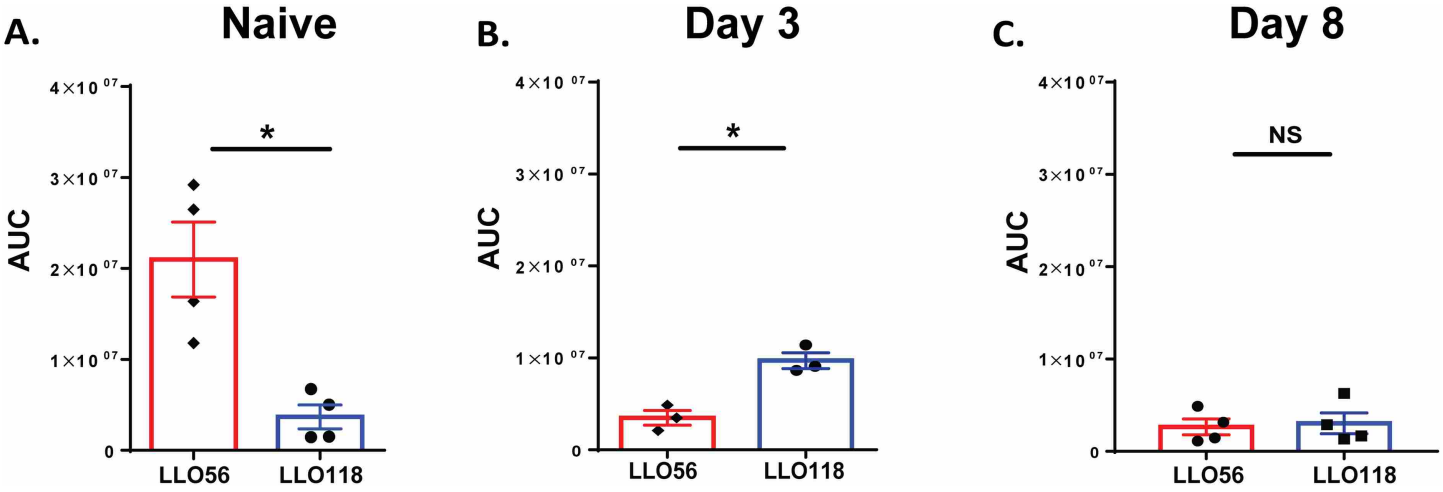

Fig 5. Flow cytometry calcium analysis confirms improved calcium mobilization for naïve LLO56 T cells and higher calcium mobilization for LLO118 T cells at day 3 post-stimulation. LLO56 and LLO118 splenocytes were isolated and cultured at different time points with the $LLO_{190\text{-}205}$ peptide. Calcium levels were quantified using the FlowJo kinetics tool to determine the area under the curve (AUC) for each sample. Calcium mobilization levels for LLO118 and LLO56 are quantified (mean ± SEM of the area under the curve). **A.** Statistical analysis of naïve LLO118 and LLO56 T helper cell calcium mobilization after activation with PMA and Ionomycin. **B.** Statistical analysis of day 3 post stimulated LLO118 and LLO56 calcium mobilization and **C.** Statistical analysis of day 8 post stimulated LLO118 and LLO56 calcium mobilization. (NS = not significant).

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Table 2. Summary of CD5 and calcium findings for LLO56 and LLO118.

	LLO56		LLO118	
	CD5 Expression	Ca^{2+} influx	CD5 Expression	Ca^{2+} mobilization
Naïve T cell	+++	+++	+	+
Day 3	++	++	+	+++
Day 8	++	++	+	++

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significantly alter LLO56 calcium mobilization. Naïve LLO118 T helper cells (CD5-low) exhibit no differences in Ca^{2+} mobilization relative to their CD5 knockout counterpart. Thus, we found naive CD5-high T cells have improved calcium mobilization to an antigen they have never seen before.

T cell development shapes the T cell population by removing strongly self-reactive cells and helping determine future immune responses. T cells that are moderately self-reactive may be

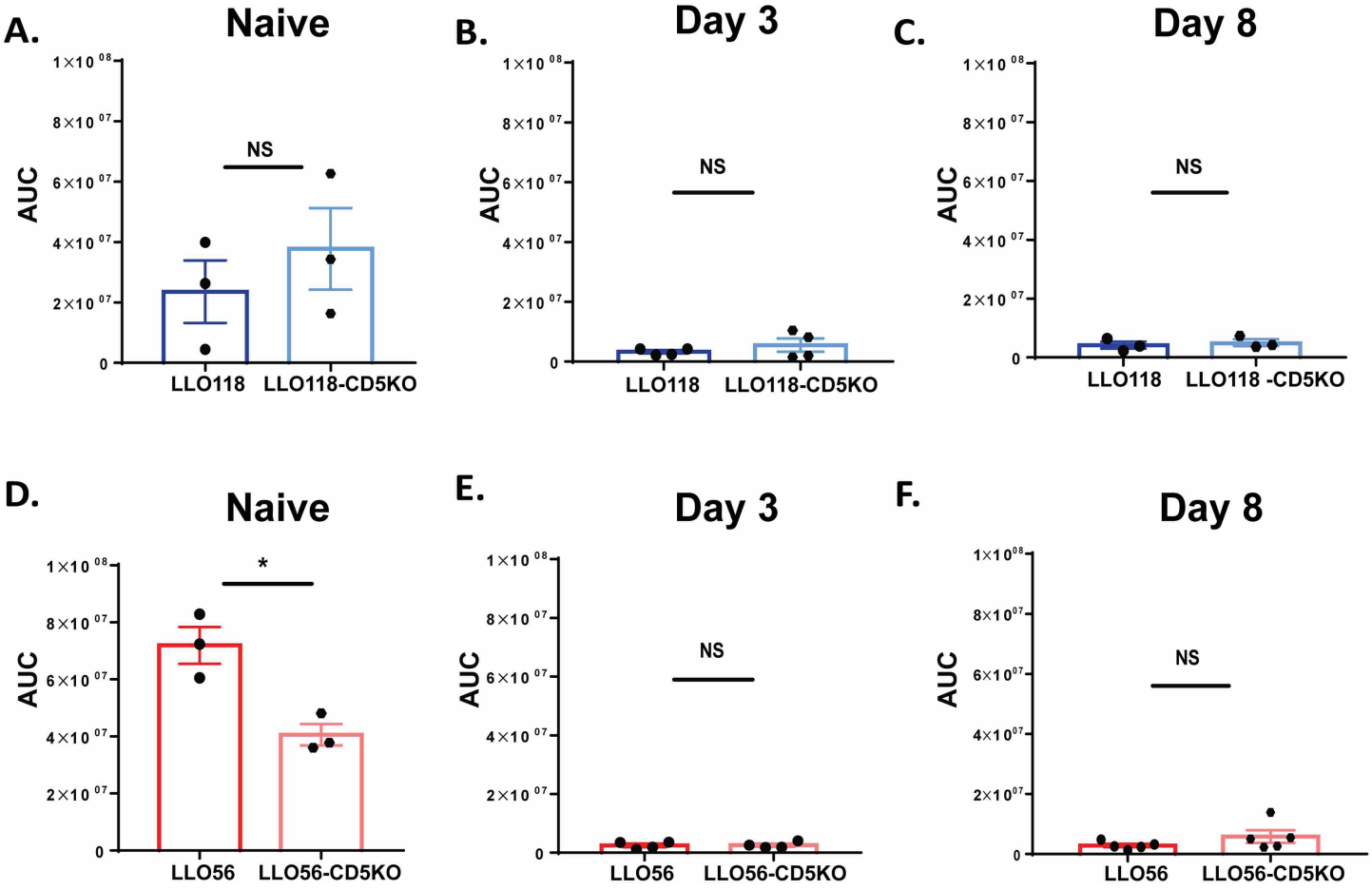

Fig 6. CD5 expression in naïve LLO56 T helper cells is correlated with higher Ca^{2+} mobilization. Flow cytometry analysis was performed to determine Ca^{2+} mobilization levels in LLO56, LLO118, LLO56-CD5 knockout and LLO118-CD5 knockout T cells stimulated with the *L. monocytogenes* peptide (naïve, day 3, and day 8 time points). Calcium levels were quantified using the FlowJo kinetics tool to determine the area under the curve (AUC) for each sample (mean ± SEM). **A-C.** Statistical analysis of calcium mobilization of LLO118 and LLO118-CD5 knockout T cells stimulated with PMA/Ionomycin. Data is shown for naive (**A**), day 3 post stimulation (**B**) and day 8 post stimulation (**C**). **D-F.** Statistical analysis of calcium mobilization of naïve LLO56 and LLO56-CD5 knockout T cells stimulated with PMA/Ionomycin. Data is shown for naive (**D**), day 3 post stimulation (**E**) and day 8 post stimulation (**F**). (* = $p < .05$; NS = not significant).

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able to pass positive selection and evade negative selection and circulate in the periphery. These self-reactive cells, marked by high levels of CD5, appear to be primed to be the best responders to foreign antigens [26]. CD5 is a known negative regulator of TCR signaling during thymocyte development and its expression is correlated to the relationship of TCR avidity for self-pMHC [25, 26, 28, 42]. Analysis in thymocytes showed that LLO56 and LLO118 CD5 knockout T cells had increased p-ERK and IL-2 production, providing additional evidence that CD5 has a negative regulatory effect in developing thymocytes [26]. However, additional work has demonstrated that CD5-high and CD5-low T cells respond differently to self and foreign antigens, suggesting that CD5 has an important role in thymocyte selection and peripheral T cell function and fate [28, 29].

Recent studies suggest that developing T cell CD5 levels affect naïve T cell responses to foreign antigens in the periphery [15, 43]. While the negative regulatory function of CD5 in the thymus does not appear to be dependent upon engagement with a ligand, the positive co-stimulatory effect of CD5 in the periphery is likely due to CD5 engagement of a ligand (CD5 or CD5L) [30–33]. As previously reported, anti-CD5 antibodies enhance TCR-mediated activation and proliferation in peripheral T cells [29, 44, 45]. This calcium difference observed in naïve T cells is supportive of the previously published finding that LLO56 T cells have significantly higher phosphorylation levels of pERK and production of IL-2 before exposure to antigen, suggesting a role for self-peptide affinity in altering CD5 levels and naive T cell responses. CD5-high T cells respond strongly upon stimulation *in vitro* and have increased IL-2 secretion and greater Erk phosphorylation compared to CD5-low T cells [26]. Since CD5 expression is set by self-peptide reactivity in the thymus, our finding that naïve CD5-high LLO56 T cells have higher calcium influx is consistent with other studies that have shown that increased reactivity to self-peptides results in T cells with improved reactivity to foreign antigens [14, 28, 46].

We found that CD5 expression plays an important role in intracellular Ca^{2+} mobilization for naïve LLO56 helper T cells (CD5-high). CD5-high T cells have stronger avidity for self-peptide. It has been suggested that the enhanced activation response to foreign pathogens of CD5-high T cells could be due to their ability to more efficiently use self-peptide as a co-agonist peptide in the periphery [15]. Additionally, CD5-high cells have better basal TCR signaling and improved functional characteristics which correlate with better response to foreign peptide [28]. Studies in naïve cytotoxic T cells suggest that the gene expression profile of CD5-high T cells transcriptionally engage into proliferative and effector functions faster than CD5-low T cells [46]. Furthermore, CD5 appears to help with CD5-high naïve T cell survival after antigen recognition [47]. In fact, T cells with high CD5 levels may maintain diversity within the memory population, which may outweigh the cost of increased self-reactivity [15]. Additionally, T cells with high CD5 expression are enriched in memory cell populations, suggesting that when designing vaccines, CD5 levels and self-peptide and foreign peptide interactions are an important consideration [14, 15].

The data presented here helps to elucidate the role that CD5 plays in regulating calcium signaling in naïve cells early after cell activation during an *in vitro* primary response. We plan to further investigate whether the unique Ca^{2+} profiles of LLO56 and LLO118 T cells are consistent in an *in vivo* model and further quantify the role CD5 plays in effector and memory T cells. These future studies will help elucidate how CD5 influences naïve T cell responses and its potential role in memory T cell generation and maintenance.

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Author Contributions

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Formal analysis: KSW CTF.

Funding acquisition: KSW.

Investigation: CTF CMR GJH.

Methodology: KSW CTF.

Project administration: KSW.

Resources: KSW.

Software: KSW.

Supervision: KSW CTF.

Validation: CTF CMR GJH.

Visualization: KSW CTF.

Writing – original draft: CTF.

Writing – review & editing: KSW CTF.

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Review

T Cell Calcium Signaling Regulation by the Co-Receptor CD5

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Abstract: Calcium influx is critical for T cell effector function and fate. T cells are activated when T cell receptors (TCRs) engage peptides presented by antigen-presenting cells (APC), causing an increase of intracellular calcium (Ca^{2+}) concentration. Co-receptors stabilize interactions between the TCR and its ligand, the peptide-major histocompatibility complex (pMHC), and enhance Ca^{2+} signaling and T cell activation. Conversely, some co-receptors can dampen Ca^{2+} signaling and inhibit T cell activation. Immune checkpoint therapies block inhibitory co-receptors, such as cytotoxic T-lymphocyte associated antigen 4 (CTLA-4) and programmed death 1 (PD-1), to increase T cell Ca^{2+} signaling and promote T cell survival. Similar to CTLA-4 and PD-1, the co-receptor CD5 has been known to act as a negative regulator of T cell activation and to alter Ca^{2+} signaling and T cell function. Though much is known about the role of CD5 in B cells, recent research has expanded our understanding of CD5 function in T cells. Here we review these recent findings and discuss how our improved understanding of CD5 Ca^{2+} signaling regulation could be useful for basic and clinical research.

Keywords: calcium signaling; T cell receptor (TCR); co-receptors; CD-5; PD-1; CTL-4

1. Introduction

T cells are a critical component of the adaptive immune system. T cell responses are influenced by signals that modulate the effects of the T cell receptor (TCR) and peptide-major histocompatibility complex (pMHC) interaction and initiate the transcription of genes involved in cytokine production, proliferation, and differentiation [1–3]. T cell activation requires multiple signals. First, the TCR engages the pMHC leading to tyrosine phosphorylation of CD3 and initiation of the Ca^{2+}/Calcineurin/Nuclear factor of activated T cells (NFAT) or Protein kinase C-theta (PKCθ)/Nuclear factor-κ-light chain enhancer of activated B cells (NF-κB) or Mitogen-activated protein kinase (MAP kinase)/AP-1 pathways [4–6]. Second, cell surface costimulatory molecules, such as co-receptor CD28, amplify TCR-pMHC complex signals and promote stronger intracellular interactions to prevent T cell anergy [7,8]. Finally, cytokines such as interleukin-12 (IL-12), interferon α (INFα), and interleukin-1 (IL-1) promote T cell proliferation, differentiation, and effector functions [6].

Co-receptors such as CD4 and CD8 interact with MHC molecules and additional co-receptors interact with surface ligands present on antigen-presenting cells (APCs) to regulate T cell homeostasis, survival, and effector functions with stimulatory or inhibitory signals [9]. Altering co-receptor levels, balance, or function dramatically affects immune responses and their dysfunction is implicated in autoimmune diseases [10]. Stimulatory co-receptors such as CD28, inducible T cell co-stimulator (ICOS), Tumor necrosis factor receptor superfamily member 9 (TNFRSF9 or 4-1BB), member of the TNR-superfamily receptor (CD134 or OX40), glucocorticoid-induced tumor necrosis factor (TNF) receptor (GITR), CD137, and CD77 promote T cell activation and protective responses [11]. Co-receptor

signaling is initiated by the phosphorylation of tyrosine residues located in immunoreceptor tyrosine-based activation motifs (ITAMs) or immunoreceptor tyrosine-based inhibitory motifs (ITIMs) [7,12]. The phosphorylated tyrosines serve as docking sites for spleen tyrosine kinase (Syk) family members such as zeta-chain-associated protein kinase 10 (ZAP-70) and Syk which activate the phospholipase C γ (PLCγ), RAS, and extracellular signal-regulated kinase (ERK) pathways in addition to mobilizing intracellular Ca^{2+} stores [13].

One of the best described T cell co-receptors, CD28, is a stimulatory T cell surface receptor from the Ig superfamily with a single Ig variable-like domain which binds to B7-1 (CD80) and B7-2 (CD86) [2]. Ligand binding phosphorylates CD28 cytoplasmic domain tyrosine motifs such as YMNM and PYAP and initiates binding and activation of phosphatidylinositide 3 kinase (PI3K) which interacts with protein kinase B (Akt) and promotes T cell proliferation and survival [1]. CD28 also activates the NFAT pathway and mobilizes intracellular Ca^{2+} stores through association with growth factor receptor-bound protein 2 (GRB2) and the production of phosphatidylinositol 4,5-bisphosphate (PIP2), the substrate of PLCγ1, respectively [2,14]. Blocking stimulatory co-receptors suppresses T cell effector function. For example, blocking stimulatory CD28 with anti-CD28 antibodies promotes regulatory T cell function and represses activation of auto- and allo-reactive T effector cells after organ transplantation [8,15].

T cells also have inhibitory co-receptors which regulate T cell responses [8]. The best characterized are immunoglobulin (Ig) superfamily members cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed cell death protein 1 (PD-1) [8,16]. CTLA-4 binds CD80 and CD86 with greater avidity than CD28, and its inhibitory role refines early phase activation signals for proliferation and cytokine production [16–19]. PD-1, another CD28/B7 family member, regulates late phase effector and memory response [20]. Inhibitory co-receptors such as CTLA-4 and PD-1, known as "immune checkpoints", block the interaction between CD28 and its ligands altering downstream secondary T cell activation signals [19]. Therefore, blocking CTLA-4 or PD-1 promotes effector T cell function in immunosuppressive environments [19,21].

There are also a number of co-receptors that have differential modulatory properties. For example, CD5, a lymphocyte glycoprotein expressed on thymocytes and all mature T cells, has contradictory roles at different time points. CD5 expression is set during thymocyte development and decreases the perceived strength of TCR-pMHC signaling in naïve T cells by clustering at the TCR-pMHC complex and reducing TCR downstream signals such as the Ca^{2+} response when its cytoplasmic pseudo-ITAM domain is phosphorylated [22–25]. The CD5 cytoplasmic domain has four tyrosine residues (Y378, Y429, Y411, and Y463), and residues Y429 and Y441 are found in a YSQP-(x8)-YPAL pseudo ITAM motif while other tyrosine residues make up a pseudo-ITIM domain [23]. Phosphorylated tyrosines recruit several effector molecules and may sequester activation kinases away from the TCR complex, effectively reducing activation signaling strength [23]. Recruited proteins include Src homology-2 protein phosphatase-1 (SHP-1), Ras GTPase protein (rasGAP), CBL, casein kinase II (CK2), zeta-chain-associated protein kinase 70 (ZAP70), and PI3K which are involved in regulating both positive and negative TCR-induced responses [26–28]. For example, ZAP-70 phosphorylates other substrates and eventually recruits effector molecules such as PLC gamma and promotes Ca^{2+} signaling and Ras activation which stimulates the ERK pathway and leads to cellular activation [13,29]. Conversely, SHP1 inhibits Ca^{2+} signaling and PKC activation via decreased tyrosine phosphorylation of PLCγ [13,26,30,31]. Further, Y463 serves as a docking site for c-Cb1, a ubiquitin ligase, which is phosphorylated upon CD3–CD5 ligation and leads to increased ubiquitylation and lysosomal/proteasomal degradation of TCR downstream signaling effectors and CD5 itself [32]. Thus, CD5 has a mix of downstream effects that both promote and inhibit T cell activation. Curiously, recent work suggests that in contrast to its initial inhibitory nature, CD5 also co-stimulates resting and mature T cells by augmenting CD3-mediated signaling [25,33–35].

Ca^{2+} is an important second messenger in many cells types, including lymphocytes, and plays a key role in shaping immune responses. In naïve T cells, intracellular Ca^{2+} is maintained at low levels, but when TCR-pMHC complexes are formed, inositol triphosphate (IP3) initiates Ca^{2+} release

from intracellular stores of the endoplasmic reticulum (ER) which opens the Ca^{2+} release-activated Ca^{2+} channels (CRAC) and initiates influx of extracellular Ca^{2+} through store-operated Ca^{2+} entry (SOCE) [36–41]. The resulting elevation of intracellular Ca^{2+} levels activates transcription factors involved in T cell proliferation, differentiation, and cytokine production (e.g., nuclear factor of activated cells (NFAT)) [36,37]. Thus, impaired Ca^{2+} mobilization affects T cell development, activation, differentiation, and function [42,43]. Examples of diseases with impaired Ca^{2+} signaling in T cells include systemic lupus erythematosus, type 1 diabetes mellitus, and others [44,45].

In this review, we will focus on CD5 co-receptor signaling and its functional effects on T cell activation. First, we will discuss how the inhibitory co-receptors CTLA-4 and PD-1 modulate T cell function. Then we will compare CTLA-4 and PD-1 function to CD5 function, examine recent findings that expand our understanding of the role of CD5, and assess how these findings apply to T cell Ca^{2+} signaling. Finally, we will consider CD5 Ca^{2+} signaling regulation in T cells and its potential physiological impact on immunometabolism, cell differentiation, homeostasis, and behavior.

2. Roles of Negative Regulatory T Cell Co-Receptors

2.1. Cytotoxic T-Lymphocyte Antigen-4 (CTLA-4)

Cytotoxic T-lymphocyte antigen-4 (CTLA-4, CD152) inhibits early stages of T cell activation by recruiting inhibitory proteins such as SHP-2 and type II serine/threonine phosphatase PP2A that interfere with T cell synapse signaling [21,46–48]. CTLA-4 binds B7, a protein on activated APCs, with higher affinity than the stimulatory co-receptor CD28; the resulting balance between inhibitory and stimulatory signals controls T cell activation or anergy [19,49]. In naïve T cells, CTLA-4 is located in intracellular vesicles which localize at TCR binding sites following antigen recognition and intracellular Ca^{2+} mobilization [19,50]. Like CD28, CTLA-4 aggregates to the central supramolecular activation complex (cSMAC) where it then extrinsically controls activation by decreasing immunological synapse contact time [51–53]. This suppresses proactivation signals by activating ligands (B7-1 and B7-2) and induces the enzyme Inoleamine 2,3-dioxygenase (IDO) which impairs Ca^{2+} mobilization and suppresses T cell activation, ultimately altering IL-2 production and other effector functions in T cells [51,54,55]. CTLA-4 also stimulates production of regulatory cytokines, such as transforming growth factor beta (TGF-β), which inhibit APC presentation and T cell effector function [47,52,53]. Compared to effector T cells (T_{eff}), CTLA-4 is highly expressed in regulatory T cells (T_{reg}) and plays a role in maintaining T_{reg} homeostasis, proliferation, and immune responses [16,56,57]. Total or partial CTLA-4 deficiency inhibits T_{reg}'s ability to control cytokine production and can cause immune dysregulation [58–61]. Thus, CTLA-4 has an important role in the T_{reg} suppressive response [60]. Additionally, CTLA-4 mutations are associated with autoimmune diseases as thoroughly reviewed by Kristiansen et al. [62].

The loss of CTLA-4 results in removal of CTLA-4 competition with CD28 for B7-1 and B7-2 and is implicated in autoimmunity and cancer [15,63]. Because CTLA-4 inhibits TCR signaling, CTLA-4 deficiency leads to T cell overactivation as measured by increased CD3ζ phosphorylation and Ca^{2+} mobilization [64]. Thus, modulating CTLA-4 signaling is an attractive target for immunotherapies that seek to boost or impair early TCR signaling for cancer and autoinflammatory diseases [65,66]. For example, Ipilimunab, an IgG1 antibody-based melanoma treatment, is a T cell potentiator that blocks CTLA-4 to stimulate T cell proliferation and stem malignant disease progression by delaying tumor progression and has been shown to significantly increase life expectancy [19,67,68]. Additionally, Tremelimumab, a noncomplement fixing IgG2 antibody, has been tested alone or in combination with other antibodies such as Durvalumab (a PD-1 inhibitor) and improves antitumor activity in patients with non-small cell lung cancer (NSCLC), melanoma, colon cancer, gastric cancer, and mesothelioma treatment [69–74].

2.2. Programmed Death 1 (PD-1)

Programmed cell death protein-1 (PD-1, CD279) is a 288-amino acid (50–55 KDa) type I transmembrane protein and a member of the B7/CD28 immunoglobulin superfamily expressed

on activated T cells, B cells, and myeloid cells [19,75,76]. PD-1 has two known ligands, PD-L1 and PD-L2, which inhibit T cell activation signals [77]. Like CTLA-4, PD-1 also inhibits T cell proliferation and cytokine production (INF-γ, TNF and IL-2) but is expressed at a later phase of T cell activation [19]. PD-1 has an extracellular single immunoglobulin (Ig) superfamily domain and a cytoplasmic domain containing an ITIM and an immunoreceptor tyrosine-based switch motif (ITSM) subunit critical for PD-1 inhibitory function [78]. Upon T cell activation, PD-1 is upregulated and initiates ITIM and ITSM tyrosine interaction with SHP-2 which mediates TCR signaling inhibition by decreasing ERK phosphorylation and intracellular Ca^{2+} mobilization [79,80]. PD-1 can block the activation signaling pathways PI3K-Akt and Ras-Mek-ERK, which inhibit or regulate T cell activation [79,81]. Thus, engagement of PD-1 by its ligand affects intracellular Ca^{2+} mobilization, IL-2 and TNF-α production, supporting PD-1's inhibitory role in TCR strength-mediated signals [82].

PD-1 signaling also affects regulatory T cell (T_{reg}) homeostasis, expansion, and function [83]. T_{reg} activation and proliferation are impacted by PD-1 expression which enhances their development and function while inhibiting T effector cells [75,84]. PD-1, PD-L, and T_{regs} help terminate immune responses [85]. Thus, PD-1 deficiency results not only in increased T cell activation, but in the breakdown of tolerance and the development of autoimmunity in diseases such as multiple sclerosis and systemic lupus erythematosus [85–89]. PD-1 and its ligands protect tissues from autoimmune attacks by regulating T cell activation and inducing and maintaining peripheral tolerance [90,91]. Studies done in PD-1-deficient mice observed the development of lupus-like glomerulonephritis and arthritis, cardiomyopathy, autoimmune hydronephrosis, and Type I diabetes, among other ailments [92–94]. PD-1 protects against autoimmunity and promotes T_{reg} function. [85]. Enhancing T_{reg} response with a PD-L1 agonist shows therapeutic potential for asthma and other autoimmune disorders [85,95]. Because PD-1 specifically modulates lymphocyte function, effective FDA-approved monoclonal antibodies targeting PD-1 are clinically available (i.e., Pembrolizumab and Nivolumab) to treat advanced malignancies [20]. Not only does blocking PD-1 decrease immunotolerance of tumor cells, it also increases cytotoxic T lymphocyte antitumor activity [20].

3. CD5: A Contradictory Co-Receptor

3.1. Overview of CD5 Signaling and Ca^{2+} Mobilization in T Cells

CD5, known as Ly-1 antigen in mice or as Leu-1 in humans, is a type I transmembrane glycoprotein (67 kDa) expressed on the surface of thymocytes, mature T cells, and a subset of B cells (B-1a) [96,97]. Although CD5 was discovered over 30 years ago, it was only in the last decade that CD5 gained attention as a key T cell activation regulator [98,99]. CD5 expression is set in the thymus during positive selection and correlates with how tightly the thymocyte TCR binds to self-peptide-MHC (self-pMHC); greater TCR affinity for self-peptide leads to increased CD5 expression in double positive (DP) thymocytes [100]. In other words, DP thymocytes that receive strong activation signals through their TCR express more CD5 than those DP thymocytes that receive weak TCR signals [100]. CD5 knockout mice ($CD5^{-/-}$) have a defective negative and positive selection process, and therefore their thymocytes are hyper-responsive to TCR stimulation with increased Ca^{2+} mobilization, proliferation, and cytokine production [23,98]. On the other hand, because of the increased TCR avidity for self-pMHC, mature T cells with high CD5 expression ($CD5^{hi}$) (peripheral or postpositive selection T cells) respond to foreign peptide with increased survival and activation compared to mature T cells with low CD5 expression ($CD5^{lo}$) [34,101]. Therefore, CD5 is a negative regulator of TCR signaling in the thymus and modulates mature T cell response in the periphery [23,34,100,102].

While CTLA-4 and PD-1 belong to the immunoglobulin (Ig) family, CD5 belongs to group B of the scavenger receptor cysteine-rich (SRCR) superfamily and contains three extracellular SRCR domains [30,96,103]. The cytoplasmic tail of CD5 contains several tyrosine residues which mediate the negative regulatory activity independent of extracellular engagement [100,104,105]. As CD5 physically associates with TCRζ/CD3 complex upon TCR and pMHC interaction, the tyrosine residues in both

TCRζ and CD5 are phosphorylated by tyrosine kinases associated with the complex [30,106–110]. This interaction is so intrinsic to T cell signaling that CD5 expression levels are proportional to the degree of TCRζ phosphorylation, IL-2 production capacity, and ERK phosphorylation which are critical for CD3-mediated signaling [33,111]. It is unknown whether posttranslational modifications, such as conserved domain 1 and domain 2 glycosylations, impact CD5 signaling [112,113]. CD5 is present in membrane lipids rafts of mature T cells where, upon activation, it helps augment TCR signaling, increases Ca^{2+} mobilization, and upregulates ZAP-70/LAT (linker for activation of T cells) activation [114–116]. This suggests that CD5 is not only a negative regulator in thymocytes, but also appears to positively influence T cell immune response to foreign antigens [117,118]. See Figure 1.

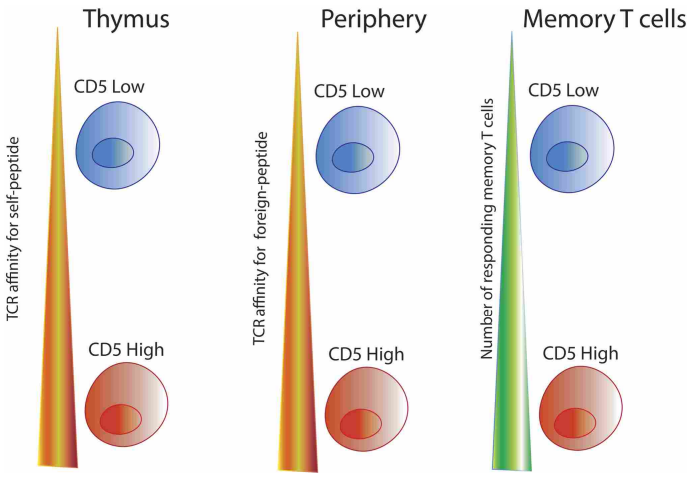

Figure 1. Effects of CD5 on different stages of T cell development. CD5 expression on thymocytes is directly proportional to the signaling intensity of the TCR:self-pMHC interaction. In the periphery, T cells with higher CD5 levels ($CD5^{hi}$) are better responders to foreign-peptide. Long-lived memory cells populations are enriched for $CD5^{hi}$ T cells [34,102,119].

CD5 has three known ligands: CD72, a glycoprotein expressed by B cells, CD5 ligand or CD5L, an activation antigen expressed on splenocytes, and CD5 itself [120–122]. Crosslinking CD5L to CD5 increases intracellular Ca^{2+} concentrations [30,120,121,123,124]. Early studies with anti-CD5 monoclonal antibodies also demonstrated enhanced Ca^{2+} mobilization and proliferation, suggesting that CD5 co-stimulates and increases the T cell activation signal [125,126]. Following TCR:pMHC interaction, CD5 cytoplasmic ITAM and ITIM like-domains are phosphorylated by p56lck and bound by Src homology 2 (SH2) domain-containing protein tyrosine phosphatase (SHP-1) [108,127,128]. However, while SHP-1 affects Ca^{2+} mobilization and is a purported down-regulator of thymocyte activation, recent findings suggest that SHP-1 is not necessary for CD5 signaling as T cells deficient in SHP-1 have normal CD5 expression and continue to signal normally [26,129]. Thus, while CD5 is not a SHP-1 substrate and SHP-1 is likely unnecessary for CD5 signaling, CD5 signaling results in increased Ca^{2+} mobilization. It has yet to be resolved how CD5 can act as an inhibiting co-receptor in the thymus and as an activating co-receptor in the periphery.

3.2. CD5 as a Ca^{2+} Signaling Modulator

As previously mentioned, CD5 expression levels are set in the thymus during T cell development and are maintained on peripheral lymphocytes [117]. CD5 expression in T cells plays an important role during development and primes naïve T cells for responsiveness in the periphery [35,111,130]. $CD5^{hi}$ T cells have the highest affinity for self-peptides and respond with increased cytokine production and proliferation to infection [101,131,132].

Our laboratory works with two TCR transgenic mouse lines with different levels of CD5 expression: LLO56 ($CD5^{hi}$) and LLO118 ($CD5^{lo}$) [111,117,130]. While LLO56 ($CD5^{hi}$) and LLO118

($CD5^{lo}$) have similar affinity for the same immunodominant epitope (listeriolysin O amino acids 190–205 or $LLO_{190–205}$) from *Listeria monocytogenes*, on day 7 of primary response, LLO118 ($CD5^{lo}$) has approximately three times the number of responding cells compared to LLO56 ($CD5^{hi}$), and conversely, on day 4 during secondary infection, LLO56 ($CD5^{hi}$) has approximately fifteen times more cells than LLO118 ($CD5^{lo}$) [130]. This difference is not due to differential proliferative capacity, rather LLO56 ($CD5^{hi}$) has higher levels of apoptosis during the primary response [130]. Thus, LLO56 $CD5^{hi}$ and LLO118 $CD5^{lo}$'s capacity to respond to infection appears to be regulated by their CD5 expression levels [117]. LLO56 ($CD5^{hi}$) thymocytes have greater affinity for self-peptide, which primes them to be highly apoptotic [130].

Recently we reported that in response to foreign peptide, LLO56 ($CD5^{hi}$) naïve T cells have higher intracellular Ca^{2+} mobilization than LLO118 ($CD5^{lo}$), which correlates with increased rate of apoptosis of LLO56 ($CD5^{hi}$), as Ca^{2+} overloaded mitochondria release cytochrome c which activates caspase and nuclease enzymes, thus initiating the apoptotic pathways [35,133,134]. LLO56 ($CD5^{hi}$) naïve T cell increased Ca^{2+} mobilization also provides additional support to the idea that $CD5^{hi}$ T cells have an enhanced response to foreign peptide [35,134]. This supports previous research that found that upon T cell activation, increased CD5 expression is correlated with greater basal TCRζ phosphorylation, increased ERK phosphorylation, and more IL-2 production [101,111].

Thus, unlike CTLA-4 and PD-1 which are expressed only on activated T cells in the periphery during early and late phases of immune response, respectively, CD5 is set during T cell development, and influences T cells both during thymic development and during postthymic immune responses [19, 101,111] (see Figure 2). CD5 not only has an important inhibitory role in the thymus, but also appears to positively influence the T cell population response; for example, more $CD5^{hi}$ T cells populate the memory T cell repertoire because $CD5^{hi}$ naïve T cells have a stronger primary response [34,135]. CD5 finetunes the sensitivity of TCR signaling to pMHC, altering intracellular Ca^{2+} mobilization and NFAT transcription, key players in T cell effector function [19,64,126]. As Ca^{2+} signaling plays a key role in T cell activation and function, controlling Ca^{2+} mobilization in T cells through CD5 expression could influence diverse areas of clinical research including metabolism, cancer treatments, and even cognitive behavior.

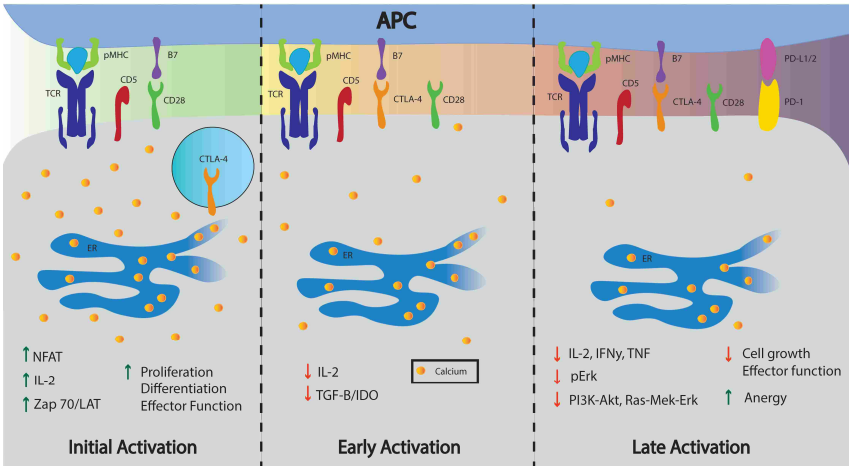

Figure 2. Inhibiting co-receptors modulate T cell activation by increasing (green arrows) or decreasing activity (red arrows). CD5 is present in naïve T cells and localizes to the TCR:pMHC complex during activation. Initial activation cascades signal for the release of CTLA-4 from vesicles to the cell surface while the transcription factor NFAT transcribes PD-1. CTLA-4 provides inhibitory signals during early activation while PD-1 is expressed later and inhibits later stages of T cell activation. The initial Ca^{2+} mobilization is decreased by CTLA-4 and PD-1 downstream signals. A more detailed illustration of the calcium signaling pathway (i.e., IP3, STIM 1/2, CRAC channel, calmodulin, etc.) is outlined in Figure 3.

4. Physiological Impact of CD5 Expression in T Cells

4.1. Metabolism

Naive T cells are in a quiescent state and rely on oxidative phosphorylation (OXPHOS) to generate ATP for survival [136,137]. Upon TCR-pMHC interaction, T cells undergo metabolic reprograming to meet energetic demands by switching from OXPHOS to glycolysis [138]. Glycolysis is a rapid source of ATP and regulates posttranscriptional production of INF-γ, a critical effector cytokine [139]. Following the immune response, most effector T cells undergo apoptosis while a subset become quiescent memory T cells. Memory T cells have lower energetic requirements and rely on OXPHOS and Fatty Acid Oxidation (FAO) to enhance mitochondrial capacity for maintenance and survival [140].

Ca^{2+} signaling is a key second messenger in T cell activation and Ca^{2+} ions also modulate T cell metabolism through CRAC channel activity and NFAT activation [3,141]. During TCR-pMHC binding Ca^{2+} is released from the endoplasmic reticulum (ER) where it is absorbed by the mitochondria and initiates an influx of extracellular Ca^{2+} [3]. First, the rise of cytoplasmic Ca^{2+} activates stromal interaction molecule 1 (STIM1) located on the ER membrane to interact with the CRAC channel located on the cell membrane [142]. The release of the ER store and resulting extracellular Ca^{2+} influx increases the intracellular Ca^{2+} concentration and promotes AMPK (adenosine monophosphates activated protein kinase) expression and CaMKK (calmodulin-dependent protein kinase kinase) activity [3,142,143]. AMPK senses cellular energy levels through the ratio of AMP to ATP and generates ATP by inhibiting ATP-dependent pathways and stimulating catabolic pathways [144]. This indirectly controls T cell fate as AMPK indirectly inhibits mTOR (mammalian target of rapamycin complex) [145]. Because mTOR coordinates the metabolic cues that control T cell homeostasis, it plays a critical role in T cell fate [146]. T cells that are TSC1 (Tuberous sclerosis complex 1)-deficient show metabolic alterations through increased glucose uptake and glycolytic flux [147].

The rise of cytoplasmic Ca^{2+} also encourages mitochondria to uptake cytoplasmic Ca^{2+} through the mitochondrial Ca^{2+} uniporter (MCU) [148]. This MCU uptake increases Ca^{2+} influx by depleting Ca^{2+} near the ER which further activates the CRAC channels and promotes STIM1 oligomerization [3,149–151]. Ca^{2+} uptake in the mitochondria also enhances the function of the tricarboxylic acid cycle (TAC), which generates more ATP through OXPHOS [152,153]. OXPHOS is maintained by a glycolysis product, phosphoenolpyruvate (PEP), which sustains TCR-mediated Ca^{2+}-NFAT signaling by inhibiting the sarcoendoplasmic reticulum (SR) calcium transport ATPase (SERCA) pump, thus promoting T cell effector function [154,155]. Downregulation of calmodulin kinase, CaMKK2, which controls NFAT signaling, decreases glycolytic flux, glucose uptake, and lactate and citrate metabolic processes [156]. Ca^{2+} may also orchestrate the metabolic reprogramming of naïve T cells by promoting glycolysis and OXPHOS through the SOCE/calcineurin pathway which controls the expression of glucose transporters GLUT1/GLUT3 and transcriptional co-regulator proteins important for the expression of electron transport chain complexes required for mitochondria respiration [141].

Co-receptor stimulation plays a pivotal role in T cell metabolism and function. A decrease in T cell Ca^{2+} signaling represses glycolysis and affects T cell effector function [152]. PD-1 and CTLA-4 depress Ca^{2+} signaling and glycolysis while promoting FAO and antibodies against CTLA-4 and PD-1 increase Ca^{2+} mobilization and glycolysis during T cell activation [157,158]. Like CTLA-4 and PD-1, CD5 modulatory function has the potential to influence T cell metabolism. Analysis of gene families modulated by CD5 in B cells found that CD5 upregulates metabolic-related genes including VEFG, Wnt signaling pathways genes, MAPK cascade genes, I-kB/NF-kB cascade genes, TGF β signaling genes, and adipogenesis process genes [159]. Therefore, proliferation differences correlated with CD5 expression in T cells may be caused by improved metabolic function as $CD5^{lo}$ T cells seem to be more quiescent than $CD5^{hi}$ T cells [160]. Although not much is known about how CD5 alters metabolic function in T cells, signaling strength differences of $CD5^{hi}$ and $CD5^{lo}$ T cell populations correlate with intracellular Ca^{2+} mobilization during activation and influence their immune response [35,111,130]. This implies that different metabolic processes may be initiated which would influence proliferation,

memory cell generation, and cytokine production. Figure 3 summarizes how Ca^{2+} may be mobilized in $CD5^{hi}$ and $CD5^{lo}$ naïve T cells and the role Ca^{2+} may play on metabolism.

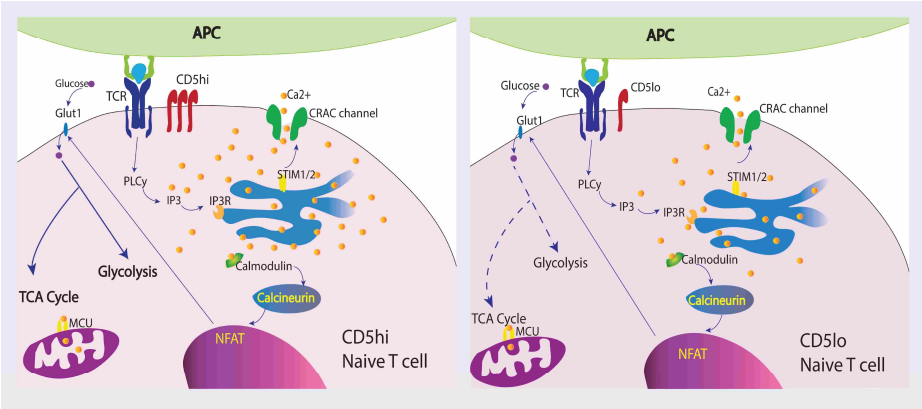

Figure 3. CD5 expression levels in naïve T cells may influence T cell metabolism and function. Differential levels of CD5 result in differences in Ca^{2+} mobilization in naïve T cells. $CD5^{hi}$ naïve T cells have higher Ca^{2+} influx than $CD5^{lo}$ naïve T cells upon TCR:pMHC interaction [35]. Ca^{2+} signaling plays a significant role in T cell activation and influences metabolism and T cell function. Differential Ca^{2+} mobilization and expression of calcineurin and NFAT affect glycolysis and mitochondrial respiration (hypothetical levels of metabolic activation are shown with dashed (low) or solid (high) arrows), suggesting CD5 expression may affect metabolic reprograming during T cell activation [141].

4.2. Neuroimmunology

The field of neuroimmunology examines the interplay between the immune system and the central nervous system (CNS) [161]. The adaptive immune system does influence the CNS as cognition is impaired by the absence of mature T cells [162]. In wild type mice, there is an increase in the number of T cells present in the meninges during the learning process, in stark contrast to mice with T helper 2 cytokine deficiencies (such as IL-4 and IL-13) who have decreased T cell recruitment and impaired learning [163]. Furthermore, regulation of T cell activation and cytokine production critically assists neuronal function and behavior, suggesting that manipulation of T cells could be a potential therapeutic target in treating neuroimmunological diseases [164,165].

T cells go through several microenvironments before reaching the CNS [166]. Many of the signal interactions present in these microenvironments affect T cell function and involve changes in intracellular Ca^{2+} levels [166,167]. In experimental autoimmune encephalitis (EAE), a model for human multiple sclerosis, autoreactive T cells have Ca^{2+} fluctuations throughout their journey to the CNS [166]. Prior to reaching the CNS, T cells interact with splenic stroma cells that do not display the cognate auto-antigen and this interaction produces short-lived low Ca^{2+} mobilization spikes [166]. Following entrance into the CNS, T cells encounter autoantigen-presenting cells and have sustained Ca^{2+} mobilization which results in NFAT translocation and T cell activation [166,168]. EAE mice display reduced social interaction and cognition demonstrating that autoimmune response impairs neuronal function and organismal behavior [169].

Inhibitory T cell co-receptors are implicated in CNS dysregulation and disease. Varicella zoster virus (VZV) infection is characterized by lifelong persistence in neurons. VZV increases the expression of CTLA-4 and PD-1 in infected T cells which reduces IL-2 production and increases T cell anergy [170,171]. PD-1-deficient mice ($Pdcd1^{-/-}$) have increased T cell activation, leading to greater intracellular Ca^{2+} mobilization, and as previously discussed, increased glycolysis [86]. PD-1 deficiency causes elevated concentration of aromatic amino acids in the serum, specifically tryptophan and tyrosine, which decreases their availability in the brain where they are important for the synthesis of neurotransmitters such as dopamine and serotonin; consequently, there is an increase in anxiety-like

behavior and fear in Pdcd1$^{-/-}$ mice [86]. Therefore, increased T cell activation caused by PD-1 deficiency can affect brain function and thus, affects cognitive behavior [86].

4.3. Cancer

T cells are critical components of the immune response to cancer. Helper T cells directly activate killer T cells to eradicate tumors and are essential in generating a strong antitumor response alone or in concert with killer T cells by promoting killer T cell activation, infiltration, persistence, and memory formation [172–177]. Tumor-specific T cells may not mount a robust response towards cancerous cells because the tumor microenvironment has numerous immunosuppressive factors; cancerous cells also downregulate cell surface co-stimulatory and MHC proteins which suppresses T cell activation [178–182]. Potent antitumor immune checkpoint blockade therapies using CTLA-4 and PD-1 monoclonal antibodies augment T cell response by suppressing the co-receptors' inhibitory signals, thereby promoting increased Ca^{2+} mobilization, glycolysis, and activation [183,184]. CTLA-4 monoclonal antibodies such as ipilimumab (Yervoy) and tremelimumab block B7-interaction and have been used to treat melanoma [47,185,186]. The monoclonal antibody pembrolizumab is highly selective for PD-1 and prevents PD-1 from engaging PD-L1 and PD-L2, thus enhancing T cell immune response [19,187,188]. Further research will address whether combining anti-CTLA-4 and anti-PD-1 antibodies will improve cancer treatments [19].

As previously mentioned, Ca^{2+} is critical for T cell activation and immune response. Manipulating Ca^{2+} signaling to enhance T cell-directed immune response against cancer is an intriguing notion, yet the means to target the Ca^{2+} response of specific cells without tampering with the metabolic processes of other cells remains elusive [189]. Antitumor activity of tumor-infiltrating lymphocytes (TIL) is inversely related to CD5 expression [99]. CD5 levels in naïve T cells are constantly tuned in the periphery by interactions with self pMHC complexes to maintain homeostasis; therefore, CD5 expression on TILs can be downregulated in response to low affinity for cancer antigens [190–192]. Thus, the majority of TILs are CD5^{lo} which increase their reactivity while CD5^{hi} TILs do not elicit a Ca^{2+} response and become anergic and are unable to eliminate malignant cells [99,192]. While downregulation of CD5 on TILs enhances antitumor T cell activity, CD5^{lo} T cells are also more likely to experience activation-induced cell death (AICD) as CD5 protects T cells from overstimulation [23]. To maximize TIL effectiveness, the inhibitory effects of CD5 could be blocked by neutralizing monoclonal antibodies or soluble CD5-Fc molecules combined with soluble FAS-Fc molecules to reduce the inherent AICD [23,193,194]. Soluble human CD5 (shCD5) may have a similar effect but avoids targeting issues by blocking CD5-mediated interaction via a "decoy receptor" effect. Mice constitutively expressing shCD5 had reduced melanoma and thyoma tumor cell growth and increased numbers of CD4^{+} and CD8^{+} T cells [195]. Wild type mice treated with an injection of recombinant shCD5 also had reduced tumor growth [195]. Finally, CD5-deficient mice engrafted with B16-F10 melanoma cells had slower tumor growth compared to wild type C57BL/6 mice [196]. This evidence suggests that CD5, along with PD-1 and CTLA-4, may be a potential target to specifically modulate T cell Ca^{2+} mobilization in an immunosuppressive tumor setting.

4.4. Microbiome

The gut microbiome, including the bacteria and their products, forms a dynamic beneficial symbiosis with the immune system influencing host genes and cellular response. The gut microbiome shapes and directs immune responses while the immune system dictates the bacterial composition of the gut microbiome [197]. As the gut is the major symbiotic system intersecting the immune system and microbiota, understanding their connection has implications for immune system development and function as the gut microbiome is involved in protecting against pathogens, influencing states of inflammation, and even affecting cancer patient outcomes [198,199].

The gut microbiome primes immune responses [200]. Alteration in the microbial composition can induce changes in T cell function in infectious disease, autoimmunity, and cancer [201]. For example,

mice treated with antibiotics which restrict or reduce the microbial environment exhibit impaired immune response because their T cells have altered TCR signaling and compromised intracellular Ca^{2+} mobilization in infectious disease and cystic fibrosis models [202–204]. In contrast, administering oral antibiotics to mice with EAE increases the frequency of $CD5^{+}$ B cell subpopulations in distal lymphoid sites and confers disease protection [205]. In cancer, the microbiome also influences patient response to immune checkpoint inhibitors such as CTLA-4 and PD-1 [206,207]. Mice and melanoma patients immunized or populated with *Bacteriodes fragilis* respond better to treatment with Ipilimumab, a monoclonal antibody against CTLA-4 [198]. Similarly, tumor-specific immunity improved when anti-PD-1/PD-L1 monoclonal antibodies where used in the presence of *Bifidobacterium* [208].

Though little is known about how CD5 influences T cell interaction with the microbiome, some tantalizing details are available. As specific bacterium promotes cancer regression during CTLA-4 and PD-1 checkpoint blockades, a CD5 blockade in conjunction with bacterial selection may also improve immune response. Such studies would lead to novel immunotherapeutic treatments for cancer and autoimmune diseases.

5. Conclusions

CD5, widely known as an inhibitory co-receptor in the thymus, appears to modulate the signaling intensity of peripheral T cells by increasing Ca^{2+} signaling activity and efficacy of $CD5^{hi}$ T cells. CD5 expression levels in the periphery correlates with intracellular Ca^{2+} mobilization, suggesting that CD5 promotes peripheral T cell activation and immune response. As such, CD5 may be a novel checkpoint therapy to regulate T cell activation and metabolism through altering Ca^{2+} mobilization, and could be used to affect neurological behavior, alter microbiome interactions, and treat cancer and autoinflammatory diseases. While this paper focuses on the role of co-receptor CD5 effects on calcium signaling and activation of T cells, CD5 itself may be regulated through posttranslational modifications, such as *N*-glycosylation, which may affect Ca^{2+} mobilization, T cell metabolism, activation, and function. In the future it would be interesting to determine the role of other posttranslational modifications (e.g., *N*-glycosylation, *S*-glutathionylation, lipidation) in CD5 signaling.

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Abbreviations

CTLA-4	Cytotoxic T-lymphocyte antigen 4
CD	Cluster of differenciation
PD-1	Programmed cell death protein 1
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
CaMKK	Calmodulin-dependent protein kinase kinase
AMPK	AMP-activated protein kinase
SOCE	Store-operated calcium channels
CRAC	$Calcium^{+}$-release-activated channel
STIM	Stromal interaction molecule
SERCA	Sarcoendoplasmic reticulum calcium transport ATPase
ER	Endoplasmic reticulum
NFAT	Nuclear factor of activated T cells
INF-γ	Interferon gamma

Abbreviations

TNF	Tumor necrosis factor
IL-2	Interleukin 2
GLUT1	Glucose transporter 1
GLUT3	Glucose transporter 3
TIL	Tumor infiltrating lymphocytes
ERK	Extracellular signal-regulated kinases

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SCIENTIFIC REPORTS

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The nuclear variant of bone morphogenetic protein 2 (nBMP2) is expressed in macrophages and alters calcium response

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We previously identified a nuclear variant of bone morphogenetic protein 2 (BMP2), named nBMP2, that is translated from an alternative start codon. Decreased nuclear localization of nBMP2 in the nBmp2NLS^{tm} mouse model leads to muscular, neurological, and immune phenotypes—all of which are consistent with aberrant intracellular calcium (Ca^{2+}) response. Ca^{2+} response in these mice, however, has yet to be measured directly. Because a prior study suggested impairment of macrophage function in nBmp2NLS^{tm} mutant mice, bone marrow derived (BMD) macrophages and splenic macrophages were isolated from wild type and nBmp2NLS^{tm} mutant mice. Immunocytochemistry revealed that nuclei of both BMD and splenic macrophages from wild type mice contain nBMP2, while the protein is decreased in nuclei of nBmp2NLS^{tm} mutant macrophages. Live-cell Ca^{2+} imaging and engulfment assays revealed that Ca^{2+} response and phagocytosis in response to bacterial supernatant are similar in BMD macrophages isolated from naïve (uninfected) nBmp2NLS^{tm} mutant mice and wild type mice, but are deficient in splenic macrophages isolated from mutant mice after secondary systemic infection with *Staphylococcus aureus*, suggesting progressive impairment as macrophages respond to infection. This direct evidence of impaired Ca^{2+} handling in nBMP2 mutant macrophages supports the hypothesis that nBMP2 plays a role in Ca^{2+} response.

Our group has reported the existence of a nuclear variant of the growth factor bone morphogenetic protein 2 (BMP2), designated nBMP2[1]. This variant protein is produced by translation from an alternative downstream start codon that eliminates the N-terminal endoplasmic reticulum signal peptide, thus preventing the protein's delivery to the secretory pathway. Instead, nBMP2 is translated in the cytoplasm and translocated to the nucleus by means of an embedded bipartite nuclear localization signal (NLS)[1]. Using immunohistochemistry, we have detected nBMP2 in skeletal muscle nuclei and in the nuclei of CA1 neurons in the hippocampus[2,3].

To examine the function of nBMP2, we generated a mutant mouse strain (nBmp2NLS^{tm}) in which a three-amino acid substitution in the NLS inhibits translocation of nBMP2 to the nucleus while still allowing normal synthesis and secretion of the conventional BMP2 growth factor[2]. The mice appear overtly normal and are fertile. They do, however, lack nBMP2 in myonuclei, and electrophysiological studies revealed that skeletal muscle relaxation is significantly slowed after stimulated twitch contraction, a process that is regulated by intracellular Ca^{2+} transport. Consistent with impaired intracellular Ca^{2+} transport, sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) activity is decreased in skeletal muscle[2]. The mutant mice also lack nBMP2 in CA1 hippocampal neurons, and electrophysiological studies revealed reduced long-term potentiation (LTP) in the hippocampus[3]. LTP is dependent on intracellular Ca^{2+} transport and is thought to be the cellular equivalent of learning and memory[4–6]. Behavioral tests revealed that the nBMP2 mutant mice have impaired object recognition memory[3].

Intracellular Ca^{2+} elevation also regulates the activation and differentiation of several different types of immune cells including T cells, B cells, dendritic cells, and macrophages[7–10]. To see if nBmp2NLS^{tm} mutants had

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compromised immune response, mice were challenged by systemic infection with *Staphylococcus aureus*. While the mutants' immune response to a primary infection appeared normal, their immune response to a secondary infection challenge 30 days later resulted in higher levels of bacteremia, increased mortality, and failure of spleens to enlarge normally[11]. Although we did not observed differences in the total number of macrophages in spleen, thymus, or lymph node from wild type compared to mutant mice, we did observe that after the secondary infection, spleen from nBmp2NLS^{tm} mutant mice showed fewer hemosiderin-laden macrophages than spleen from wild type mice[11]. Macrophages in the spleen accumulate hemosiderin by phagocytosing damaged red blood cells and hemoglobin, which would be present in the blood stream of *S. aureus*-challenged mice due to the hemolysins that *S. aureus* expresses[12–14]. The observation of fewer hemosiderin-laden macrophages in the spleens of mutant mice after a secondary infection suggested to us that macrophage phagocytic activity might be impaired in the absence of nBMP2, potentially providing us with an accessible cell type in which to directly test our hypothesis that intracellular Ca^{2+} response is disrupted in the absence of nBMP2.

To interrogate if nBMP2 might play a role in Ca^{2+} response, we isolated macrophages from wild type and nBmp2NLS^{tm} mutant mice. These macrophages included bone marrow derived (BMD) macrophages from uninfected mice, and splenic macrophages from mice that had undergone primary and secondary infections with *S. aureus*[15]. Live-cell Ca^{2+} imaging as well as bead engulfment assays were performed to measure intracellular Ca^{2+} response and phagocytic activity. These analyses revealed deficient Ca^{2+} response and phagocytosis in splenic macrophages isolated from mutant mice after secondary systemic infection with *S. aureus*, but not in BMD macrophages from naïve mice, suggesting that as nBmp2NLS^{tm} mutant cells respond to infection over time, Ca^{2+} response is progressively impaired.

Results

The nuclear variant nBMP2 is expressed in BMD and splenic macrophages from wild type mice. To determine whether nBMP2 is expressed in macrophages, BMD macrophages and splenic macrophages were isolated from naïve (uninfected) wild type and nBmp2NLS^{tm} mutant mice and differentiated *in vitro*, and immunocytochemistry was performed using an anti-BMP2 antibody that binds to both BMP2 and nBMP2. Consistent with our prior observation of impaired immune response in nBmp2NLS^{tm} mutant mice[11], nBMP2 was detected in the nuclei of wild type BMD (Fig. 1a) and splenic (Fig. 1b) macrophages. As expected, nBMP2 was significantly decreased in macrophage nuclei from nBmp2NLS^{tm} mutant mice (Fig. 1a,b, mutant). ImageJ software quantification of immunofluorescence images showed that the density of nuclear BMP2 staining was significantly more intense in wild type compared to mutant macrophages in both BMD macrophages ($p = 0.0005$) and splenic macrophages ($p < 0.0001$) (Fig. 2). BMP2 staining was visible throughout the cytoplasm of both wild type and mutant macrophages, as expected, given that nBMP2 is synthesized in the cytosol before being translocated to the nucleus and that the conventional BMP2 growth factor is synthesized in the rough ER and translocated through the Golgi before being secreted from the cell.

BMD macrophages from uninfected nBmp2NLS^{tm} mutant mice and wild type mice have similar Ca^{2+} response. Naïve BMD macrophages isolated from femurs and tibias of uninfected mice were differentiated and activated *in vitro* then plated for live-cell Ca^{2+} imaging. Plated cells were loaded with Fura-2AM, a UV-excitable ratiometric calcium indicator that changes its excitation in response to Ca^{2+} binding; Fura-2AM emits at 380 nm when Ca^{2+} is not bound, and at 340 nm when Ca^{2+} binds to the dye. The fluorescence ratio (F340/F380), increases as cytosolic Ca^{2+} levels increase[16]. At the 2 min time point, supernatant from *Escherichia coli* (ECS) cultures was added to stimulate Ca^{2+} flux (Fig. 3a)[17–19]. Following this stimulation, there were no observable differences between naïve mutant and wild type BMD macrophages in peak Ca^{2+} response (Fig. 3b) or sustained Ca^{2+} levels (Fig. 3c).

Splenic macrophages isolated from nBmp2NLS^{tm} mutant mice after secondary infection show impaired Ca^{2+} response. In our prior study, immune deficiencies in nBMP2NLS^{tm} mice were detectable only after the mice received a secondary infection with *S. aureus*[11]. Because our current experiments revealed no significant differences in Ca^{2+} response in naïve BMD macrophages from mutant compared to wild type mice, we decided to replicate the *in vivo* conditions of our previous work by examining splenic macrophage harvested from mice after a secondary infection with *S. aureus*, and by using *S. aureus* supernatant as the stimulus to trigger Ca^{2+} flux[11]. Although *S. aureus* is a gram positive bacteria that does not produce LPS, it does produce liphoteichoic acid (LTA), which is similarly able to activate macrophages[20,21]. Thirty-five days after primary systemic *S. aureus* infections, mice were given a second injection of *S. aureus*, and splenic macrophages were isolated 3 days later.

After one week *in vitro* maturation, splenic macrophages were loaded with Fura-2AM for live-cell Ca^{2+} imaging experiments. *S. aureus* supernatant (SAS) was used to stimulate Ca^{2+} flux at the 2-min time point (Fig. 4a). Compared to the lack of a difference in naïve BMD macrophages, it is particularly striking that peak Ca^{2+} response was significantly decreased ($p = 0.0335$) in mutant splenic macrophages after secondary infection (Fig. 4b). Sustained Ca^{2+} levels as measured by the area under the curve (AUC) from minutes 3–10 was also significantly decreased ($p = 0.0008$) (Fig. 4c).

BMD macrophages from uninfected nBmp2NLS^{tm} mutant mice and wild type mice show similar phagocytic activity. To test phagocytic activity of naïve BMD macrophages (meaning macrophages that were isolated from uninfected mice) from nBmp2NLS^{tm} mutant compared to wild type mice, we measured fluorescent bead engulfment by CD11b and F4/80 positive cells with flow cytometry (Fig. 5a)[22–28]. We observed no differences in the phagocytic activity of naïve BMD macrophages from nBmp2NLS^{tm} mutant compared to wild type mice (Fig. 5b–e).

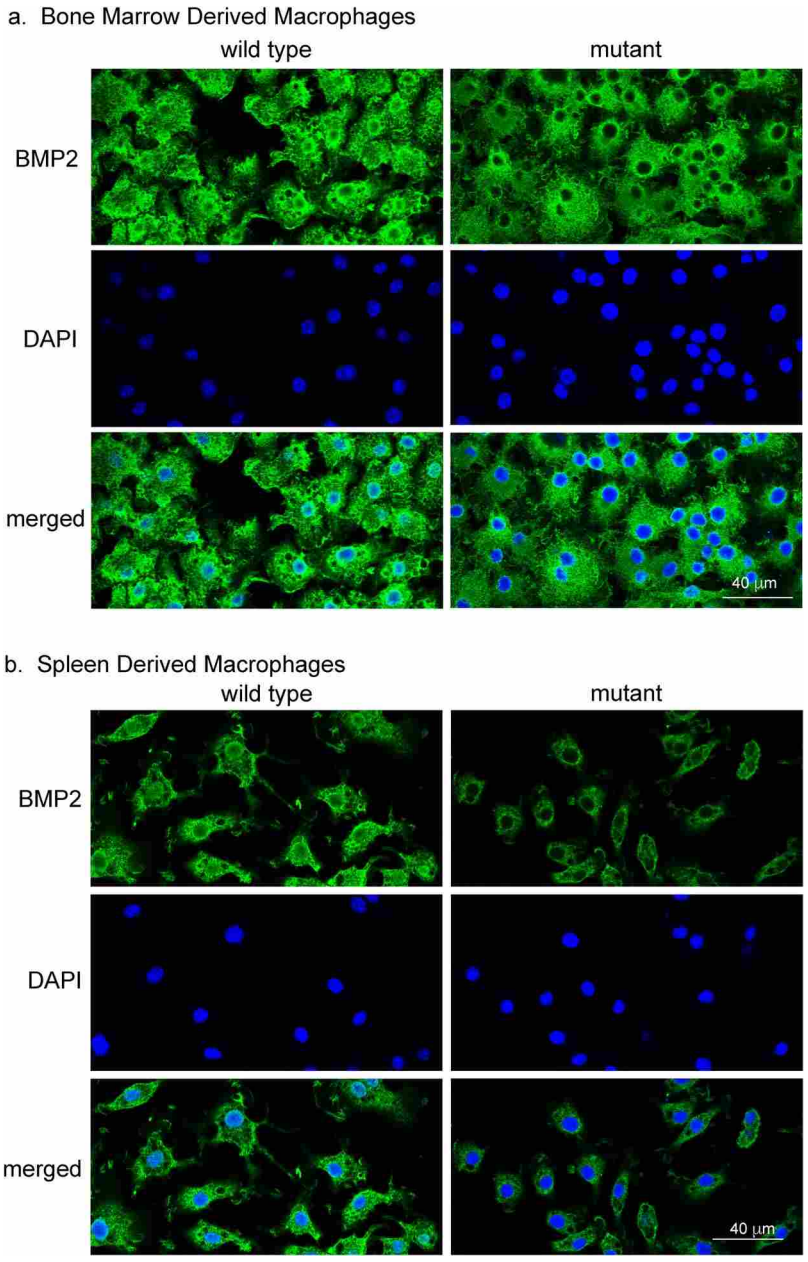

Figure 1. BMD macrophages and splenic macrophages express nBMP2, which is decreased in the nuclei of nBmp2NLS^{tm} mutant macrophages. (**a**) BMD macrophages and (**b**) splenic macrophages were stained with anti-BMP2 antibody (green) and counterstained with DAPI (blue), demonstrating that nBMP2 is expressed and localized to the nucleus in wild type macrophages, and that nuclear translocation of nBMP2 is inhibited in mutant macrophages. BMP2 labeling within the cytoplasm is present in both wild type and mutant cells as expected, because the targeted mutation allows translation of nBMP2 in the cytoplasm but inhibits nuclear translocation, and it allows normal synthesis and secretion of conventional BMP2.

Splenic macrophages from nBmp2NLS^{tm} mutant mice show impaired phagocytic activity. To test phagocytic activity in macrophages isolated from mice after secondary infection, splenic macrophages were isolated from wild type and nBmp2NLS^{tm} mutant mice 3 days after mice received a second systemic infection with *S. aureus*, and fluorescent bead engulfment was measured as described above. While differences between wild type and mutant macrophages did not reach significance when subgroups that engulfed 1, 2, or 3 or more beads were analyzed individually (Fig. 6a–c), there was a significant reduction in overall mutant phagocytic activity ($p = 0.0176$) when the subgroups were pooled (Fig. 6d). These data suggest a possible relationship between the decreased Ca^{2+} response and reduced phagocytosis in nBmp2NLS^{tm} mutant splenic macrophages.

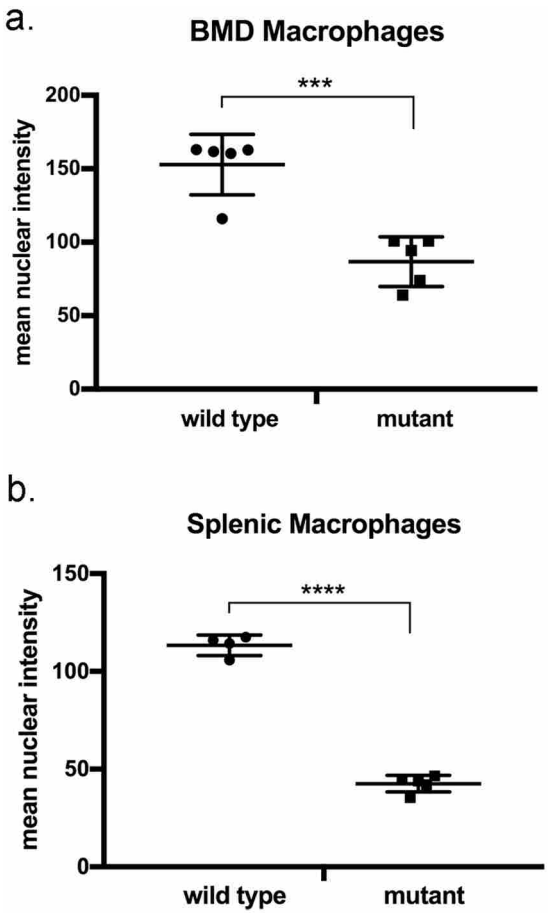

Figure 2. Quantification of nBMP2 nuclear staining intensity. Five images each were analyzed for wild type and mutant BMD macrophages and for mutant splenic macrophages. Four images were analyzed for wild type splenic macrophages. Each image contained between 10 and 93 cells, and the number of cells analyzed per group ranged from 100 to 337. ImageJ was used to outline DAPI-stained regions and quantify BMP2 immunostaining as the sum of pixel intensities within each nucleus. The mean density of BMP2 immunostaining was then calculated for all nuclei in an image. An unpaired, two-tailed t-test was performed to compare nuclear staining between wild type and mutant cells. For BMD wild type vs. mutant macrophages, $p = 0.0005$. For splenic wild type vs mutant macrophages, $p < 0.0001$.

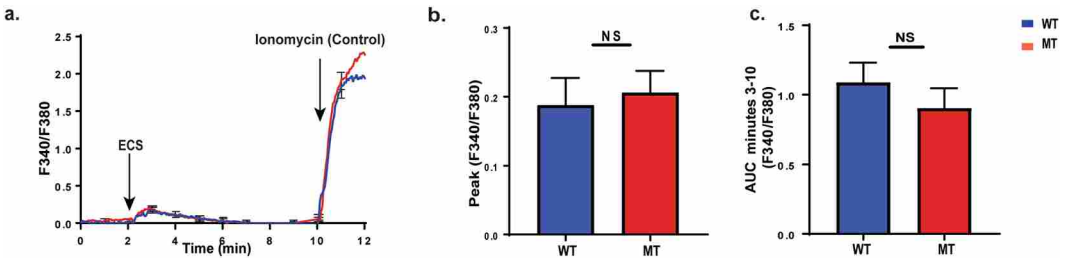

Figure 3. Naïve bone marrow derived (BMD) macrophages from nBmp2NLS^{tm} mutant mice and wild type mice have a similar Ca^{2+} response. Naïve BMD macrophages from wild type (WT) and nBmp2NLS^{tm} mutant (MT) mice were loaded with Fura-2AM for live-cell Ca^{2+} imaging. During imaging, cells were stimulated at 2 min with *E. coli* supernatant (ECS), then at 10 min with ionomycin as a positive control. (**a**) Average curves showing intracellular Ca^{2+} response in wild type and nBmp2NLS^{tm} mutant BMD macrophages. Fluorescence ratios (F340/F380) were measured at 3 sec intervals from 0–12 min ($n = 38$ cells). Error bars (s.e.m.) are shown at one min intervals. (**b**) Average (±s.e.m.) of peak Ca^{2+} influx (F340/F380) in wild type and nBmp2NLS^{tm} mutant BMD macrophages ($n = 38$ cells). (**c**) Area under the curve (AUC) of F340/F380 ratios from minutes 3 to 10 min shows sustained intracellular Ca^{2+} levels ($n = 38$ cells). NS, not significant.

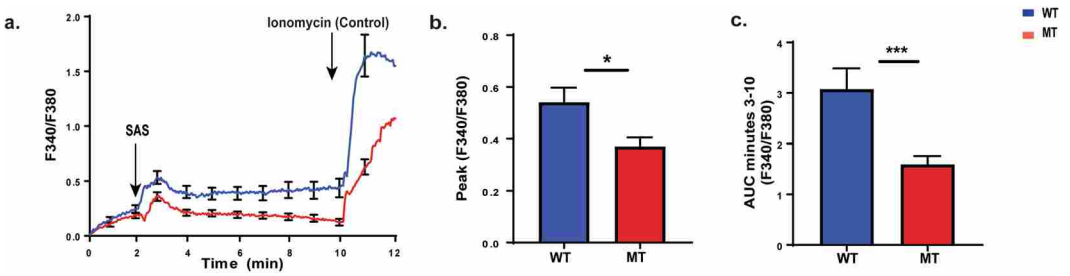

Figure 4. Splenic macrophages collected from nBmp2NLS^{tm} mutant mice after secondary infection have an impaired Ca^{2+} response. Splenic macrophages from wild type (WT) and nBmp2NLS^{tm} mutant (MT) mice were loaded with Fura-2AM for live-cell Ca^{2+} imaging. During imaging, cells were stimulated at 2 min with *S. aureus* supernatant (SAS), then at 10 min with ionomycin as a positive control. (**a**) Average curves showing intracellular Ca^{2+} response in wild type and nBmp2NLS^{tm} mutant splenic macrophages. Fluorescence ratios (F340/F380) were measured at 3 sec intervals from 0-12 min (n = 44 cells). Error bars (s.e.m.) are shown at one min intervals. (**b**) Average ± s.e.m. of peak Ca^{2+} influx (F340/F380) in wild type and nBmp2NLS^{tm} mutant splenic macrophages shows a significant difference (n = 44 cells). (**c**) AUC of F340/F380 ratios from minutes 3 to 10 min shows a significant difference in sustained intracellular Ca^{2+} levels (n = 44 cells). *$p < 0.05$, **$p < 0.01$, ***$p < 0.0001$.

Discussion

The role of BMP2 in macrophages is unknown and remains an area of active research. BMP2 has been reported to be constitutively expressed in M1 (inflammatory) macrophages[29]. Other studies have shown that BMP2 expression is upregulated as macrophages shift toward the pro-healing/anti-inflammatory M2 phenotype[30,31]. BMP2 secretion by macrophages promotes migration of vascular smooth muscle cells, and macrophages in the intestinal muscularis secrete BMP2 to signal enteric neurons[32,33]. Reports of BMP2 expression by hematopoietic cells, in particular macrophages, are relevant to this study because nBMP2 can be produced from the same mRNA as the conventional secreted BMP2 growth factor—any time BMP2 mRNA or BMP2 growth factor is detected, the potential for nBMP2 synthesis exists[1]. Accordingly, we have demonstrated by immunofluorescence that both BMD macrophages and splenic macrophages express the nuclear variant of BMP2, nBMP2, and that nBMP2 is decreased in the nuclei of macrophages from nBmp2NLS^{tm} mutant mice.

Previously, we demonstrated that deficiency of nBMP2 in the nucleus impairs secondary immune response as evidenced by diminished spleen enlargement, poor clearance of *S. aureus* from the bloodstream, and increased mortality after secondary infection[11]. We have also shown that deficiency of nBMP2 in myonuclei is correlated with slowed skeletal muscle relaxation after contraction, and deficiency of nBMP2 in the nuclei of hippocampal neurons is correlated with learning/memory deficits[2,3]. Each of these phenotypes is consistent with deficiencies in intracellular Ca^{2+} transport, but until now, no direct measurements of intracellular Ca^{2+} have been performed in cells from nBmp2NLS^{tm} mutant mice. The discovery that macrophages express nBMP2 (Fig. 1) provided an accessible cell type in which to directly address the question of whether nBMP2 plays a role in intracellular Ca^{2+} response.

We found that intracellular Ca^{2+} response was impaired in mutant splenic macrophages after secondary infection with *S. aureus*, but not in mutant BMD macrophages isolated from uninfected mice, even though both macrophage types expressed nBMP2. Recent work has revealed that innate immune cells can undergo memory-like adaptive responses to increasing pathogen load, and the deficient Ca^{2+} response in splenic macrophages after secondary infection might represent a failure of those adaptive responses[34,35]. Alternatively, it may be that the effects of nBMP2 deficiency in the nucleus are simply cumulative, causing a Ca^{2+}-handling phenotype that becomes progressively more severe as cells differentiate and mature. A progressive phenotype is consistent with our previously reported observation that hippocampal long-term potentiation (LTP) was normal in 3-week-old nBmp2NLS^{tm} mutant mice but deficient in 3-month-old mice[3]. Progressive impairment of intracellular Ca^{2+} response has received attention recently as a potential mechanism for both brain and muscle aging[36–38], suggesting that nBMP2 dysfunction could contribute to premature aging or aging-related diseases.

Deficiency of nBMP2 in the nucleus also produced a significant decrease in the total phagocytic activity of splenic macrophages from nBmp2NLS^{tm} mutant mice, suggesting that mutant cells may be less effective at clearing pathogens from the blood stream. This is consistent with prior studies suggesting that intracellular Ca^{2+} mobilization plays a role in macrophage phagocytic activity. For example, impaired Ca^{2+} response in macrophages from Trpm4(−/−) mutant mice led to decreased phagocytic activity, resulting in bacterial overgrowth and translocation to the bloodstream[39]. Intracellular Ca^{2+} levels increase during Fcγ receptor (FcR)-mediated phagocytosis[40–42], and the loss of CaMKK2, a calcium-dependent kinase, left macrophages unable to phagocytose bacteria or synthesize cytokines in response to bacterial lipopolysaccharide (LPS)[43].

Although evidence supports the involvement of Ca^{2+} response in macrophage phagocytic activity, the scale of the decreased phagocytosis by splenic macrophages observed in our study seems insufficient to account for the markedly increased mortality of nBmp2NLS^{tm} mutant mice after secondary infection[3]. We cannot rule out the possibility that the bead engulfment assay did not fully reflect the severity of phagocytosis impairment in splenic macrophages. Liver macrophages also play a role in bacterial clearance, and it is possible that the absence of nBMP2 in the nucleus affects their function more severely[44,45]. In addition, the absence of nBMP2 in the

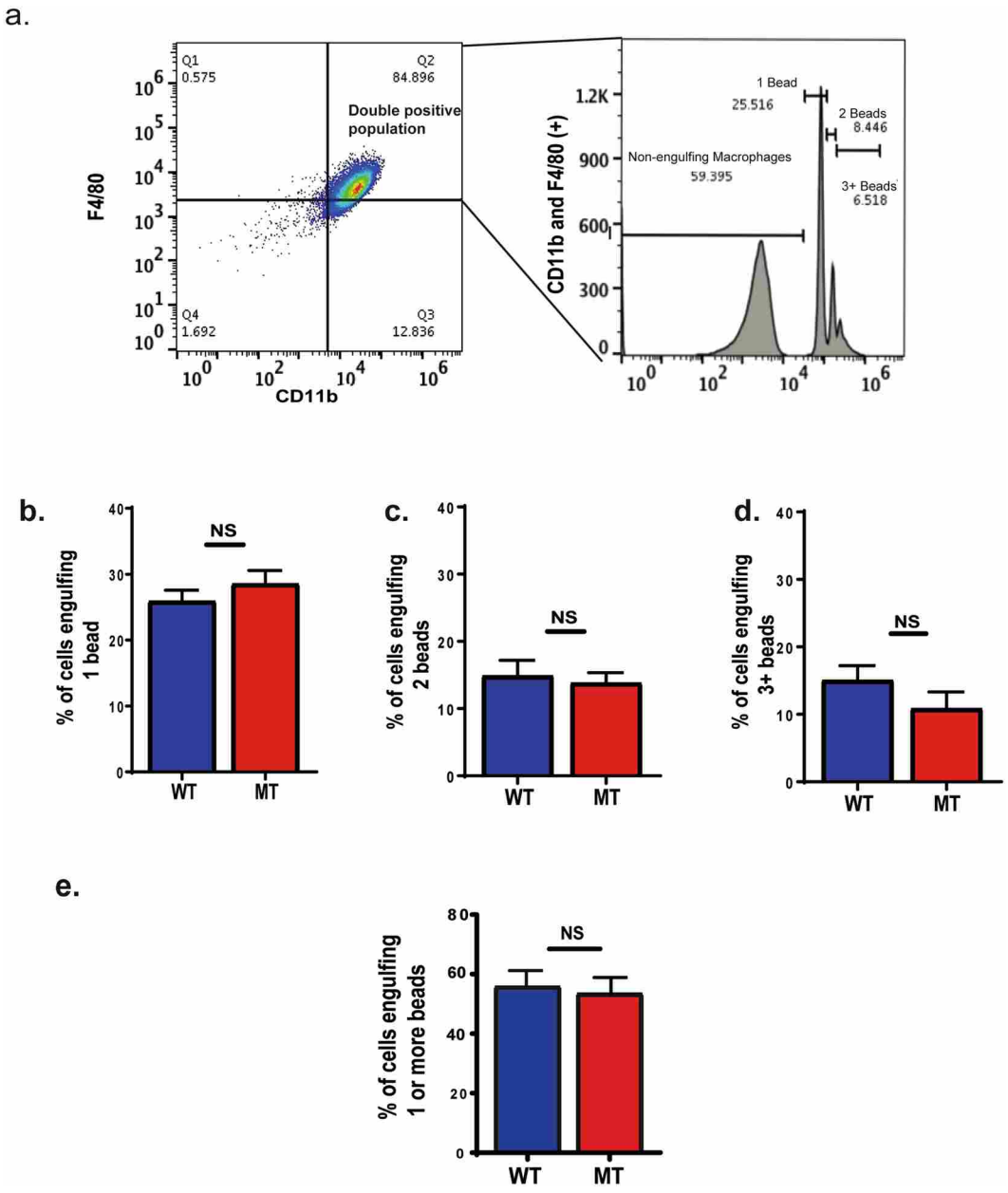

Figure 5. Naïve bone marrow derived (BMD) macrophages from nBmp2NLS^{tm} mutant mice and wild type mice show similar phagocytic activity. After incubation with fluorescent microspheres, macrophages were analyzed by flow cytometry. (**a**) A representative analysis is shown. The F4/80 and CD11b double positive population was selected, and from this gate a histogram was produced to identify macrophages that had engulfed 1, 2, or 3 or more beads. The percentages of total double positive cells represented within each peak are indicated. (**b**) Percent of cells engulfing 1 bead, (**c**) percent of cells engulfing 2 beads, and (**d**) percent of cells engulfing 3 or more beads. (**e**) Percent of cells engulfing one or more beads. N = 3 pairs of wild type and 3 pairs of mutant mice. NS, not significant.

nucleus might affect other immune system cell types besides macrophages, and it is possible that another cell type, or perhaps several cell types together, account for the increased mortality of nBmp2NLS^{tm} mutant mice after secondary infection[3]. Indeed, BMP2 (and therefore potentially nBMP2) is expressed by a specialized endothelial population in the early embryo, termed hemogenic endothelium, that gives rise to hematopoietic stem cells[46]. The absence of nBMP2 at the earliest stages of hemogenesis could therefore impact a wide range of immune cell types. BMP2 is also expressed in human cord blood cells, including those that express CD34, a hematopoietic progenitor cell antigen[47], and acute bleeding triggers upregulation of BMP2 expression in hematopoietic stem cells[48]. BMP2 expression is also found in mature B cells, where it is upregulated in response to infection with *Aggregatibacter actinomycetemcomitans*[49]. It is possible, therefore, that nBMP2 impacts the activation or function of other immune cell types in addition to macrophages, and the combined functional deficits account for the increased mortality in nBmp2NLS^{tm} mutant mice after secondary infection.

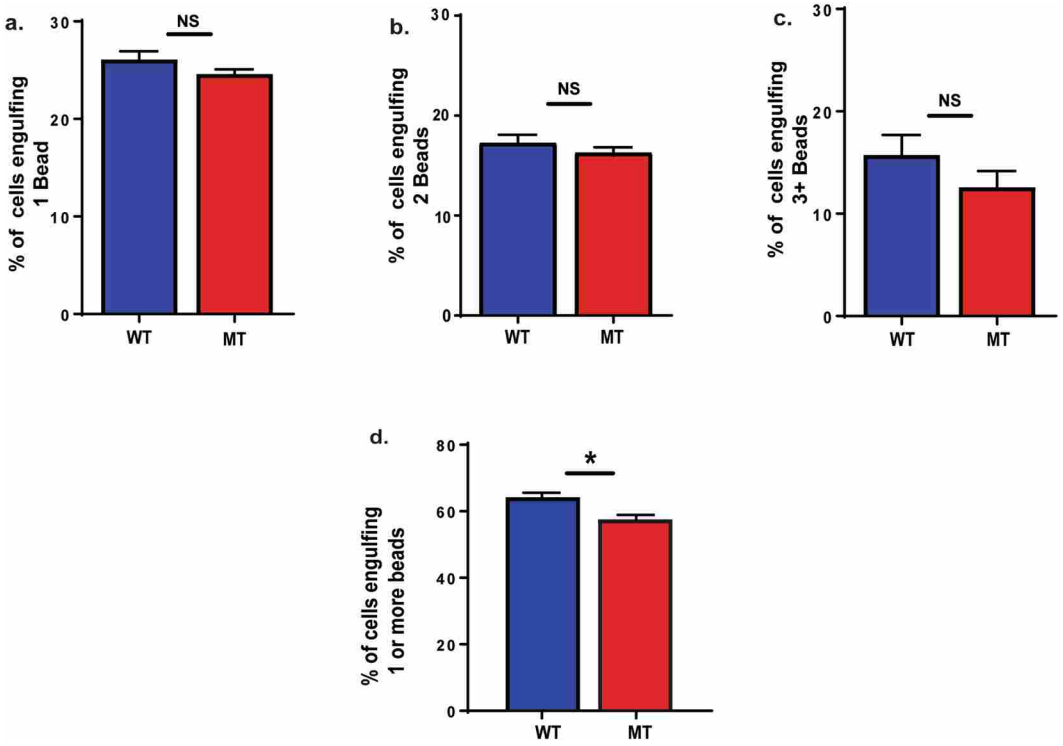

Figure 6. Splenic macrophages collected from nBmp2NLS^{tm} mutant mice after secondary infection show impaired engulfment activity. After incubation with fluorescent microspheres, macrophages were analyzed by flow cytometry as described in Fig. 3. (**a**) Percent of cells engulfing 1 bead, (**b**) percent of cells engulfing 2 beads and, (**c**) percent of cells engulfing 3 or more beads. (**d**) Percent of cells engulfing one or more beads. N = 3 pairs of wild type and 3 pairs of mutant mice. NS, not significant. *$p < 0.05$.

It will be important, in future work, to elucidate the molecular mechanisms underlying the Ca^{2+} response differences between macrophages from wild type and nBMP2 mutant mice. Differences may stem from impaired uptake or release of Ca^{2+} from endoplasmic reticulum stores, as suggested by the decreased SERCA activity observed in skeletal muscle of nBMP2 mutant mice[2]. Alternatively, transport of Ca^{2+} could be impaired at the macrophage cell membrane, consistent with observations that increasing extracellular Ca^{2+} levels can improve phagocytosis[50,51]. Neurons and muscle cells are excitable cells and are therefore equipped with a different set of ion channels and transporters than are macrophages, and so it will be important to examine molecular details of the Ca^{2+} handling defect in all three cell types. This work has thus opened the way for future studies into the molecular interactions and activities of nBMP2.

Questions about how nBMP2 functions from inside the nucleus to affect Ca^{2+} response also remain to be answered. The novel protein nBMP2 was first identified from among nuclear proteins that had been isolated using DNA affinity chromatography, but subsequent experiments failed to show direct binding of nBMP2 to DNA, and the amino acid sequence of nBMP2 contains no predicted DNA-binding domain[1]. It is possible that nBMP2 interacts indirectly with DNA through a transcription factor, and future studies of nBMP2's impact on the expression of genes involved in Ca^{2+} signaling will be informative.

In summary, this study supports our working hypothesis that aberrant intracellular Ca^{2+} response is the mechanism that unites the otherwise disparate muscle, neurological, and immune phenotypes observed in nBmp2NLS^{tm} mutant mice[2,3,11,52–54]. In doing so, this study has paved the way for future work to elucidate the precise molecular nature of the Ca^{2+} signaling disruptions in nBMP2 mutant cells and to understand how nBMP2's interactions in the nucleus impact Ca^{2+} signaling.

Materials and Methods

Research Animals. This study was carried out in strict accordance with recommendations in the Guide for the Care and Use of Laboratory Animals[55]. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Brigham Young University (protocol numbers 15-0107 and 15-0603).

Mice were housed in a temperature-controlled (21–22 °C) room with a 12:12 hr light-dark cycle and fed standard rodent chow and water *ad libitum*. The nBmp2NLS^{tm} mice were constructed on a Bl6/129 background, as described[2]. The homozygous wild type and mutant mice used in this study were obtained by breeding heterozygotes, and genotyping was performed as previously described[3]. All experiments were performed with male mice at least 6 months of age.

BMD and Splenic Macrophage Isolation. BMD macrophages were obtained from femurs and tibias of wild type and nBmp2NLS^{tm} mutant mice and were differentiated in culture at 37 °C with 5% CO_2 for 7 days in macrophage medium (DMEM (HyClone), 10% fetal bovine serum (FBS) (HyClone), 20% supernatant from L929 mouse fibroblast as a source of macrophage colony-stimulating factor (M-CSF), 5% heat inactivated horse serum (Sigma), 1 mM sodium pyruvate (Gibco by Life Technologies), 1.5 mM L-glutamine (Thermofisher), 10 u/ml penicillin, 10 μg/ml streptomycin (Gibco by Life Technologies)) prior to plating for immunocytochemistry, Ca^{2+} imaging or engulfment assays.

Spleens from wild type and nBmp2NLS^{tm} mutant mice were homogenized in phosphate buffered saline (PBS). The homogenate was filtered, pelleted at 450 × g for 5 min, suspended in lysis buffer (155 mM NH_2Cl, 10 mM $KHCO_3$, 0.1 mM EDTA) on ice for 3–5 min to lyse erythrocytes, and then washed with 37 °C macrophage media and plated in macrophage medium in 6-well plates. After 3 days of culture at 37 °C in 5% CO_2, medium was replaced to remove non-adherent cells[56]. On day 4, 100 ng/ml lipopolysaccharide (LPS) was added to the culture medium to stimulate differentiation, and cells were incubated for 3–4 more days[57]. Differentiated cells were then plated for immunocytochemistry, Ca^{2+} imaging, or engulfment assays.

Immunocytochemistry. Immunocytochemistry was performed using BMD and splenic macrophages. Following macrophage isolation and 7-day differentiation as described above, cells were plated on coverslips that were pre-treated with 0.025% HCl in PBS for 20 min to facilitate cell attachment. Cells were cultured for 1–2 days to reach 70–90% confluence, then fixed at 37 °C in 4% paraformaldehyde for 10 min. Epitopes were exposed through antigen retrieval using 5% sodium citrate and 0.25% Tween-20 in ddH2O, pH 6.0, at 95 °C for 10 min. Cells were permeabilized using 0.1% Triton X-100 then blocked for 1.5 hr at room temperature (RT) using SEA BLOCK blocking buffer (ThermoFisher Scientific, 37527). The samples were then probed with 1:50 anti-BMP2 antibody (Novus Biologicals, NBP1-19751) diluted in 10% SEA BLOCK blocking buffer in 0.1% Tween-20/PBS (PBS-T), overnight at 4 °C. The probed slides were then stained with anti-rabbit Alexa Fluor 488 (ThermoFisher Scientific, A-11034) for 1 hr at RT. Afterwards, nuclei were stained by incubating the slides in 1:5000 DAPI in PBS-T for 15 min., then slides were mounted using Prolong^{TM} Gold Antifade Mountant (Life Technologies, P10144) and cured overnight prior to microscopic imaging. Cells were imaged using a Leica TCS-SP8 confocal microscope with 63X magnification, using the same laser intensities for all samples. Appropriate laser lines were used such as 405 nm for DAPI and 488 nm for BMP2-Alexa Fluor 488.

Comparison of nuclear BMP2 staining intensity between wild type and mutant cells was performed on tiff versions of confocal microscope images using ImageJ to create tracings of DAPI-stained regions and to calculate the mean pixel intensity of nBMP2 staining within each nucleus. Mean nuclear staining intensity was calculated for each image, and groups were compared using an unpaired, two-tailed t-test in GraphPad Prism.

***S. aureus* Bacterial Infections.** *S. aureus* ATCC strain 12600 was cultured in tryptic soy broth liquid culture alternating with standard streak plating on mannitol salt agar (Thermo Fisher Scientific) for counting. To prepare bacteria for injections, 100 μl of overnight liquid culture was transferred into a new 15 ml broth culture and grown until OD_{600} reached 1.0, then pelleted and resuspended in 15 ml of PBS with 20% glycerol, aliquoted, and stored at −80 °C for 3 weeks before injection. Frozen stock concentration was verified one day before the infection by thawing a single aliquot and performing standard serial dilution plate counts. On the day of infection, *S. aureus* was diluted from the frozen stock to the desired concentration in PBS, and mice received a 200 μl retroorbital injection using a 1 ml syringe and 27-gauge needle. The injected volume contained a priming dose of 1×10^4 CFU/g body weight on day 0 (primary infection), and a dose of 3×10^5 CFU/g body weight on day 35 (secondary infection). Macrophages were harvested three days later.

Bacterial Supernatant Preparation. Bacterial supernatant obtained from *E. coli* K12 and *S. aureus* 12600 was used to stimulate Ca^{2+} fluxes in BMD and splenic macrophages[19,58]. A single colony was picked from an agar plate and inoculated into liquid broth overnight culture. The next day, 1 ml of the overnight culture was inoculated into 15 ml liquid broth and incubated with shaking at 37 °C until culture reach an OD_{600} of 1–1.3. Cells were then pelleted by centrifugation at 1,800 × g for 12 min at 4 °C, and supernatant was collected.

Calcium Imaging. BMD and splenic macrophages were isolated and differentiated in culture for 7 days as described above, then seeded on 8-chambered coverglasses (Nunc 155411, Thermo Scientific) and incubated overnight in macrophage medium at 37 °C in 5% CO_2. For BMD macrophages, 10 ng/ml LPS from E. coli O55:B5 (Sigma) was included in the overnight incubation to activate cells. The next day, cells were loaded with 3 μM Fura-2AM (Invitrogen) in Ringers solution containing Ca^{2+} to be used as an extracellular source during the Ca^{2+} imaging assay (150 mM NaCl, 10 mM glucose, 5 mM HEPES, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, pH 7.4) for 30 min at 37 °C in 5% CO_2, washed with Ringers solution, then incubated for another 30 minutes at 37 °C in Ringers solution. Calcium imaging was performed at room temperature using an Olympus IX51 inverted microscope equipped with a xenon arc lamp. Fura-2AM loaded macrophages were excited using 340 nm and 380 nm excitation filters, and images of 340 nm, 380 nm, and transmitted light were capture using a florescence microscope camera (Q Imaging Exi Blue) with a 20x objective (N.A. 0.75) at 3-sec intervals. At the 2-min time point in each imaging protocol, 20 μl of bacterial supernatant was added to stimulate Ca^{2+} flux. Ionomycin (1 μM final concentration) was added at the 10-min time point as a positive control. 10–20 representative cells were selected as regions of interest in each frame, and F340:F380 ratios were calculated and analyzed using CellSens software from Olympus. Each individual cell's fluorescence was normalized to its first recorded value according to the equation (F-Fo)/Fo, where F is the fluorescence at the specific time point, and Fo is the fluorescence value at time 0[19,59].

Engulfment Assay. BMD and splenic macrophages were isolated and differentiated in culture for 7 days as described above, then seeded in 12-well culture plates for flow cytometry-based engulfment assays[22–28]. 100% FBS was used to resuspend 2.0 μm phycoerythrin-conjugated polychromatic red latex microspheres (Polysciences, Inc.) to prevent beads from sticking to the cell membranes during engulfment[23]. The ~10^9 particles/ml concentration was chosen to ensure that beads were not a limiting factor in phagocytosis rates[23]. Macrophages were then activated by adding LPS from *E. coli* O55:B5 (Sigma) to a final concentration of 10 ng/ml and incubated for 1 hour at 37 °C and 5% CO_2. Media was removed and cells were rinsed with cold PBS, then collected and analyzed by flow cytometry using an Attune flow cytometer (Applied Biosystems by Life technologies). Cells were pre-treated with anti-CD16/32 antibodies (14-0161-85 eBioscience) to prevent non-specific antibody binding, then surface stained with APC-conjugated anti-CD11b antibodies (17-0112-82 eBioscience) and FITC-conjugated anti-F4/80 antibodies (11-4801-82 eBioscience). Doublets were removed based on forward scatter width (FSC-W)/forward scatter area (FSC-A), and the F4/80 and CD11b double positive population was selected. From within this gate, engulfing macrophages were distinguished from non-engulfing macrophages based on phycoerythrin fluorescence, and macrophages could be further distinguished based on the engulfment of one, two, or three or more beads. Results were analyzed using FlowJo software (Tree Star).

Data Analysis. All assays were performed as at least three independent repeats, each in triplicate. Area under the curve (AUC) was determined using GraphPad Prism. Statistical significance was assessed using unpaired two-tailed Students T test in GraphPad Prism.

Data Availability

All data generated or analyzed during this study are included in this published article. Biological reagents will be made available on request.

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Author Contributions
L.C.B. and K.S.W. designed the project and obtained funding; L.C.B., K.S.W. and C.T.F. developed experiments and methodology; H.R.B. bred and maintained experimental animals; J.L.A. and J.C.V. directed and performed immunocytochemistry experiments; C.T.F., G.J.H., C.M.R., T.D.C., H.R.B. and D.K.J. performed remaining experiments; L.C.B., K.S.W. and C.T.F. performed data analysis and wrote the manuscript. All authors helped with manuscript revisions.

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Common gut microbial metabolites of dietary flavonoids exert potent protective activities in β-cells and skeletal muscle cells☆

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Abstract

Flavonoids are dietary compounds with potential anti-diabetes activities. Many flavonoids have poor bioavailability and thus low circulating concentrations. Unabsorbed flavonoids are metabolized by the gut microbiota to smaller metabolites, which are more bioavailable than their precursors. The activities of these metabolites may be partly responsible for associations between flavonoids and health. However, these activities remain poorly understood. We investigated bioactivities of flavonoid microbial metabolites [hippuric acid (HA), homovanillic acid (HVA), and 5-phenylvaleric acid (5PVA)] in primary skeletal muscle and β-cells compared to a native flavonoid [(−)-epicatechin, EC]. In muscle, EC was the most potent stimulator of glucose oxidation, while 5PVA and HA simulated glucose metabolism at 25 μM, and all compounds preserved mitochondrial function after insult. However, EC and the metabolites did not uncouple mitochonndrial respiration, with the exception of 5PVA at10 μM. In β-cells, all metabolites more potently enhanced glucose-stimulated insulin secretion (GSIS) compared to EC. Unlike EC, the metabolites appear to enhance GSIS without enhancing β-cell mitochondrial respiration or increasing expression of mitochondrial electron transport chain components, and with varying effects on β-cell insulin content. The present results demonstrate the activities of flavonoid microbial metabolites for preservation of β-cell function and glucose utilization. Additionally, our data suggest that metabolites and native compounds may act by distinct mechanisms, suggesting complementary and synergistic activities in vivo which warrant further investigation. This raises the intriguing prospect that bioavailability of native dietary flavonoids may not be as critical of a limiting factor to bioactivity as previously thought.

Keywords: Hippuric acid; Homovanillic acid; 5-Phenylvaleric acid; (−)-Epicatechin; Insulin; Respiration

1. Introduction

Incidence rates of type-2 diabetes and obesity are rising worldwide. In addition to traditional medical interventions, complementary lifestyle strategies such as diet and exercise are needed to blunt this epidemic. Flavonoids from cocoa, fruit, tea and other sources have been identified as dietary bioactive compounds with potential anti-obesity and anti-diabetes activities. Many of these flavonoids, such as quercetin [1] and procyanidins [2], have poor oral bioavailability and thus low circulating concentrations. Non-extractable/bound flavonoids (from cocoa, *etc.*) and oxidized flavonoids, such as theaflavins and thearubigins from oolong and black teas, have extremely limited oral bioavailability [3,4] and vanishing low circulating concentrations. As an extreme example, consumption of 700 mg theaflavins (equivalent to~30 cups of black tea), produced maximal blood concentrations of only 1 μg/L (~1.8 nM) in humans [3]. Therefore,

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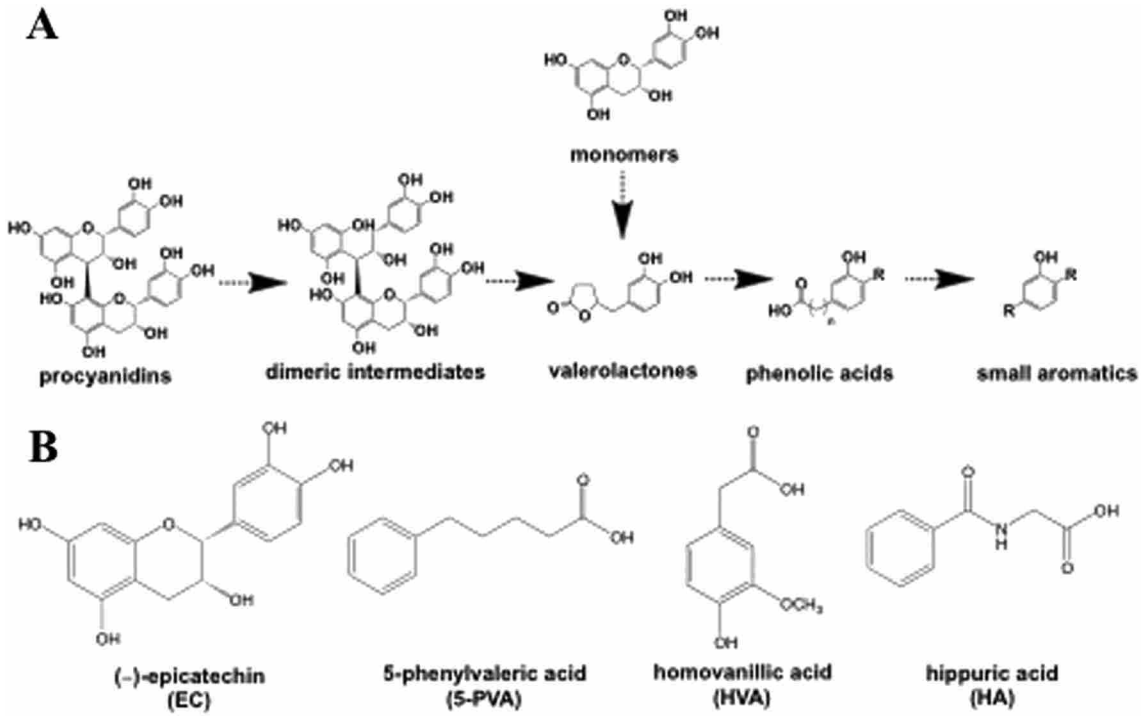

Fig. 1. (A) Schematic showing representative sequential metabolism of representative flavonoids [a dimeric procyanidin, and (−)-epicatechin monomer] by the gut microbiota. (B) Structures of (−)-epicatechin and the three representative flavonoid microbial metabolites employed in this present study.

circulating concentrations of the native species may represent a very small fraction of the ingested dose, whereas the majority reaches the colon unabsorbed. Unabsorbed flavonoids are extensively metabolized by the gut microbiota to a series of smaller metabolites such as valerolactones, phenylalkyl acids, and smaller aromatics (Fig. 1A) [4–9]. While some metabolites are unique to individual flavonoid compounds or subclasses, dozens of metabolites are common to most flavonoids [10,11]. These metabolites are comparatively more bioavailable than their native flavonoid precursors, and in many cases represent the predominant circulating forms following flavonoid consumption [10]. For example, a recent study of pharmacokinetics following consumption of grape pomace demonstrated that anthocyanins and procyanidins were not detected in blood and catechins and their phase-II conjugates exhibited maximum blood levels of 7–136 nM (with only 1 compound reaching at least 100 nM), while microbial metabolites exhibited maximum blood levels of 3–1170 nM (with 8 compounds reaching at least 100 nM) [12]. In an extreme example, consumption of 6 cups of green or black tea resulted in circulating metabolite levels in the mM range (hippuric acid, HA, reached 2.3 mM) [13]. This highlights the comparative importance of these metabolites as potential bioactives in circulation following the consumption of flavonoids.

Even flavonoids with comparatively high bioavailability (monomeric catechins, etc.) are only present in the bloodstream at nM to very low μM levels following consumption of typical doses in foods and supplements [14,15]. These doses are generally lower than the range of concentrations typically used to study mechanisms in cell culture models (1–100 μM, or sometimes higher). Despite poor bioavailability and low circulating concentrations, many of these compounds (and foods rich in them) appear to effectively prevent or ameliorate metabolic syndrome even at low dietary doses in animals [16] and humans [17]. Dietary efficacy, despite poor bioavailability and/or low circulating concentrations of the native forms, suggests three mechanisms by which ingested flavonoids exert their activities: (1) native flavonoids primarily exert their activities in the gut lumen (inhibition of digestive enzymes, alteration of microbiome composition and function, *etc.*) [18,19] and/or epithelium (improving barrier function, immune development, etc.) [20] where they are at highest concentrations (μM-mM range), (2) native flavonoids primarily exert their activity in peripheral tissues even at the very low (pM-low μM range) circulating levels achieved, or (3) microbial metabolites of flavonoids generated by commensal microbiota in the lower gut exert activities locally in the gut and systemically [21,22].

Considering the relatively high concentrations of microbial metabolites documented in plasma compared to the native compounds, it is plausible that these metabolites may be responsible, at least in part, for observed associations between dietary flavonoids and health outcomes. While all of the three possible scenarios identified above likely occur simultaneously, the potential anti-diabetic and anti-obesity activities of microbial metabolites formed from unabsorbed flavonoids remain poorly understood.

Recent provocative evidence has strengthened the argument that native flavonoids may exert their effects independent of systemic bioavailability: either directly on the microbiota, or by formation of bioavailable microbial metabolites that then act in peripheral tissues [23]. *In vitro*, 3-(3-hydroxyphenyl)propionoic acid (a microbial metabolite common to many flavonoids) prevented loss of insulin-stimulated nitric oxide synthesis and activity under high glucose concentrations in human aortic endothelial cells [24]. In human skeletal muscle myotubes, various microbial metabolites stimulated glucose and oleic acid uptake [25]. Recent studies demonstrated that phenylacetic and phenylpropionic acid have protective activities in pancreatic β-cells and islets [26,27] and protect hepatocytes from acetaminophen injury [28]. Two recent studies demonstrated that valerolactones inhibited monocyte adhesion to endothelial cells [29]. A key animal study demonstrated that administration of antibiotics (depletion of gut microbiota and their associated metabolites) abolished the ability of procyanidin-rich grape seed extract to prevent inflammation, insulin resistance, hyperglycemia and weight gain in a high-fat feeding mouse model [30]. Furthermore, antibiotic

administration reversed the ability of blackcurrant anthocyanins to ameliorate diet-induced obesity in mice [31]. Finally, digestion and microbial metabolism of berry flavonoids did not diminish their protective activities against colon cancer [32]. While the in vivo studies did not measure metabolite production, they strongly suggest that these effects are mediated by the microbiota and/or their metabolites produced from the native dietary flavonoids. Perhaps the most well-known microbial metabolites, the phenylalkyl acids (phenylacetic, phenyl propionic, and phenylvaleric acids) have not been well studied, and the phenylvaleric acids have not been studied at all to our knowledge. Some compounds that are microbial metabolites have been studied, but only because they also exist as native compounds in foods, such as the cinnamic acids and small aromatics such as vanillic acid. These compounds have been shown to possess anti-diabetic and anti-obesity activities in β-cell, skeletal muscle, hepatocyte and adipose models (see Supplementary Information). Finally, some microbial metabolites of flavonoids have been shown to possess enhanced anti-tumor and anti-platelet aggregation activities compared to the native forms [33].

Despite these promising findings, relatively little work has been done to characterize the effects of these metabolites in cell or animal models, in comparison to the exhaustive body of literature on the bioactivities of native flavonoids. The majority of research that does exist on these metabolites has focused on their formation, but not their activities nor mechanisms of action. Our objectives were therefore to 1) investigate the anti-diabetic activities of microbial flavonoid metabolites (including a poorly-studied class, phenylvaleric acids) in β-cells and primary skeletal muscle cells, 2) compare these activities to those of a control native flavonoid, and 3) suggest potential mechanisms by which these activities may occur. Our findings demonstrate that these metabolites possess potent bioactivities, and may contribute to the observed peripheral tissue effects of dietary flavonoids.

2. Materials and methods

2.1. Materials

Three representative metabolites representative of three distinct classes of metabolites common to a variety of dietary flavonoids were selected for investigation: hippuric acid (HA, 98%), homovanillic acid (HVA), and 5-phenylvaleric acid (5PVA, 99%) were obtained from Sigma (St. Louis, MO). A native flavanol, (−)-epicatechin (EC, Sigma), was used as a positive control; note that the three selected metabolites can be obtained by metabolism of EC and related compounds [9]. Structures of these compounds are shown in Fig. 1B. All compounds were tested over a range of 0–100 μM (depending upon the specific assay) in water or DMSO, with equal final concentrations of DMSO in cell media for all treatments. Generally, doses of 5–25 μM were employed, which are easily obtainable in circulation for metabolites but which represent the extreme upper end of what is attainable for native flavonoids [13,34]. Microbial metabolites, similar to those of native flavonoids, exhibit pharmacokinetic curves that depend on a variety of factors and circulating concentrations necessarily fluctuate over time based on consumption frequency. The levels employed herein are attainable following flavonoid consumption but are not continuously present, similar to those of native dietary flavonoids. Furthermore, while compounds and doses were uniform across experiments, differences in some aspects (treatment times, *etc.*) were necessary due to the use of established, robust experimental protocols for each model system.

2.2. Skeletal muscle experiments

Skeletal muscle metabolism experiments were conducted per previously published methods [35,36], with modifications. Primary human muscle cells were cultured for measuring palmitate and glucose oxidation. Cultures of primary human muscle cells were obtained from a singler subject who provided written informed consent under an approved protocol by Virginia Polytechnic Institute and State University Institutional Review Board (approval #11–770). The subject was a healthy Caucasian male, age 22 years, with a BMI of 23.6 and 20.9% body fat.

2.2.1. Skeletal muscle substrate metabolism

Cells were grown in low glucose DMEM supplemented with 10% fetal bovine serum and SkGM SingleQuots (Lonza, Walkersville, MD, USA). Upon reaching~80% confluence in standard 12-well plates, cells were differentiated for 7 days in 2% horse serum. All experiments were performed on day 7 of differentiation following overnight serum deprivation. The compounds tested were treated for 24 h prior to assessment of substrate metabolism. Fatty acid oxidation was assessed by measuring and summing $^{14}CO_2$ production (complete) and ^{14}C-labeled acid-soluble metabolites (incomplete) from the oxidation of [1-^{14}C] palmitic acid (American Radiolabeled Chemicals, St. Louis, MO, USA). Briefly, cells were incubated in media containing radiolabeled substrate along with the compound at 5 or 10 μM, or vehicle only (0 μM, 0.1% DMSO) for 3 h at 37 °C, 5% CO_2. Following incubation media was removed and acidified with 45% perchloric acid to elute gaseous $^{14}CO_2$. $^{14}CO_2$ was trapped in 1 M NaOH over the course of 1 h. The NaOH was then placed in a liquid scintillation counter and counted. Data were expressed as means±S.E.M. and is normalized to total protein content. Glucose oxidation was assessed by measuring $^{14}CO_2$ production from the oxidation of [U-^{14}C] glucose (American Radiolabeled Chemicals, St. Louis, MO, USA) in a manner similar to fatty acid oxidation expect for the substitution of glucose in place of palmitic acid. Compounds were tested at 10 and 25 μM.

2.2.2. Skeletal muscle cell respiration

Oxygen consumption rate (OCR) was measured with our established protocols [37] using a XF96 Seahorse Extracellular Flux Analyzer (Agilent Technologies, Santa Clara, CA, USA). C2C12 myoblast studies are commonly used by our groups as a fast and practical model to screen for compound efficacy. Because differentiating cells into myotubes takes 7 days continuously in the SeaHorse plate, we utilized the myoblasts as a more feasible approach. Cultured C2C12 muscle cells were seeded at a density of 1.5×10^4 per well in supplemented DMEM media [4.5 g/L D-Glucose, L-Glutamine, and 110 mg/L Sodium Pyruvate supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin Streptomycin (PSA)] on a Seahorse XF96 Cell Culture Microplate. Cells were then incubated overnight at 37 °C in 5% CO_2 to allow for adherence. Following adherence, cells were pretreated for 4 h with 10% FBS/1% PSA DMEM containing the test compounds (5 and 10 μM) or vehicle only (≤0.1% DMSO). After the 4-h pretreatment, 500 μM H_2O_2 was added to injure the cells, and the microplate was subsequently incubated for an additional 4 h. Following incubation, the cells were washed with supplemented XF media (XF base media plus 1 mM pyruvate, 2 mM glutamine, 10 mM glucose) twice before adding a final volume of 180 μL per well. A XF Cell Mitochondrial Stress Test was completed to assess the bioenergetic status of the cells by injecting ATP synthase inhibitor oligomycin (1 μg/mL), inner membrane uncoupler fluorocarbonyl cyanide (FCCP, 2 μM), and complex III inhibitor antimycin A (2 μM). Oxygen consumption rate data were normalized by subtracting non-mitochondrial rates of respiration (after antimycin A), and are expressed as pmol O_2 per minute per 1.5 x 10^4 cells. Mitochondrial coupling efficiency was calculated by taking the ATP-dependent respiration (baseline-oligomycin) and dividing by the basal rates for internal normalization.

2.3. β-Cell experiments

β-cell metabolism experiments were conducted per previously published methods, with modifications [38,39].

2.3.1. INS-1832/13 β-cell culture

Cell culture was performed per our established protocols [40–44]. The INS-1 derived 832/13 rat β-cell line was maintained in complete RPMI 1640 medium with L-glutamine and 11.2 mM glucose supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin, 10 mM HEPES, 10% fetal bovine serum, and INS-1 supplement, as previously described. For all glucose-stimulated insulin secretion and respiration assays using the 832/13 β-cells, cells were plated at 0 h, treated with test compounds at 24 h, and harvested at 48 h. Stock solutions of test compounds were made at 100 mM, and diluted in media for assays at final concentrations of 0–100 μM (0.1% DMSO in all treatments).

2.3.2. Glucose-stimulated insulin secretion

Glucose-stimulated insulin secretion (GSIS) was performed as previously described [40]. Briefly, INS-1832/13 β-cells were plated and grown to confluency in standard 24-well plates. Upon reaching confluency, cells were cultured with test compounds at 0–100 μM in complete media for 24 h. Following the 24 h treatment, cells were washed with PBS and preincubated in secretion assay buffer (SAB) for 1.5 h (114 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4 1.16 mM $MgSO_4$, 20 mM HEPES, 2.5 mM $CaCl_2$, 0.2% BSA, pH 7.2) containing 2.5 mM glucose. GSIS was performed by incubating quadruplicate replicate wells of cells previously cultured with test compounds in SAB containing 2.5 mM glucose for 1 h (basal), followed by 1 h in SAB with 16.7 mM glucose (glucose stimulation), followed by collection of the respective buffers, as previously described. For total insulin content, β-cells stimulated with 16.7 mM glucose for 1 h were lysed in RIPA buffer with protease inhibitors (Life Technologies). Secreted insulin and total insulin was measured in SAB using a rat insulin RIA kit (MP Biomedicals), and normalized to total cellular protein concentration (determined by BCA assay), as previously described.

2.3.3. INS-1832/13 β-cell oxygen consumption rate

Oxygen consumption rate (OCR) was measured using an XFp Extracellular Flux Analyzer (Agilent Technologies). INS-1832/13 β-cells were seeded at 2.0 x 10^4 cells/well in complete 832/13 RPMI 1640 medium (L-glutamine, 11.2 mM glucose supplemented,

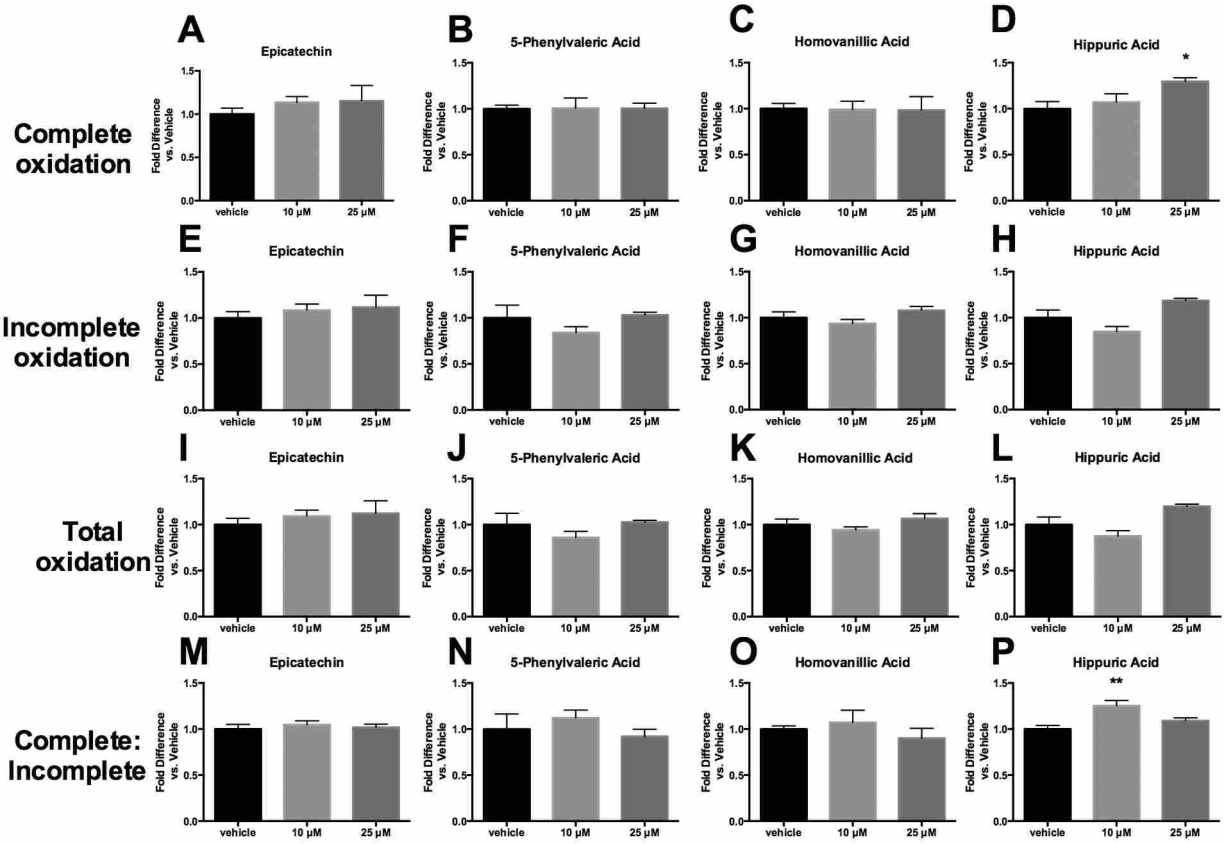

Fig. 2. Fatty acid oxidation in primary human skeletal muscle cells treated with either hippuric acid, homovanillic acid, 5-phenylvaleric acid, or epicatechin. Complete oxidation represents evolution of $^{14}CO_2$ from ^{14}C-labeled palmitate. Incomplete oxidation represents production of ^{14}C-labeled acid-soluble metabolites (ASMs) from ^{14}C-labeled palmitate. Total oxidation represents the sum of complete and incomplete oxidation. Values represent mean±S.E.M. from $n=4$ replicates, normalized to vehicle (vehicle expressed as 1). Data were analyzed by one-way ANOVA. If a significant treatment effect was detected, Dunnett's post hoc test was performed within each compound to compare each dose to the vehicle control. Significance vs. vehicle control is indicated by: $^{*}P \le 0.05$, $^{**}P \le 0.01$.

50 U/ml penicillin, 50 μg/ml streptomycin, 10 mM HEPES, 10% fetal bovine serum, and INS-1 supplement) on a Seahorse XFp Cell Culture Microplate. Cells were incubated overnight and then treated with test compounds at 10 μM, 5 μM or 0 μM in complete RPMI 1640 media. Following 24 h of culture with the compounds, cells were incubated in 2.5 mM glucose SAB for 3 h. Following incubation, buffer was exchanged for 180 μL fresh pre-warmed 2.5 mM glucose SAB per well. A XF Cell Mitochondrial Stress Test was completed to assess the bioenergetic status of the cells by injecting glucose (16.7 mM, in order to examine respiration under glucose stimulation), oligomycin (4 μM), FCCP (2.5 μM), and antimycin A with rotenone (2.5 μM). Residual oxygen consumption was determined following inhibition of complex III with the addition of rotenone and antimycin A. This state of residual oxygen consumption served as a baseline correction for all of the other states. All data were normalized to protein content of each well, determined by BCA assay.

2.3.4. Western blotting

832/13 beta cells were plated in standard 6-welll plates, grown to confluency, and cultured overnight in media containing each test compound at 10 μM or vehicle control (0.1% DMSO in both). Cells were washed in PBS and harvested in RIPA buffer followed by sonication. Protein concentration was quantified by BCA, and 30 μg was run per sample. Western blotting and transfer was performed as previously described [38,40,41]. Blot were probed using the Anti Rt/Ms. Total OxPhos Complex Kit (1:250, Life Technologies, Carlsbad, CA) which contains a cocktail of antibodies for the electron transport chain (ETC) components ATP5A (Complex V), UQCR2 (Complex III), MTCO1 (Complex IV), SDHB (Complex II) and NDUFB8 (Complex I). Blot was imaged in the linear range using a LI-COR Odyssey CLx (LI-COR Biotechnology, Lincoln, NE). Blotting was performed on triplicate samples.

2.4. Statistics

All results are expressed as mean±S.E.M. For activity assays, data were analyzed by 1- or 2-way ANOVA as appropriate. For 2-way ANOVAs, if a significant main effect of treatment compound dose was detected, Dunnett's post hoc test was performed within the high-glucose treatments to compare each dose to the vehicle (0 μM) control. For one-way ANOVAs, if a significant treatment effect was detected, Dunnett's post hoc test was performed within each compound to compare each dose to the vehicle controls. Significance was defined *a priori* as $P<.05$. Statistical analyses were performed on Prism v6.0f (GraphPad, La Jolla, CA).

3. Results and discussion

3.1. Skeletal muscle

3.1.1. Skeletal muscle metabolism

The ability of EC (+ control, native flavonoid) and three representative metabolites (HA, HVA and 5PVA) to influence fatty acid or glucose uptake and metabolism was examined in primary human skeletal muscle cells. As shown in Fig. 2, these compounds exhibited minimal ability to alter fatty acid oxidation. The only statistically significant findings were that HA was able to increase complete fatty acid oxidation at 25 μM (Fig. 2D) and increase the ratio of complete:incomplete oxidation at 10 μM (Fig. 2P). While these results suggest that HA has more potent activities than EC, overall the enhancement of fatty acid oxidation does not seem to be a significant mechanism of action for these metabolites. These results suggest that, despite a reported finding that metabolites increased oleic acid uptake in human skeletal muscle myotubes [25], alteration of fatty acid oxidation in skeletal muscle may not be a primary mechanism by which flavonoid microbial metabolites exert anti-diabetic and anti-obesity activities.

Glucose oxidation results (Fig. 3) were more promising than fatty acid oxidation. EC appeared to be the most potent stimulator of glucose utilization, increasing activity at both 10 and 25 μM (Fig. 3A).

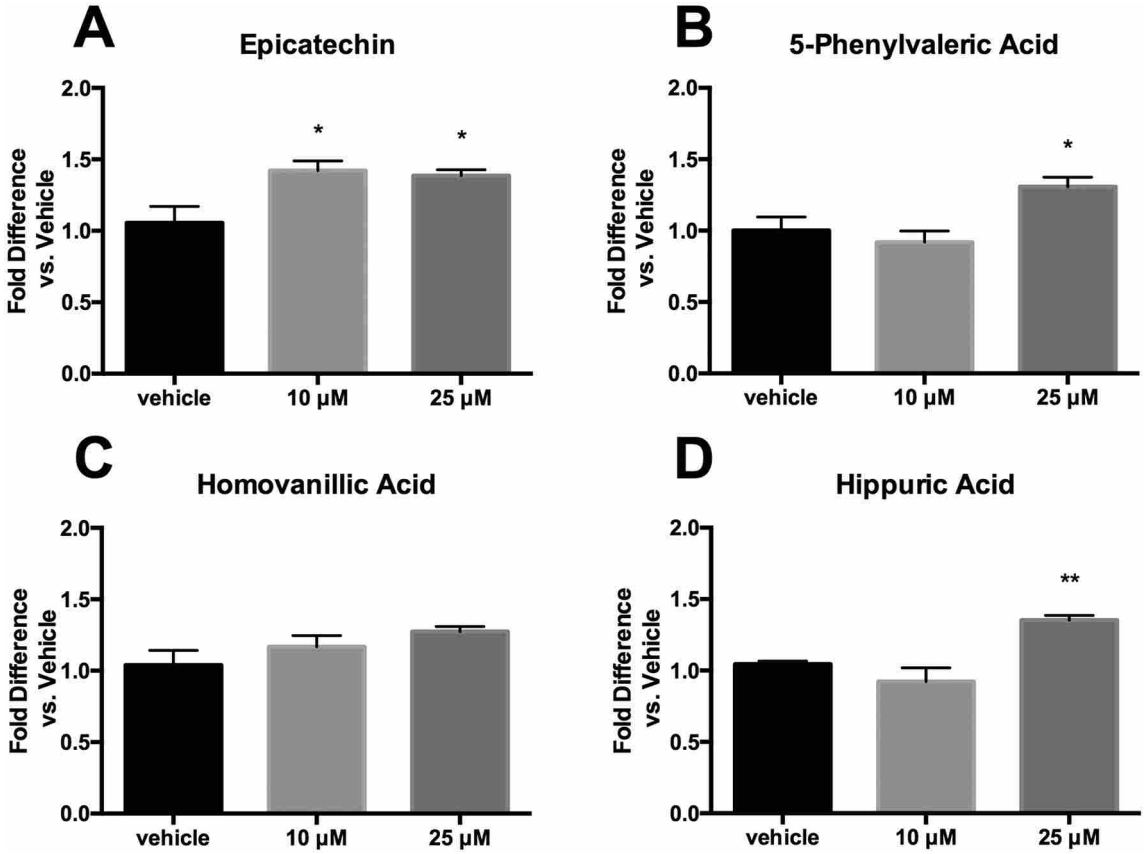

Fig. 3. Glucose oxidation in primary human skeletal muscle cells treated with either hippuric acid, homovanillic acid, 5-phenylvaleric acid, or epicatechin. Oxidation represents evolution of $^{14}CO_2$ from ^{14}C-labeled glucose. Values represent mean±S.E.M. from $n=4$ replicates, normalized to vehicle (vehicle expressed as 1). Data were analyzed by one-way ANOVA. If a significant treatment effect was detected, Dunnett's post hoc test was performed within each compound to compare each dose to the vehicle control. Significance vs. vehicle control is indicated by: $^{*}P \le 0.05$, $^{**}P \le 0.01$, $^{***}P \le 0.001$, $^{****}P \le 0.0001$.

While HVA had no apparent activity, both 5PVA and HA were able to simulate glucose metabolism at 25 μM (Fig. 3B-D). While the EC activity at lower concentrations suggests that it is more potent than the metabolites on an equal concentration basis, it is important to keep in mind that the metabolites tend to exist in circulation at higher levels than the native forms. Thus, the observed increase in glucose oxidation for 5PVA and HA, combined with previous reports that microbial metabolites stimulate glucose uptake [25], suggest promise for the ability of these metabolites to exert significant benefits on blood glucose levels in vivo.

3.1.2. Skeletal muscle cell respiration

The effects of EC and the three metabolites on respiration in normal, uninjured C2C12 cells are shown in Fig. 4. We utilized a peroxide stress paradigm since heightened mitochondrial ROS burdens are observed in skeletal muscle from humans and animal models of diabetes, often before the onset of overt systemic hyperglycemia [45]. Respiration curves for controls and each dose, including basal, leak (oligomycin) and maximal (FCCP) respiration, are shown in Fig. 4A and B. None of the compounds tested significantly enhanced basal respiration (Fig. 4C), ATP-dependent respiration (Fig. 4E), maximal respiration (Fig. 4F), or respiratory reserve (the difference between basal and maximal respiration, which reflects reserve bioenergetic capacity available to the cell, Fig. 4G) compared to the control at either 5 or 10 μM compared to vehicle control. Coupling efficiency was not influenced by any of the compounds at any concentration, with the exception of 5PVA at 10 μM (Fig. 4H). HA and 5PVA both modestly enhanced 'leak' respiration at 10 μM (Fig. 4D), suggesting either slight mitochondrial injury (potentially due to minor pro-oxidant effects at these higher doses) or mitochondrial uncoupling. The data in uninjured cells generally suggest that EC and the metabolites do not alter skeletal muscle respiration under normal conditions at low doses, and indicate that do not appear to acutely uncouple mitochondria or partially inhibit the respiratory chain (both of which have been postulated as a strategy to treat obesity/diabetes for decades) with the possible exception of HA and 5PVA at high doses [46,47].

The effects of EC and the metabolites on C2C12 cells exposed to peroxide challenge (*i.e.* injured) are presented in Fig. 5. Peroxide treatment induced mitochondrial injury as assessed by increased 'leak' respiration (respiration after oligomycin roughly doubled) (Fig. 5D) and lower rates of maximal respiration (FCCP), ATP-dependent respiration (Fig. 5E), respiratory reserve capacity (Fig. 5G), and coupling efficiency (Fig. 5H) for H_2O_2 treated cells (red bars) compared to control (blue bars). While there were some differences in basal respiration, this can be due to slight respiratory uncoupling due to the injury and should be interpreted with caution. Each of the compounds studied significantly protected against peroxide-mediated injury at 5 μM, reflected by reduced leak respiration, and preserved maximal respiration respiratory reserve and/or coupling efficiency at the same level as the uninjured control despite peroxide challenge (Fig. 5D-H). As observed for uninjured cells, one metabolite actually worsened cell injury as measured by leak respiration, although in this case it was 10 μM HVA (as opposed to HA and PVA in uninjured cells), again suggesting either cellular injury or uncoupling. Interestingly, while HA and 5PVA increased leak

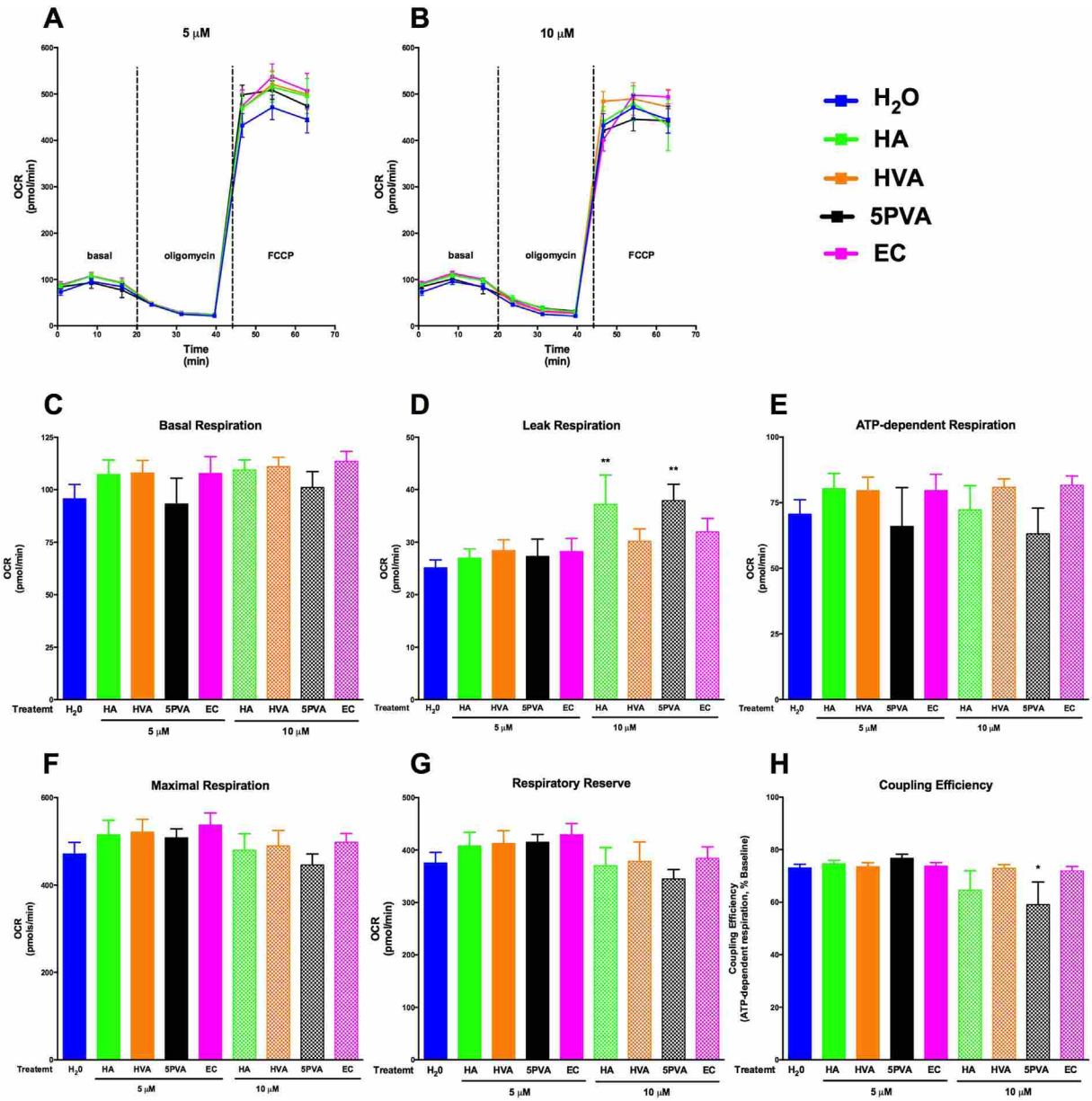

Fig. 4. Corrected mitochondrial respiration data for C2C12 cells cultured acutely (4 h) in the presence of hippuric acid (HA), homovanillic acid (HVA), 5-phenylvaleric acid (5PVA), or epicatechin (EC): oxygen consumption rate (OCR) curves for treatments at 5 μM (**A**) and 10 μM (**B**), basal respiration (**C**), leak respiration (after oligomycin, **D**), ATP-dependent respiration (**E**), maximal respiration (after FCCP, **F**) respiratory reserve (maximal − basal, **G**), and coupling efficiency (ATP-dependent respiration/basal respiration, **H**). Oxygen consumption rate data were normalized by subtracting non-mitochondrial rates of respiration (after antimycin A, not shown), and are expressed as pmol O_2 per minute per 1.5×10^4 cells. Values represent mean±S.E.M. from $n=8$ replicates. Data were analyzed by one-way ANOVA. If a significant treatment effect was detected, Dunnett's post hoc test was performed within each compound to compare each dose to the vehicle control (H_2O). Significance vs. vehicle control is indicated by: $^*P \le 0.05$, $^{**}P \le 0.01$, $^{***}P \le 0.001$, $^{****}P \le 0.0001$.

respiration in the absence of H_2O_2, there was only a slight additional increase in leak respiration with H_2O_2 treatment. These data indicate that HA and 5PVA may have pro-oxidant effects similar to H_2O_2, but that these metabolites did not exacerbate leak respiration when combined with H_2O_2 stress. Future studies that further examine the effects of HA and 5PVA will advance our understanding of these compounds on mitochondrial bioenergetics. The 10 μM dose was generally ineffective for all compounds except HA, which partly preserved respiratory reserve (Fig. 5E). These results suggest that EC and the flavonoid microbial metabolites preserve skeletal mitochondrial function after oxidative insult, notably at lower micromolar concentrations.

3.2. β-Cells

3.2.1. β-Cell glucose-stimulated insulin secretion

In addition to substrate utilization in skeletal muscle, β-cell function is a critical target at all stages of diabetes development. We sought to examine the impact of EC and representative flavonoid metabolites on GSIS in a β-cell model (Fig. 6). We have previously demonstrated that the epicatechin-rich fraction from cocoa enhances β-cell GSIS at 25 μg/ml [38]. In the present experiment, EC was able to enhance GSIS in INS-1832/13 β-cells but only at 100 μM (Fig. 6A), which is not physiologically relevant, suggesting minimal relevance for activity in vivo. Interestingly, all three microbial metabolites

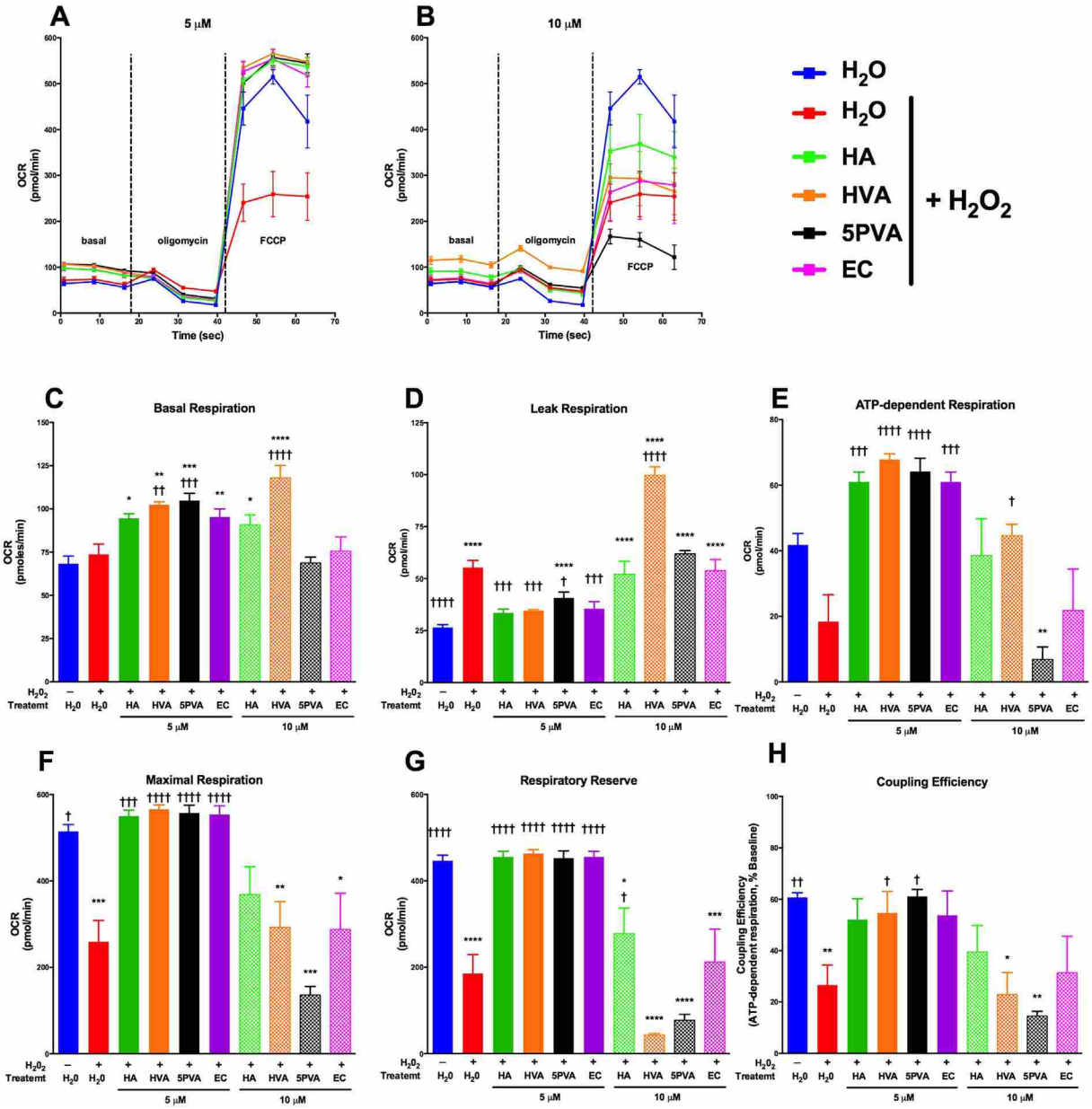

Fig. 5. Corrected mitochondrial respiration data for H_2O_2-injured C2C12 cells cultured acutely (4 h) in the presence of hippuric acid (HA), homovanillic acid (HVA), 5-phenylvaleric acid (5PVA), or epicatechin (EC): oxygen consumption rate (OCR) curves for treatments at 5 μM (**A**) and 10 μM (**B**), basal respiration (**C**), leak respiration (after oligomycin, **D**), ATP-dependent respiration (**E**), maximal respiration (after FCCP, **F**) respiratory reserve (maximal − basal, **G**), and coupling efficiency (ATP-dependent respiration/basal respiration, **H**). Oxygen consumption rate data were normalized by subtracting non-mitochondrial rates of respiration (after antimycin A, not shown), and are expressed as pmol O_2 per minute per 1.5×10^4 cells. Values represent mean±S.E.M. from $n=8$ replicates. Data were analyzed by one-way ANOVA. If a significant treatment effect was detected, Dunnett's post hoc test was performed within each compound to compare each dose to the vehicle control (H_2O) as well as injury control ($H_2O + H_2O_2$). Significance vs. vehicle control is indicated by: $^*P\leq0.05$, $^{**}P\leq0.01$, $^{***}P\leq0.001$, $^{****}P\leq0.0001$; significance vs. injury control is indicated by: †$P \leq 0.05$, ††$P \leq 0.01$, †††$P \leq 0.001$, ††††††$P \leq 0.0001$.

demonstrated significant induction of GSIS at concentrations from 5–100 μM (Fig. 6B-D) except HA, which induced GSIS at 5–50 μM but not 100 μM). These data demonstrate that the metabolites increase GSIS at much lower (and physiologically relevant) concentrations compared to EC, suggesting that the metabolites are more potent stimulators of GSIS than native EC. This fact, combined with the greater bioavailability of microbial metabolites than the parent compound, point towards the potential contribution of microbial metabolites to the observed effects of dietary flavanoids.

To further investigate the effects of these compounds on INS-1832/13 β-cells, we examined the cellular insulin content under stimulatory conditions (16.7 mM glucose) to determine if treatment impacted insulin expression (Fig. 7). An increase in insulin content, concomitant with an increase in insulin secretion would indicate greater insulin expression, while a decrease in insulin content with no change in insulin secretion would indicate an impediment in insulin production. EC exhibited small increases in insulin content (Fig. 7A), but the effect was inconsistent across doses. Interestingly, increases in insulin content were vastly different across the metabolites (Fig. 7B-D), despite similarities observed in GSIS. 5PVA and HVA stimulated greater insulin content, particularly at lower doses. HA exhibited a slight increase in insulin content at 50 μM. These results are intriguing, as they suggest distinct mechanism at play that impinges on β-cell insulin secretion. The results for EC are consistent with our previous

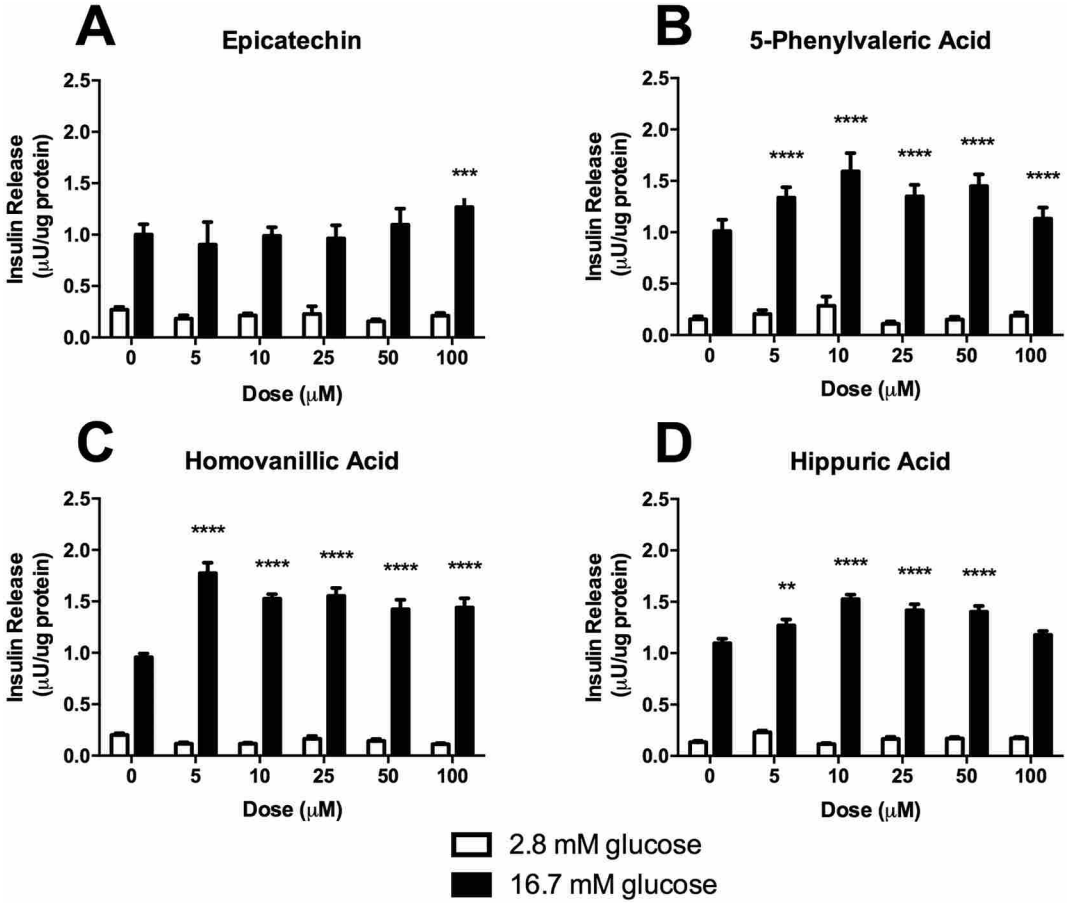

Fig. 6. Glucose-stimulated insulin secretion in INS-1 derived 832/13 rat β-cells treated with either hippuric acid, homovanillic acid, 5-phenylvaleric acid, or epicatechin. Values represent mean±S.E.M. from $n=6$ replicates. Data were analyzed by 2-way ANOVA. If a significant main effect of treatment compound dose was detected, Dunnett's post hoc test was performed within the high-glucose treatments to compare each dose to the untreated (0 μM) control. Significance vs. untreated control is indicated by: $^{*}P \le 0.05$, $^{**}P \le 0.01$, $^{***}P \le 0.001$, $^{****}P \le 0.0001$.

results demonstrating increased insulin secretion at high doses, without concurrent increase in insulin content [38]. For 5PVA and HVA we observed increased GSIS and increased cellular insulin content. The increased insulin content could be due to greater insulin gene expression, enhanced insulin processing, or improved insulin stability. As has been previously shown, increased cellular insulin content can be sufficient to enhance GSIS [48]. Therefore, the enhanced insulin secretion from β-cells treated with these metabolites, particularly at lower doses, may be due to an increased insulin load, rather than modulation of the β-cell glucose sensing machinery. The GSIS observed by HA occurs with minimal changes to insulin content. The data suggest that flavonoid microbial metabolites may exert significant effects on β-cell function by increasing both β-cell insulin production and insulin secretion. These distinct mechanisms suggest complementary and synergistic activities of various metabolites present simultaneously following flavonoid consumption, and thus warrant further investigation in vitro and in vivo.

3.2.2. β-Cell respiration

Given our previous data demonstrating enhanced β-cell mitochondrial respiration due to exposure to EC from cocoa [38,39], we sought to define the effect of culture in the presence of EC and microbial metabolites on β-cell mitochondrial respiration under basal conditions (low glucose) and glucose stimulation (Fig. 8). Basal respiration rate was significantly increased by 10 μM EC, and appeared to be somewhat reduced (albeit not statistically significantly) by 5 and 10 μM HA (Fig. 8C). The same results were also observed under glucose stimulation and maximal respiration (although the level of glucose induced respiration is surprisingly less than what has been observed in other studies) (Fig. 8D-E). None of the compounds tested significantly affected respiratory reserve (Fig. 8F). It is important to note that the low means and comparatively high S.E.M.'s for respiratory reserve in this case are indicative of the fact that these cells were essentially already operating near maximal respiration in the basal state (Fig. 8A-B, F). Note that uncoupling and ATP-dependent respiration were not plotted individually from these data due to differences in the question being asked between the β-cells (do these compounds enhance respiration as a means to improve β-cell function?) vs. the skeletal muscle cells (do these compounds enhance respiration *via* uncoupling as a means to improve energy expenditure, and do they protect from injury?). The finding that EC enhances respiration is consistent with our previous data [38,39]. Coupled with the GSIS data (Fig. 6), these respiration data suggest several novel findings. First, EC does not enhance GSIS except at extremely high doses despite enhancing β-cell respiration at lower doses. Second, HA enhances GSIS despite inhibition of β-cell respiration (although these reductions were not statistically significant, this trend appears to be of practical significance as suggested by Fig. 8C-E). Third, HVA and 5PVA enhance GSIS despite not affecting β-cell respiration. Thus, these data demonstrate that while each of the epicatechin metabolites enhances GSIS; their individual mechanisms do not all increase insulin release through modulating mitochondrial respiration. Therefore, the mechanisms by which these compounds exert their effects are likely distinct and thus warrant further investigation.

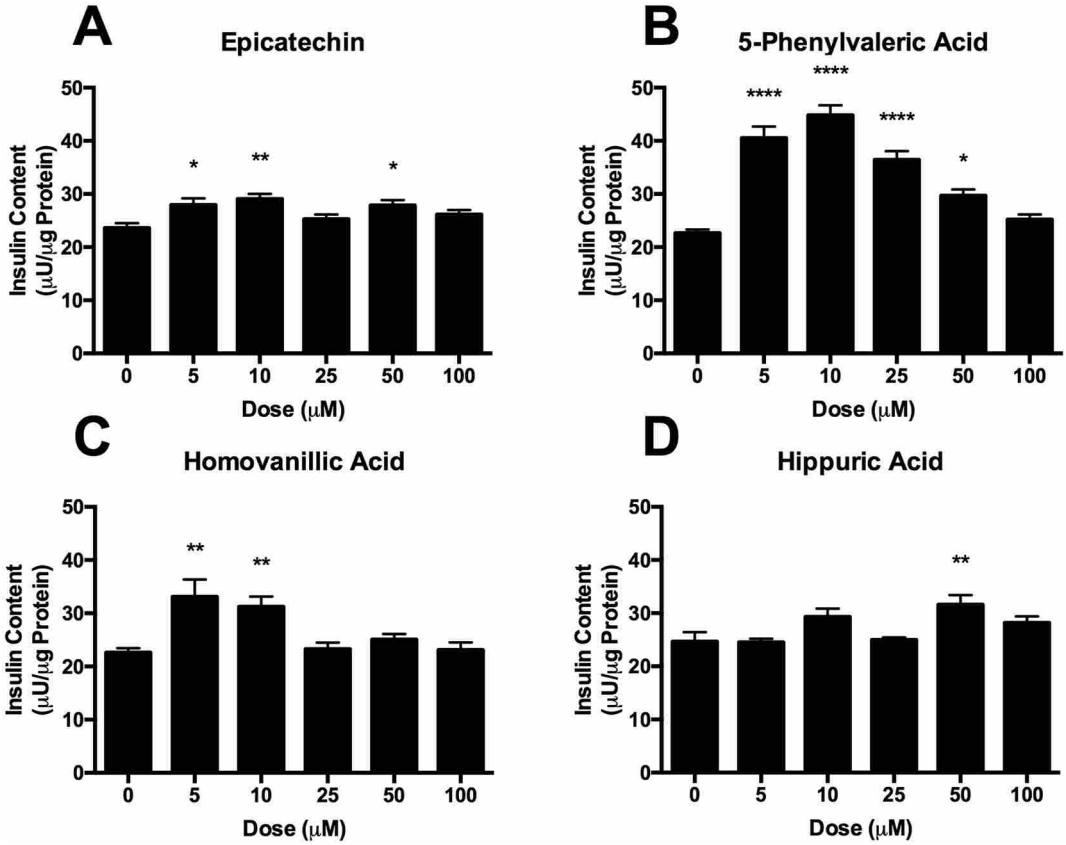

Fig. 7. Total insulin content of INS-1 derived 832/13 rat β-cells cultured in 16.7 mM glucose treated with either hippuric acid, homovanillic acid, 5-phenylvaleric acid, or epicatechin. Values represent mean±S.E.M. from $n=6$ replicates. Data were analyzed by one-way ANOVA. If a significant treatment effect was detected, Dunnett's post hoc test was performed to compare each dose to the untreated (0 μM) control. Significance vs. untreated control is indicated by: $^{*}P \le 0.05$, $^{**}P \le 0.01$, $^{***}P \le 0.001$, $^{****}P \le 0.0001$.

3.2.3. Expression of ETC components

To validate the changes that we observed in β-cell respiration after treatment with EC or the gut metabolites, we measured protein levels of select ETC components (Fig. 9). Similar to what was observed in our mitochondrial respiration studies, only treatment with EC changed protein levels of ETC components. These data validate our previous findings that while the metabolites do enhance glucose stimulated insulin secretion, it appears to be through extra mitochondrial modifications.

3.3. Discussion

The premise of this study was to explore the possibility that the unique activities of microbial flavonoid metabolites on peripheral tissues may contribute to the observed bioactivities of native dietary flavonoids. In other words, can dietary flavonoids exert significant bioactivities despite poor bioavailability, or is bioavailability of the native dietary species at peripheral target tissues indeed the primary limiting factor for bioactivity in vivo? Our central hypothesis, spanning this study and others in progress, is that the systemic, peripheral tissue activities of microbial metabolites may account for a significant portion of observed bioactivity following dietary flavonoid exposure in vivo.

The present results demonstrate the potent activities of flavonoid microbial metabolites, particularly for preservation of β-cell function, enhancement of skeletal muscle glucose utilization and protection of skeletal muscle respiratory function from oxidative injury, Therefore, these data suggest that further investigation of the anti-diabetic activities of flavonoid microbial metabolites is warranted. Additionally, our data suggest that metabolites and native compounds may act by distinct mechanisms, suggesting complementary and synergistic activities in vivo. Specifically, our data demonstrate that the gut metabolites enhance β-cell glucose stimulated insulin secretion more effectively than EC. Furthermore, unlike EC, these metabolites appear to do this without enhancing mitochondrial respiration or increasing expression of mitochondrial electron transport chain components, and with varying effects on β-cell insulin content. Insulin secretion is dependent on ATP production in the β-cell due to glycolysis, TCA cycle and the *ETC*. In addition, the increases in ATP closes K^+ channels which cause membrane depolarization and opening of Ca^{2+} channels which allow Ca^{2+} influx. The modulation of these two channels is an area of future interest in determining how the metabolites enhance glucose stimulated insulin secretion. In skeletal muscle, these compounds appear to enhance glucose utilization, but do not appear to enhance respiration under normal conditions. Therefore, mitochondrial uncoupling does not appear to be a mechanism by which these compounds can prevent obesity and glucose intolerance, with the exception of HA and 5PVA at high doses. However, they do appear to significantly protect respiratory function against oxidative injury. The objective of these respiration experiments was to evaluate the impacts of the selected compounds on overall respiration. Future mechanistic experiments, including use of ETC complex inhibitors as well as comparing intact cells, permeabilized cells and isolated mitochondria, will be useful to elucidate the specific mechanisms by which the microbial metabolites exert these effects on respiration. Future work will also provide new insight that address some of the current study

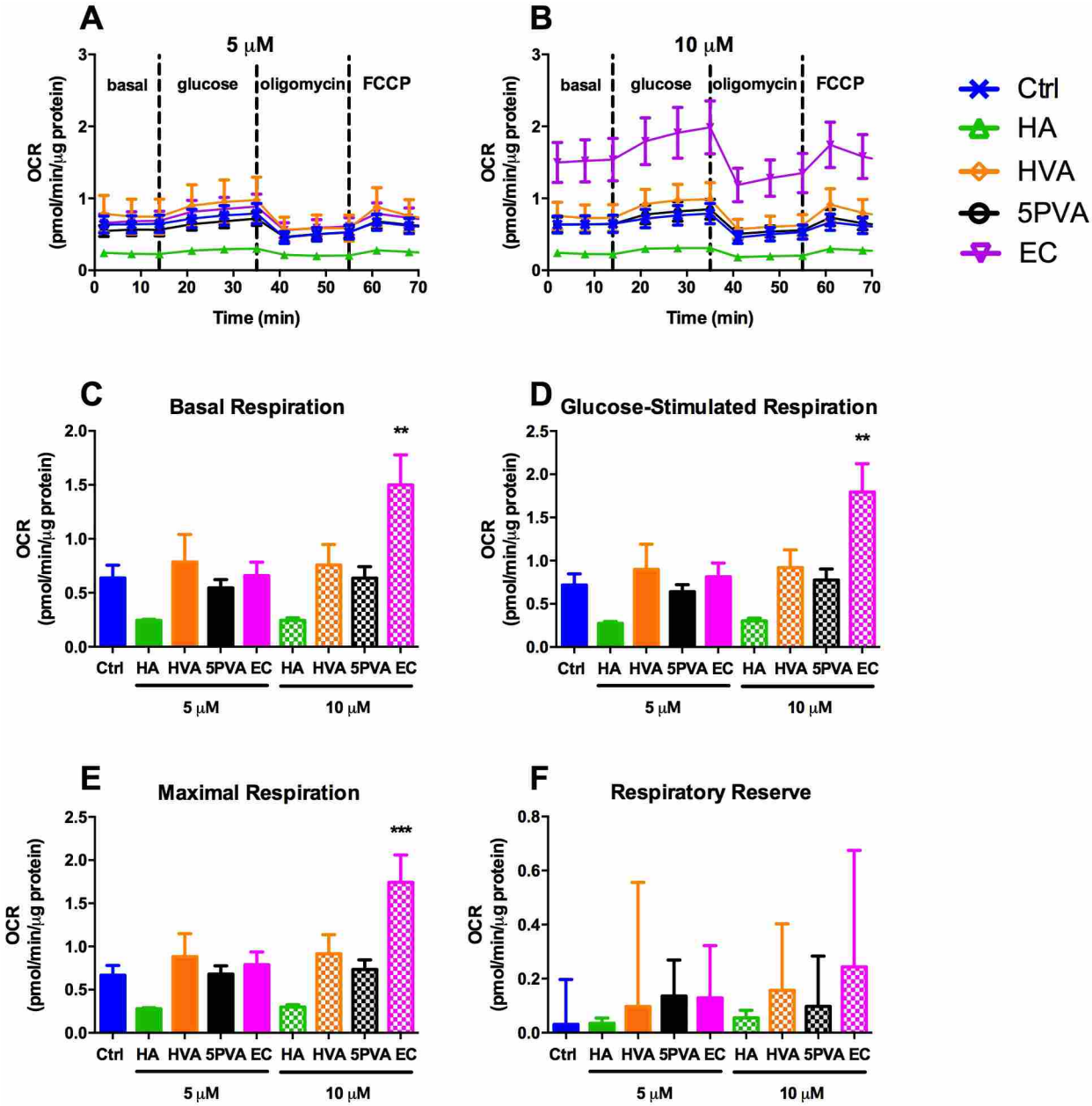

Fig. 8. Corrected mitochondrial respiration measured after culturing INS-1832/13 β-cells for 24 h in the presence of 0, 5 or 10 μM hippuric acid (HA), homovanillic acid (HVA), 5-phenylvaleric acid (5PVA), or epicatechin (EC): (**A**) 0 (Ctrl) and 5 μM, (**B**) 0 (Ctrl) and 10 μM, (**C**) Basal respiration (2 min), (**D**) glucose-stimulated respiration (21 min), (**E**) maximal respiration (61 min) and (**F**) respiratory reserve (maximal − basal). Oxygen consumption rate data were normalized by subtracting non-mitochondrial rates of respiration (after antimycin A, not shown), and are expressed as pmol O_2 per minute, normalizer per μg protein. Values represent mean±S.E.M. from $n=5$ replicates. Significance vs. untreated control is indicated by: $^{*}P \le 0.05$, $^{**}P \le 0.01$, $^{***}P \le 0.001$, $^{****}P \le 0.0001$.

limitations, such as examining compound efficacy in differentiated muscle myotubes from mouse and human (to compliment myoblast studies that were conducted herein).

The results presented here make significant additions to the small, yet growing, body of published data indicating that flavonoid microbial metabolites likely account for a significant fraction of many observed bioactivities of dietary flavonoids, particularly those with poor oral bioavailability of the native forms. These data help to explain epidemiological and experimental data suggesting that some dietary flavonoids (and potentially other classes of compounds, such as curcuminoids)possess potent bioactivities despite poor oral bioavailability. These results also suggest that the metabolites may be equally important to, if not more important than (in some cases), the native forms for in vitro mechanistic studies in cell culture models that attempt to recapitulate effects in peripheral tissues (hepatic, adipose, pancreatic, skeletal muscle, endothelial and other cell models). This is particularly true at compound doses in the mid to high μM range, which are commonly used for bioactives in cell culture but which are much more likely to be obtained by the microbial metabolites than the native dietary forms.

Moving forward, there is a need to further identify the most active individual metabolites (or metabolite profiles) that confer systemic benefits, to understand the characteristics of the microbiome that facilitate generation of these profiles, and to understand how inter-individual variability in microbial metabolism affects subsequent metabolite profiles and bioactivities [49]. This knowledge will be

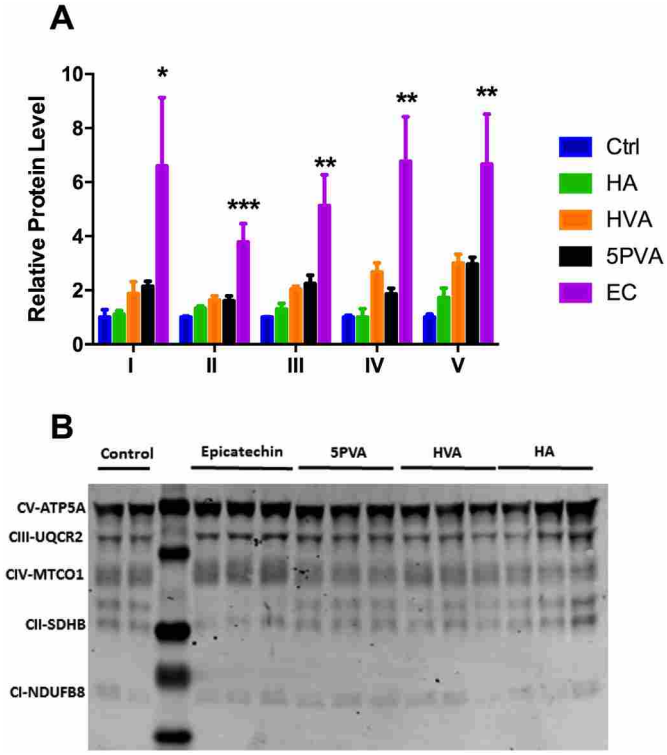

Fig. 9. (A) Expression levels of electron transport chain components ATP5A (Complex V), UQCR2 (Complex III), MTCO1 (Complex IV), SDHB (Complex II) and NDUFB8 (Complex I) as quantified by Western blotting. Values are presented as mean±S.E.M. from $n=3$ replicates per condition. Data were analyzed by one-way ANOVA. If a significant treatment effect was detected, Dunnett's post hoc test was performed to compare each dose to the untreated (0 μM) control. Significance vs. untreated control is indicated by: $^{*}P \le 0.05$, $^{**}P \le 0.01$, $^{***}P \le 0.001$, $^{****}P \le 0.0001$. (B) Representative Western blot.

critical for development of strategies to fully exploit the potential health benefits of dietary flavonoids. While initial studies have used antibiotics to eliminate the effect of the microbiome and microbiome-derived metabolites [30,31], germ-free and other gnotobiotic models will be instrumental in elucidation of the role of the microbiome in mediating the beneficial effects of poorly-bioavailable flavonoids. Furthermore, large-scale screening of several dozen (if not libraries of several hundred) microbial metabolites in peripheral tissue cell culture models will need to be performed in order to understand the tissue-specific mechanisms by which these compounds exert their activities. This will require advances in commercial availability of some metabolites, specifically the valerolactones, which to our knowledge are not currently available. It will also be important to conduct full dose-dependence studies of these metabolites. Furthermore, in vitro anaerobic fecal fermentations of flavonoids, with assessment of the bioactivity before and after fermentation in vitro and in vivo (*via* i.p. administration of filter-sterilized supernatants) will be useful to identify broad effects of microbial transformation.

It is important to note that we did not study valerolactones, which are among the early microbial metabolites of flavonoids. These compounds are present in high concentrations in circulation following flavonoid intake, and represent important compounds that may possess significant bioactivities. We did not study these compounds due to the lack of commercial availability, which is a significant obstacle for understanding their activities. Due to the provocative data in the present work, future work is needed to generate, isolate, and elucidate the activity of valerolactones. Two possible approaches include isolation from in vivo or ex vivo fecal fermentation mixtures, as well as synthetic approaches. These will need to be performed in order to complete our understanding of the potential bioactivities of flavonoid microbial metabolites.

It is also important to note that these microbial metabolites exist in circulation in the unconjugated forms studied, as well as Phase-II conjugates (sulfate, *O*-methyl and glucuronide forms) produced in enterocytes and hepatocytes following their absorption [50]. While the present work focused on the unconjugated forms, future work needs to be performed to elucidate the bioactivities of the conjugated forms. Such transformations can be performed using enterocytes, hepatocytes, liver microsomes, or isolated conjugating enzymes. Such studies will further advance the overall objective of the present work which is to understand the bioactivities of the actual circulating profile of compounds (unconjugated and phase-II conjugates of both native dietary flavonoids and their microbial metabolites) as opposed to just the native, unconjugated forms (*i.e.* the majority of existing studies).

4. Conclusion

In summary, our data demonstrate that flavonoid microbial metabolites stimulate β-cell function, as well as glucose utilization and mitochondrial respiration in skeletal muscle. These data support the hypothesis that dietary flavonoids may exert significant activity despite poor bioavailability *via* their microbial metabolites. This raises the intriguing prospect that bioavailability of native flavonoids may not be as critical of a limiting factor to bioactivity as previously thought. If, in fact, bioavailability of native flavonoids is not as crucial as currently thought, this would represent a paradigm shift in the thinking regarding how to exploit the activities of flavonoids in the diet. While development of strategies to enhance bioavailability of native compounds should not be discontinued, exploration of strategies that do not require bioavailability should receive extensive consideration as a parallel complementary approach to solving the same problem. Our overall logic for the proposed experiments moving forward is that we are quickly approaching an asymptote (diminishing novel returns) in terms of what we can learn from further studies focusing on the activities of native flavonoids. New approaches are now needed to answer the complex questions remaining.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jnutbio.2018.09.004.

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