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The Effects of Immune Regulation and Dysregulation: Helper T Cell Receptor Affinity, Systemic Lupus Erythematosus and Cancer Risk, and Vaccine Hesitancy

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A dissertation submitted to the faculty of
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ABSTRACT

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Helper T cells direct the immunological response to foreign pathogens and cancer. To become activated, helper T cells must recognize unique peptides presented on major histocompatibility complex II (pMHCII) by antigen presenting cells (APCs) with their T cell receptor (TCR). While much is known about helper T cell activation signaling cascades and the subsequent roles of helper T cell subsets, the initiation of helper T cell activation by the TCR and other co-receptors is less well understood. Specifically, the affinity of the TCR for its pMHCII can change helper T cell subset fate, proliferation, and alter the risk for activation induced cell death. High affinity TCRs are attractive targets for immunotherapies, but little is known about how helper T cells respond to high affinity TCRs. Here we describe high affinity TCR activation thresholds for both full length TCRs and chimeric antigen receptor TCRs both with and without the presence of the coreceptor CD4 and propose a mechanism whereby CD4 inhibits T cell activation via Lck sequestration and a CD4-independent method.

Dysregulated helper T cells play critical roles in the development and perpetuation of systemic lupus erythematosus (SLE), a systemic autoimmune disease that causes widespread inflammation and organ damage throughout the body. Chronic inflammation in SLE affects the immune response to viruses and the risk of developing cancer. However, in SLE patients, it is unclear if viruses initiate the development of cancer directly or if the effects are non-interacting and concomitant. Here we describe the interactions between SLE, viruses, and cancer risk revealing that viruses and SLE do interact to increase the both the overall cancer risk and the risk for hematological malignancies.

Due to vaccine efficacy, vaccine preventable diseases (VPDs) are no longer commonly experienced or understood by the public. Vaccines are a victim of their own success and according to the World Health Organization (WHO), vaccine hesitancy (VH) is one of the top threats to global health. VH is the refusal to accept vaccinations and the reasons for VH vary across time, place, and vaccine. Refuting VH is difficult as directly confronting false assumptions can cause individuals to become more entrenched in their position resulting in confirmation bias. Adults with VH attitudes are often motivated by concerns over personal liberty, harm, independence, and body purity. Here we describe the results of a VPD interview- and education-based intervention geared towards promoting positive vaccine attitudes for young adults and demonstrate that education focused on VPDs is more effective than vaccine safety.

Keywords: Helper T cell, $CD4^+$ T cell, CD4, T cell receptor (TCR), Lck, IL-2, T cell activation, TCR single chain signaling (TCR-SCS), chimeric antigen receptor (CAR), full length TCR (flTCR), high affinity TCRs, systemic lupus erythematosus (SLE), cancer-risk, viruses, vaccine hesitancy (VH), vaccine preventable diseases (VPDs), vaccines

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ABBREVIATIONS

4-1BB—CD137

AICD—Activation induced cell death

Akt—Protein kinase B (also known as PKB)

Anti-dsDNA—Anti-double stranded DNA antibodies

AP-1—Activator protein 1

APC—Antigen presenting cell

ATP—Adenosine triphosphate

BAFF—B cell activating factor

Bcl-2—B cell lymphoma 2

Bcl-XL—B cell lymphoma-extra large

Bfl-1—Member of Bcl-2 family

Bim—Bcl-2-like protein 11

BLI—Bio-layer interferometry

BRCA2—Breast cancer susceptibility gene 2

Ca^{2+}—Calcium ion

cAMP—Cyclic adenosine monophosphate

CaMKIV—Calcium/calmodulin kinase IC

CAR—Chimeric antigen receptor

CD(x) —Cluster of differentiation (#)

CD1-d—Non-classical MHC

CDR3β—Complementarity determining regions β

CI—Confidence interval

CI diff—Confidence interval difference

CMV—Cytomegalovirus

CNS—Central nervous system

CRAC channel—Calcium release-activated channels

CREMα—cAMP responsive element modulator α

DN—Double negative

EBV—Epstein Barr virus

EC_{50}—Half maximal effective concentration

ER—Endoplasmic reticulum

FcRγ—Fragment constant region γ

flTCR—Full length T cell receptor

GFP—Green fluorescing protein

GM1—Monosialotetrahexosylganglioside

Grb2/Sos—Growth factor receptor-bound protein 2/Son of Sevenless

HA—Hemagglutinin

HEP—Hepatitis

HPV—Human papilloma virus

HSP-27—Heat shock protein 27

$I\text{-}A^b$—Major histocompatibility complex II $I\text{-}A^b$

ICOS—Inducible T-cell COStimulator

IFN—Interferon

IL—Interleukin

Int—Intermediate

ITAMS—Tyrosine based activation motif

JNK—c-Jun N-terminal kinase

k_a—Association constant

k_d—Dissociation constant

K_D—Equilibrium dissociation constant

LAT—Linker for activation of T cells

Lck—Lymphocyte-specific protein tyrosine kinase

LLO—Listeria lysine O

MAPK—Mitogen-activated protein kinase

MHC—Major histocompatibility complex

MFI—Mean fluorescent intensity

MS—Multiple sclerosis

mRNA—Messenger ribonucleic acid

mTOR—Mammalian target of rapamycin

NFAT—Nuclear factor of activated T-cells

NFκB—Nuclear Factor kappa-light-chain-enhancer of activated B cells

NKT—Natural killer T cell

NME—Non-medical exemption

pMHC—Peptide MHC complex

pMHCI—Peptide MHCI complex

pMHCII—Peptide MHCII complex

OX40—CD134

PI3K—Phosphatidylinositol-3-kinase

PIP2—Phosphatidylinositol (4,5)-bisphosphate

PIP3—Phosphatidylinositol (3,4,5)-triphosphate

PLCγ1—Phosphoinositide phospholipase C

PP2a—Protein phosphatase 2a

PV—Pro-vaccine

ROCK—Rho associated protein kinase

ROI—Reactive oxygen intermediates

SCS—Single chain signaling

scTCR—Single chain T cell receptor

SH-2—Src homology 2

SLE—Systemic lupus erythematosus

SLP-76—Lymphocyte cytosolic protein or SH2 domain containing leukocyte protein of 74kDa

Syk—spleen tyrosine kinase

$t_{1/2}$—Half-life

TAA—Tumor associated antigens

TCR—T cell receptor

TCR-pMHC—T cell receptor peptide major histocompatibility complex

TCR-SCS—T cell receptor single chain signaling

TGFβ—Tumor growth factor β

T_h1—T helper 1

T_h2—T helper 2

T_h17—T helper 17

T_{fh}—Follicular helper T cell

TRAF—TNF receptor associated factors

TNF—Tumor necrosis factor

T_{reg}—Regulatory helper T cell

Vα2—T cell receptor variable domain alpha 2

VAS—Vaccine Attitude Score

Vβ2—T cell receptor variable domain beta 2

VH—Vaccine hesitant

VPDs—Vaccine preventable diseases

VUMC SD—Vanderbilt University Medical Center Synthetic Derivative

WHO—World Health Organization

WT—Wild type

ZAP-70—Zeta-chain-associated protein kinase 70

CHAPTER 1: Immune Functions in Immunotherapy and Disease

1.1 Introduction: The role of helper T cells in immune responses

Helper T cells coordinate the immune system's cellular and humoral response to cancer or infectious disease [1-3]. Each helper T cell has a unique T cell receptor (TCR) which recognizes a specific antigen, a short intracellular polypeptide presented on the major histocompatibility complex II (MHCII) by antigen presenting cells (APC) such as macrophages, dendritic cells, and B cells [2, 4, 5]. APCs phagocytose, digest and present foreign material, including mutated cancer cell proteins. This ability to recognize foreign peptides or mutated intracellular targets makes TCRs important assets for cancer and infectious disease immunotherapies [3, 5, 6].

T cells originate in bone marrow as hematopoietic stem cells and mature in the thymus. Similar to B cell antibodies, TCRs are stitched together from several multiple-copy gene fragments to generate a staggering array of TCR diversity. The mature "classic" TCR is a heterodimer comprised of an α and a β chain. Once naïve helper T cells display a mature TCR, they undergo several rounds of selection [7]. To become a circulating peripheral helper T cell, naïve helper T cells must be able to recognize MHCII while not binding too tightly to self-peptide-MHCII [8]. If the naïve helper T cell fails either test it will undergo apoptosis, thus reducing the likelihood of self-reactive helper T cells reaching the periphery and causing autoimmunity [8]. As such, most peripheral helper T cells that make it through thymic selection have affinity for foreign pMHC in the low range, with K_D values of approximately 5-90 μM [9, 10].

Once activated, helper T cells play a critical role in coordinating immune system cell function. To respond effectively to unique immunological threats such as viruses, bacteria, and

parasites, activated helper T cells can differentiate into distinct effector subtypes (Figure 1). These subtypes modulate the immune response by secreting specific cytokines (intercellular immune cell signaling proteins) to stimulate, as needed, innate immune system cells, B cells, cytotoxic T cells, and non-immune cells, and can even suppress the immune reaction [11]. Helper T cell differentiation into subsets is controlled by a complex network of specific cytokine signaling, TCR affinity for peptide, and transcription factors, followed by epigenetic modifications [11].

Two helper T cell subsets, T_h1 and T_h2, can illustrate this point. T_h1 cells organize the immune response to intracellular pathogens by stimulating cytotoxic T cells, macrophages, and microglial cells to kill infected body cells [12, 13]. T_h2 cells assist the immune response to extracellular parasites including helminths, by promoting the activation of antibody producing B-cells, and anti-parasite eosinophils, neutrophils, and mast cells [14]. A naïve helper T cell is stimulated by foreign peptide-MHCII (pMHCII) while simultaneously receiving cytokine signals from the APC. To stimulate T_h1 development, APCs produce cytokines IL-12 and IFN-γ [15], whereas cytokines IL-4 and IL-2 augment T_h2 development [16]. These cytokines induce activation of exclusive transcription factors in the T cell (T_h1: T-bet and STAT4; T_h2: STAT6, GATA3, and STAT5) [17-25], which aid in the upregulation of genes required for each subset function while actively inhibiting the activation of other subset functions [26]. T_h1 and T_h2 further differentiate and express their own cytokines, which promote recruitment and activation of specific immune cell subsets. For example, T_h1 produce IFN-γ and IL-2 which respectively enhances phagocytic activities of macrophages and microglial cells, or promotes killer T cell proliferation, cytotoxic capacities and memory responses [13, 27, 28]. Contrastingly, T_h2 produce a milieu of cytokines (IL-4, IL-5, IL-9, IL-10, IL-13, IL-25 and amphiregulin) that can

promote mast cell responses [29], activate eosinophils [30], B cells, neutrophils and airway epithelial cells [14], and inhibit T_h1 responses [31-33]. This complex system of initial inputs influencing downstream T cell behavior, allows for nuanced control of immune responses.

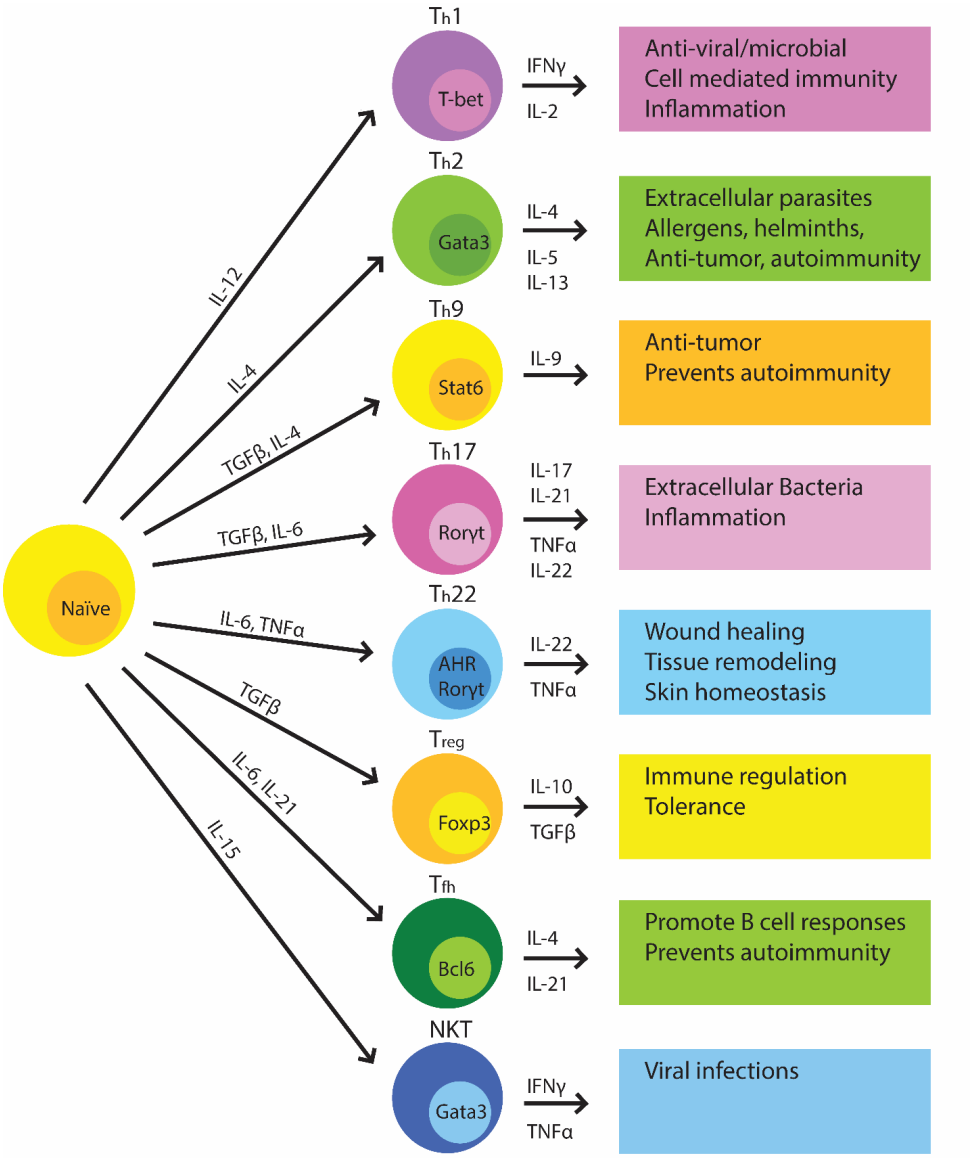

Figure 1. Naïve helper T cells become unique subsets

Naïve helper T cells receive unique signals upon activation to become unique helper T cell subsets with distinct immune responses. Cytokines produced by APCs (far left arrows) influence the activation of master transcription factors in T cells that determine helper T cell subset fate and T cell cytokine production, which influences the immune response. This includes activating specific immune cell subsets, influencing behavior of non-immune cells, or suppress the immune response (Treg) [34, 35].

1.1.1 TCR signaling

The most crucial step to downstream helper T cell activation behavior is TCR recognition of pMHCII. TCR molecular architecture must relay information from the TCR-pMHCII interface to internal signaling molecules that then recruit kinases to interact, initiate and sustain activation signaling. TCR signaling is crucial throughout the life of the T cell including, T cell development, survival, cell fate decisions [8], and T cell memory response [36-39]. Despite the many functions of TCR signaling, the central T cell biology question: "How does TCR signaling begin?" remains difficult to answer simply [40]. Downstream T cell responses are clearly modulated by the strength of TCR signaling thru diverse factors including TCR-pMHC affinity, antigen concentration, the quality of peptide presented, and co-stimulatory molecule signaling combinations [36], however, it is poorly understood how each unique contribution interacts to produce helper T cell responses.

To appreciate the complexity of TCR signaling, consider basic T cell activation as it is currently understood (Figure 2). Each TCR signaling apparatus is a conglomerate of several proteins including multiple CD3 subunits and coreceptor CD4 with its cytoplasmic partner Src-family tyrosine kinase Lck (Lck). TCRs cannot signal directly into the cell and rely on CD3 chains to transmit the transmembrane signal. Once the TCR recognizes its cognate pMHCII, coreceptor CD4 recognizes MHCII and brings Lck into close proximity with the CD3 subunits. Lck phosphorylates ITAMs (immunoreceptor tyrosine-based activation motif) on the cytosolic tails of the CD3 subunits, which then serve as high affinity docking sites for SH2 domains of the ZAP-70 kinase. Lck also phosphorylates and binds to ZAP-70, which induces full activation [41]. The ZAP-70/Lck complex recruits LAT adaptor protein and the ZAP-70 substrates LAT and SLP-76 [42]. Once LAT is phosphorylated by ZAP-70 it forms a signalosome with

signaling molecules (SLP-76, Grb2/Sos, PLCγ1, and Vav1) and regulates downstream effector events required for T cell activation [43]. This downstream signaling is well understood (see T_h1 and T_h2 discussion), and includes activating MAPK and Ca^{+2} signaling pathways, cytoskeletal reorganization, integrin activation, and the eventual activation and translocation to the nucleus of critical transcription factors NFAT, NFκB, and AP-1 which control IL-2 expression [44, 45]. Other coreceptors like CD28, 4-1BB, and CD5, provide secondary costimulatory signals that either promote the activation signal or prevent cell death from overstimulation [16] (Figure 2). To explore further the nuances of helper T cell activation, the next section will discuss the contributions of coreceptors CD4, CD28, and 4-1BB to promoting or attenuating activation signals.

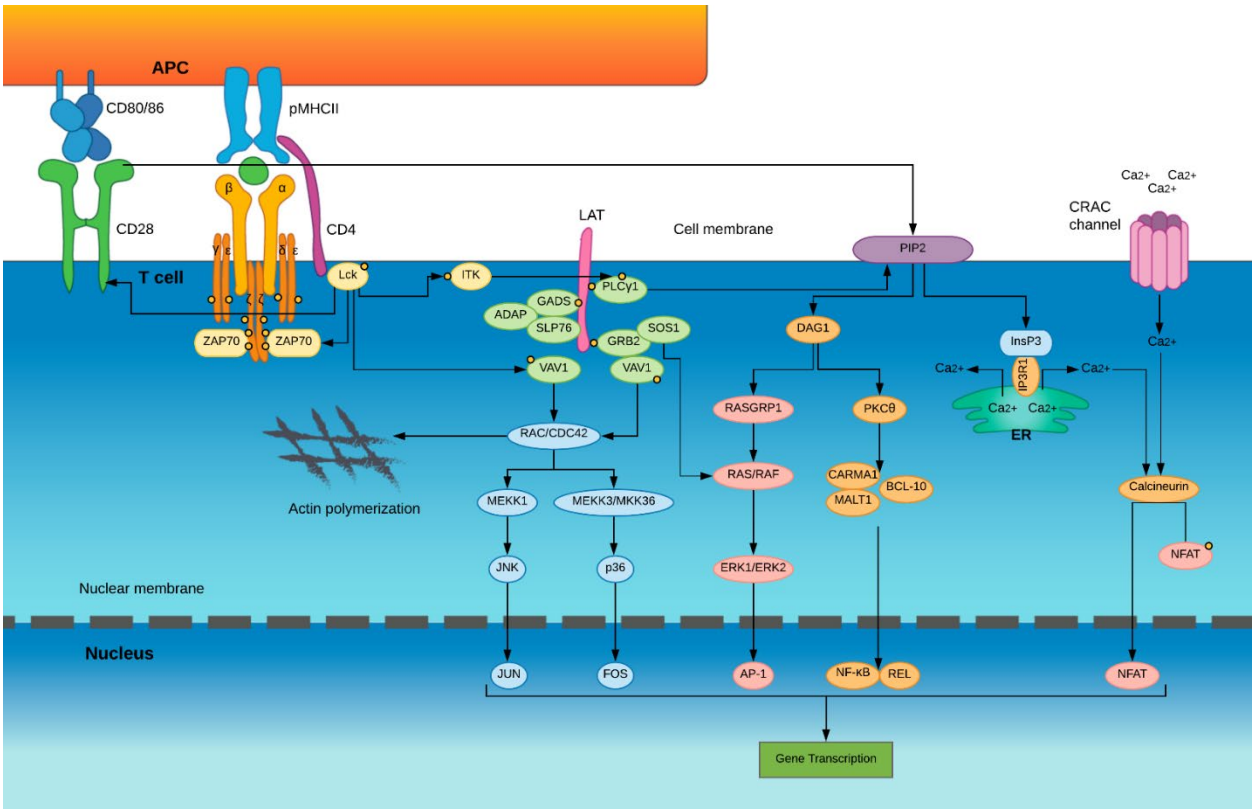

Figure 2. Helper T cell activation signaling cascade

Following TCR recognition of pMHC, CD4 relays signals to Lck located on its cytoplasmic tail. Lck then starts a phosphorylation cascade (phosphate groups denoted by small yellow circles) which results in actin polymerization, translocation of transcription factors and subsequent upregulation of activation genes, and calcium influx from the intracellular (endoplasmic reticulum (ER)) and extracellular (CRAC channel) sources [46]. CD28 is also phosphorylated by Lck and provides a secondary set of signals through PIP2 to further promote calcium influx, cell survival, cytokine production, and upregulation of other costimulatory receptors such as 4-1BB [47].

1.1.2 T cell coreceptors modulate TCR-activation signaling

Coreceptor CD4 is expressed on helper T cells and subsets of innate immune system cells including natural killer cells, monocytes and macrophages [11]. CD4 expression is crucial throughout the life of the T cell from thymic development and antigen recognition in the periphery [48] to differentiation, migration and cytokine expression [49, 50]. As previously mentioned, following colocalization to the TCR, CD4 signals via Lck which is associated with

the cytoplasmic tail of CD4 (Figure 2) [51, 52]. Lck phosphorylates ITAMs of the CD3 subunits of the TCR complex, which then initiates other early signaling machinery of the T cell [51, 53]. Interestingly, unlike the cytotoxic T cell cognate receptor CD8, CD4 has negligible effect on TCR-pMHCII interaction and affinity [54, 55]. However, CD4 enhances T cell sensitivity for antigen by 30-100 fold [56-59], and reduces the number of antigenic peptides required for sustained TCR signaling by 10-fold [60]. When CD4 is present, only 10 pMHCII are required for full T cell activation [61]. In contrast, 30 pMHCII are required to activate helper T cells when CD4 is blocked from the immunological synapse [61]. This is because when CD4 is not recruited to the TCR-pMHC synapse it sequesters Lck away from the activation complex, inhibiting the efficiency of the CD3-mediated activation phosphorylation cascade, which attenuates T cell activation and IL-2 production [62]. Thus, delayed Lck accumulation reduces T cell responsiveness [63]. Intriguingly, ligated CD4 can also send an inhibitory signal independent of Lck, which attenuates IL-2 production, suggesting that CD4 ligation after activation is inhibitory [64].

Coreceptor CD28 is activated by binding to CD80 or CD86 expressed by the activated APC [65], and provides crucial co-stimulation to promote T cell proliferation, cytokine production (via gene transcription and mRNA stability), cell survival and cellular metabolism (Figure 2) [47]. CD28 early signals may stimulate the expression of other pro-activation coreceptors such as ICOS, OX40, and 4-1BB, which sustain or prolong an immune response and assist memory T cell formation [66]. CD28 ligation with CD80/86 leads to multifactorial downstream effects. First, PI3K associates with CD28 then catalytically converts PIP2 to PIP3. PIP3 becomes a docking site for PDK1 and its target Akt. Akt phosphorylates multiple proteins that affect numerous cellular responses such as NFκB, which promotes cell survival and inhibits

transcription factors responsible for cell cycle arrest [47]. Akt is also responsible for increasing glucose uptake and glycolysis, critical for proliferation and cytokine expression, by upregulating cell surface expression of insulin transporter Glut1 [67, 68]. Finally, Akt promotes IL-2 production by optimizing transcription of NFAT regulatory genes [69]. Other proteins that dock to PIP3, such as Itk, enhance Ca^{2+} flux critical for activation [70]. Independent of Akt, CD28 induces arginine methylation, which is also correlated with IL-2 production, although a direct connection is not currently known [47].

4-1BB is induced by CD28 activation. Like CD28, once activated by its APC ligand (4-1BBL), induces PI3K/Akt, NFκB, JNK, and p38 MAPK [66]. However, unlike CD28, 4-1BB does not directly associate with protein kinases, instead signaling through TRAF family of adaptor proteins [71]. The linking proteins between 4-1BB and the adaptor proteins are unknown [71]. Whereas CD28 creates powerful initial activation signals, 4-1BB promotes T cell longevity and establishment of memory by upregulating pro-survival genes surviving, Bcl-2, Bcl-XL, and Bfl-1 while reducing the expression of pro-apoptotic Bim [72-74]. 4-1BB is more active in killer T cells than helper T cells *in vitro* [75] and *in vivo* [76, 77].

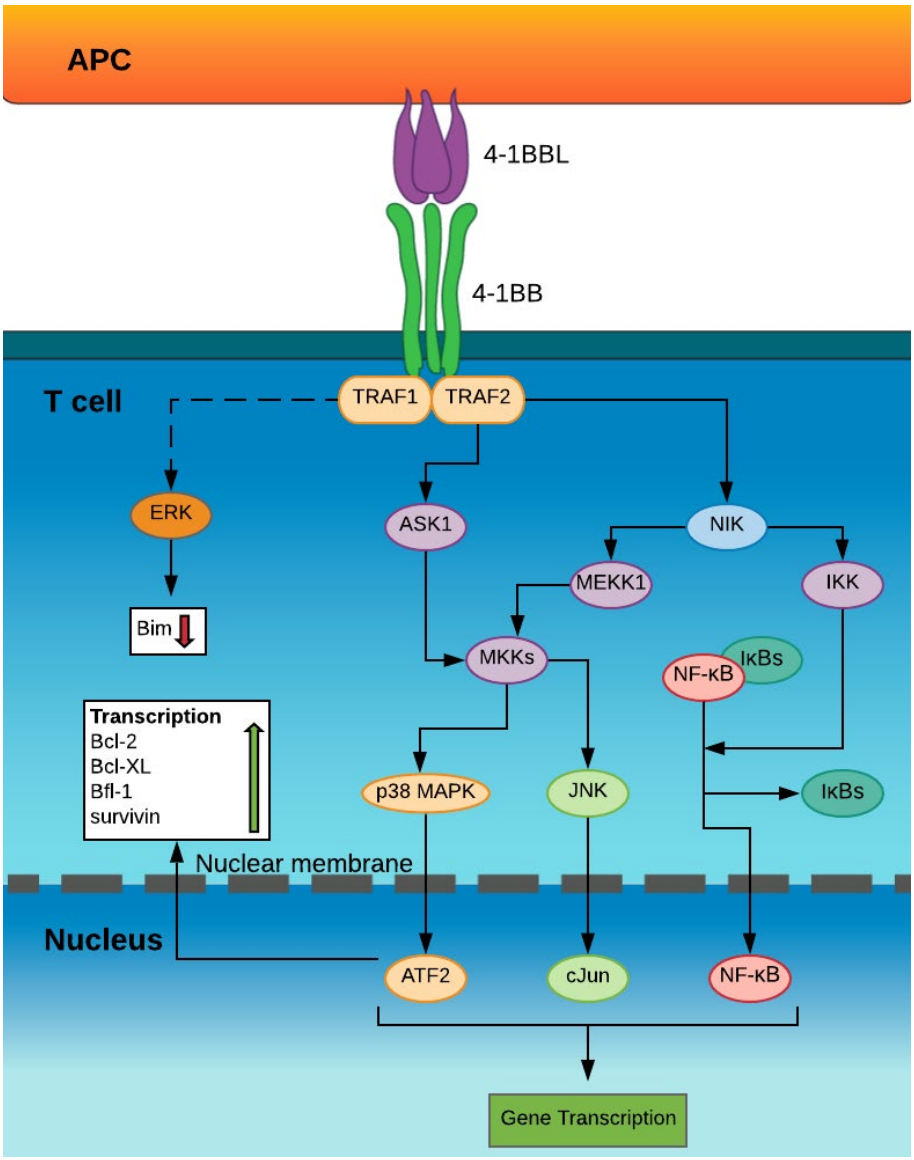

Figure 3. 4-1BB signaling cascade

Stimulatory co-receptor 4-1BB is upregulated by CD28 stimulation and promotes T cell longevity by decreasing cell cycle regulator Bim, and upregulating cell proliferation and survival genes Bcl-2, Bcl-XL, Bfl-1 and surviving [78].

1.1.3 TCR affinity effects on activation

In addition to coreceptor modulations, TCR signaling is affected by the affinity of the TCR for its cognate pMHCII. These interactions maintain high sensitivity and specificity in spite of their low KD values (~1-100 μM) and short half-lives (3-30s) [79-82]. TCR-pMHC

interactions are intrinsically low affinity because high-affinity clones are selected against during T cell development in the thymus, resulting in a self-tolerant population of T cells that can still recognize and react to foreign pMHC [83] (Figure 4A). Interestingly, the mature T cell repertoire is skewed towards T cells that are more highly reactive to self-antigen, indicating that T cells with high affinity for self-antigen are better responders to infection [84]. While the lowest-affinity T cells can induce proliferation, cytokine production, and memory formation, higher-affinity T cells form the bulk of memory responses [84-86] (Figure 4B). Whether high or low avidity T cells respond to an antigen best may be influenced by the amount of antigen, the mode of antigen delivery, the kind of antigen presenting cell presenting the antigen, T cell subtype competition, and the level of TCR expression and its ability to initiate a signaling cascade [87].

As previously discussed, T cell activation is initiated by the TCR-pMHC interaction, followed by an ordered phosphorylation of ITAMs [88]. Conflicting activation models suggest different roles for TCR-pMHC affinity from an optimal dwell time for a single pMHC which facilitates serial interactions to a simpler model that suggests that longer half-lives increase activation [89-91]. Efforts to find a single affinity parameter that is predictive of T cell activation has been difficult [92-94], however, several recent studies of helper and killer T cells report clearer correlations between T cell activation and 2D-acquired affinity and off-rates compared to 3D-acquired kinetics [93, 95-97]. Using mathematical modeling of the current affinity data, kinetic proofreading with limited signaling incorporates aspects of both the occupancy and kinetic proofreading models and was the most predictive [98]. This model assumes that TCR signaling is limited in response to pMHC stimulation and thus multiple TCRs must be activated serially for functional T cell response, implying that high-affinity TCRs with long dissociation times may have impaired signaling [98]. However, engineered TCRs with

exceptionally long dissociation times have been shown to mediate sensitive T cell responses [99].

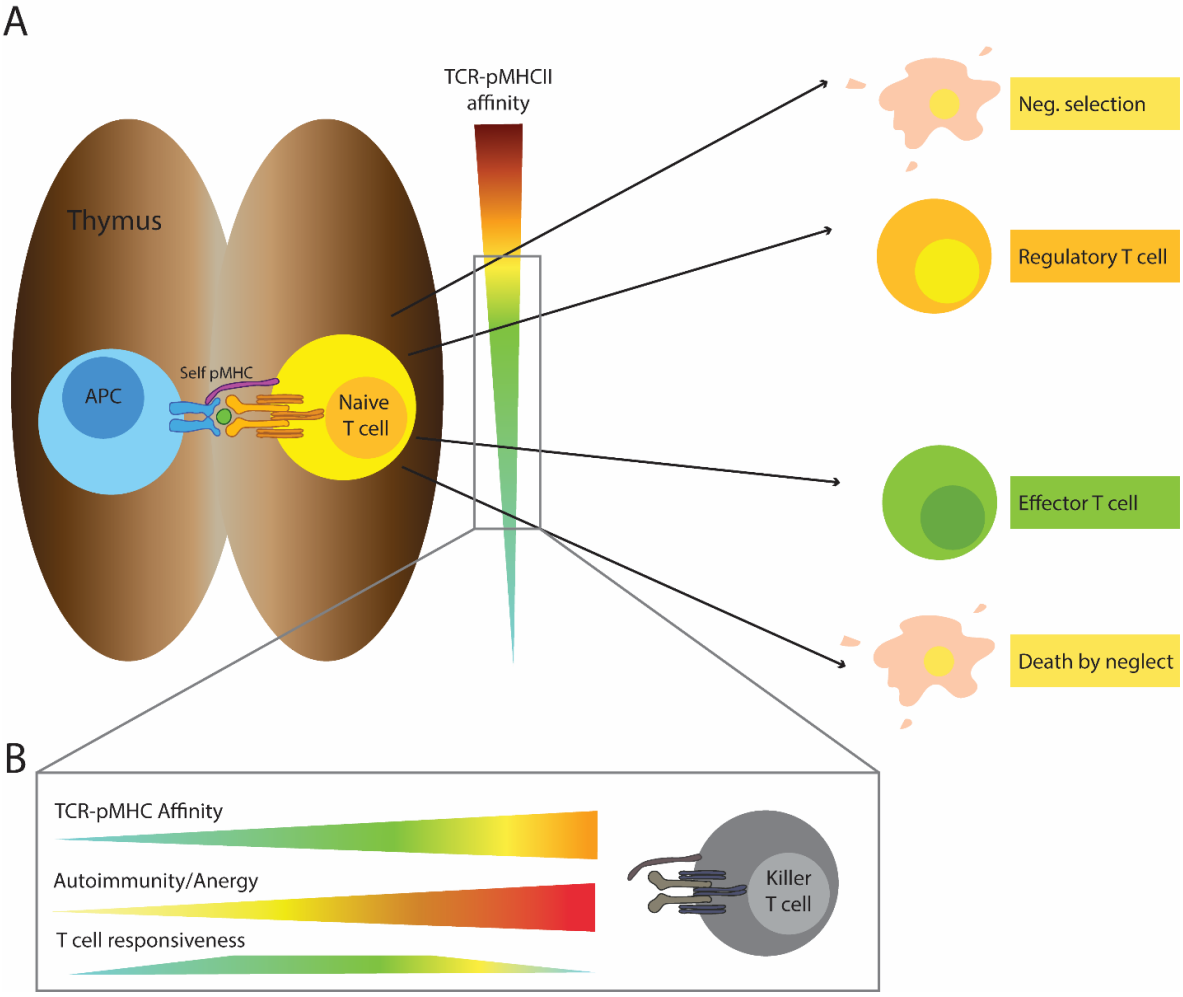

Figure 4. TCR affinity during selection and periphery stimulation

A. Naïve helper T cells are selected in the thymus through TCR binding to self-pMHC. If naïve T cells bind too tightly, they are negatively selected and die by apoptosis. Conversely, naïve T cells that bind too weakly to self-pMHC die from neglect. Naïve helper T cells that are within an optimal window of affinity for TCR-pMHC receive confirmatory signals and are released into the peripheral immune system and secondary lymphoid organs (spleen, lymph nodes, etc) to await activation by foreign pMHC. T cells that bind tightly to self-pMHC are likely to become regulatory T cells to prevent autoimmunity, and T cells that bind less tightly to self-pMHC are fated to become effector T cells responding to foreign pMHC [100]. B. T cell responses vary within the defined window of affinity. More is known about killer T cell affinity responses than helper T cell affinity responses. Autoimmunity and T cell propensity for anergy (stimulated cell death) increases as affinity for TCR-pMHC increases. T cell responsiveness increases as TCR-pMHC affinity increases, however at a certain high affinity, T cell responsiveness decreases as high affinity cells are prone to anergy from overstimulation.

Because T cells have a naturally low affinity for antigen and function in an environment where there are many copies of the TCR on the cell surface, their affinity is often best measured through avidity, a multimeric measurement of TCR affinity. Both high-avidity MHCI and MHCII-specific TCRs have some general and conflicting response trends. In some studies, higher-avidity T cells responded more sensitively to antigen, produced more cytokines, proliferated better than their lower-avidity counterparts, and were preferentially selected during memory formation [85, 101-105]. Conversely, other studies have demonstrated that high-avidity T cells can also be severely limited in their biological activity and have reduced peptide specificity [82, 86, 91, 106, 107]. Thus, it is possible that during an immune response intermediate or low affinity clones are advantageous while the highest affinity clones may be impaired [82, 108].

Engineered high affinity TCRs have been used to tease out basic T cell functions such as identifying important conserved pMHC contact residues and investigating the effects of altered peptide ligands on activation, and have also been used as multimers to locate MHC-specific peptides [109-113]. Additionally, high affinity TCRs are attractive immunotherapeutics because TCRs can target intracellular antigens, respond robustly to as few as 1-10 cognate pMHC, and utilize endogenous T cell signaling machinery [61, 114-116]. Naturally weak TCR-pMHC interactions have short half-lives which are inadequate for successful therapeutic interactions [117]. Furthermore, high affinity TCRs can be used to uncover potential safety concerns even if lower affinity TCRs are used in clinical settings [118, 119]. Taking these considerations into account, understanding the peptide specificity and functional avidity effects of high-affinity class II specific TCRs is of great interest not only to better understand basic T cell biology, but also

because of the potential use of high-affinity class II specific TCRs in diagnostics and immunotherapies.

1.1.4 TCR signaling in T cell-based immunotherapies

Helper T cells are critical responders to infection and cancer [2]. Recent research indicates that helper T cells are the most frequent responders to cancer antigens [120]. Tumor environments suppress immune responders by secreting anti-inflammatory cytokines, downregulating co-stimulatory molecules and encouraging the development of regulatory T cells [121, 122]. 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 and upregulate co-inhibitory proteins, all of which suppress T cell activation [123-127]. 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 their activation, infiltration, persistence, and memory formation (Figure 5) [128-133]. In one study, 70-95% of tumor-specific antigens elicited a helper T cell response rather than a killer T cell response and were presented by MHC II significantly better than non-immunogenic antigens [6].

A promising new molecular means of circumventing this suppressive environment is the recent development of chimeric antigen receptors (CARs). CARs typically consist of antibody binding domains specific for a cancer antigen connected to T cell signaling molecules such as CD3, CD28, and 4-1BB. Transfection of CARs into T cells creates high affinity cancer-specific effector cells [134]. This technology has had some spectacular results. In one study, 90% of acute lymphoblastic leukemia patients achieved immediate remission and 67% of the patients had sustained remission after six months [4]. Antibodies generally recognize extracellular

antigens while TCRs can recognize intracellular antigens presented by MHC. Furthermore, helper T cell TCR-CARs are advantageous because they can target low density antigens, maintain polyfunctional responses, and persist in both helper and killer T cells [135]. CAR immunotherapy can also be designed to use high affinity tumor-directed TCRs which are truncated to the peptide recognition portions (Vα and Vβ) and fused to the intracellular signaling proteins CD3 and CD28 to produce a single-chain TCR construct [136]. This prevents TCR chain mispairing, takes advantage of TCR intracellular targeting, and conveys important signaling advantages to combat cancer evasion strategies [135], [137]. As such, high affinity TCRs are attractive targets for immunotherapy development. However, TCR selection in the thymus results in deletion of high affinity TCRs and a natural mature T cell repertoire with low TCR affinity [138]. Thus, the functional activity and peptide specificity of high affinity T cells are unknown. Intriguingly, T cells with the greatest affinity for self-antigen make up the largest and most active responders to infection. This pattern is seen in killer T cells, Treg cells, and helper T cells [139-141]. This compellingly suggests that TCR self-antigen affinity is an important determinant of T cell responsiveness to foreign or cancer pMHC.

To date, almost all clinical trials utilizing TCRs, except one, have focused on killer TCRs that recognize pMHCI targets [142]. Mounting evidence suggests that helper TCRs targeting pMHCII may be especially advantageous as they can contribute to indirect effects (activate innate immune cells) and direct effects (cytotoxicity for MHCII positive tumors) on immune response [143-145] and may be less toxic against overexpressed tumor associated antigens (TAA) [146]. Additionally, because CD4 TCRs do not rely on CD4 for enhanced affinity, they may function without CD8 or CD4 coreceptor in cell lines like cultured natural killer cell lines [147].

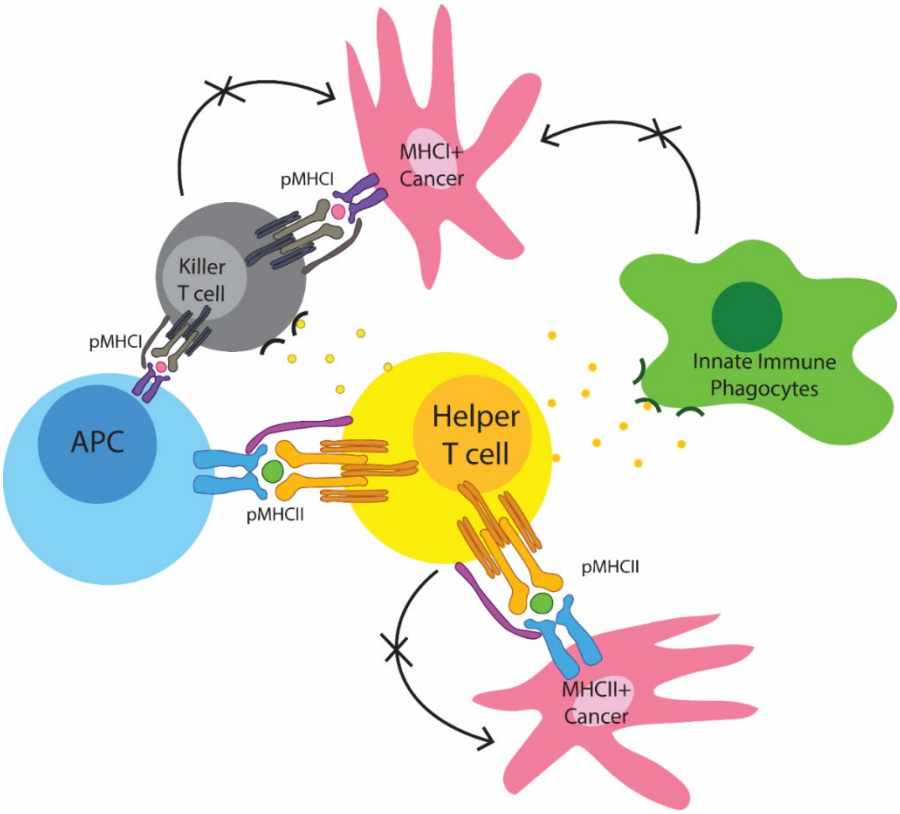

Figure 5. Helper T cells influence the immune response to cancer both directly and indirectly

Helper T cells can directly kill (arrow with cross) tumors expressing pMHCII (APC-derived cancers). Indirectly, helper T cells can stimulate natural killer cells and phagocytic cells including neutrophils and macrophages with cytokines to aggressively attack cancer cells. Helper T cell can also promote killer T cell activity with IL-2 to attack MHCI+ cancer cells (most body cells).

1.1.5 Summary and questions (LLO118 and LLO56)

There remains much to learn about the role of TCR affinity and co-receptors on T cell activation. The second chapter of this dissertation entitled "CD4 inhibits helper T cell activation at lower affinity threshold for full-length T cell receptors than single chain signaling constructs" asks the following questions: 1. How does helper TCR affinity contribute to helper T cell activation? 2. What is the contribution of CD4 to high affinity TCR T cell activation? 3. Does CD4-Lck sequestration affect high affinity TCR T cell activation? 4. How do high affinity TCRs function in multiple CAR formats such as CD28, 4-1BB, and 3rd generation CARs?

To answer these questions, we used helper T cell transgenic mouse lines, LLO56 and LLO118, to generate a panel of low, intermediate, and high affinity TCRs. LLO56 and LLO118 recognize a *Listeria monocytogenes* peptide from protein listeriolysin O bound to MHCII molecule I-A^b (I-A^b/$LLO_{190-201}$ or I-A^b/$LLO_{190-205}$) [148]. LLO118 and LLO56 TCRs differ by 15 amino acids and have the same affinity for I-A^b/$LLO_{190-201}$ *in vitro* but respond very differently *in vivo*. LLO118 has a better response to primary infection while LLO56 has a better response to secondary infection [149]. Comparisons of LLO56 and LLO118 revealed that LLO56 expresses higher levels of co-receptor CD5 than LLO118 suggesting that LLO56 binds more strongly to self peptide:MHC [22]. The affinity clones were transfected into T cell hybridomas with and without CD4 expression as full length TCRs and second and third generation single chain TCR-CARs to explore the effects of affinity and co-receptor CD4 on functional activity and binding specificity. While our system specifically targets an infectious disease peptide, CAR T cell research is strongly informed by early work from HIV-1/AIDs research [35-38]. Our system is the first known model to explore MHC II-specific TCR CARs effects on infectious disease and may have direct cancer therapy applications.

1.2 Systemic lupus erythematosus

Systemic Lupus Erythematosus (SLE) afflicts 0.3-241/100,000 people globally [150, 151]. In the United States, SLE incidence is between 20-150 per 100,000 people, and has increased prevalence in the southern US [152-155]. SLE is more common in urban than rural areas [153, 156]. SLE prevalence and incidence is greatly influenced by race, ethnicity, socioeconomic status and sex [157-159]. Compared to Caucasians, non-Caucasian populations (Hispanics, African descendants, and Asians) develop SLE more frequently and have heightened disease activity and damage accrual [160]. Additionally, improved survival was correlated with

increased household income [161]. Furthermore, females are much more likely than males to develop SLE [162, 163]. This sex ratio ranges from 2:1 [164] to 15:1 [165]. The 15-year survival rate is approximately 80% [166] and this increased risk for all-cause mortality applies to SLE patients with cancer [167-170].

Autoimmunity occurs when the immune system mistakes cellular markers or proteins for foreign antigens and damages healthy tissues. A combination of immunologic, genetic, and environmental factors (including infectious agents) are required to develop an autoimmune disorder (Figure 6) [171]. While basic etiology of SLE cause and progression understanding has improved over the last decade, the underlying triggering events and pathological mechanisms of symptoms for SLE are still undefined. SLE is a complex autoimmune disease characterized by defects in cellular apoptotic debris clearance, interferon (IFN) expression signature in peripheral lymphocytes and autoantibodies produced by a breakdown of peripheral tolerance mechanisms which produces irreversible damage to tissues and organs through cellular infiltration and local activation of complement [172, 173]. These autoreactive polyclonal antibodies usually appear years before disease clinical manifestation [174]. SLE chronically affects multiple body systems including the skin, joints, central nervous system (CNS), lungs, kidneys, digestive tract, and hemopoietic system [175]. Because SLE is systemic rather than localized, SLE patients exhibit a wide diversity of symptoms which makes diagnosing SLE difficult. Symptoms include cutaneous manifestations (malar rash), photosensitivity, discoid lesions, oral ulcers, joint pain and swelling, serositis, renal disorders, hemolytic anemia, leukopenia, neuropsychiatric disorders, and poor vascularization [176]. SLE flares and remission are unpredictable and no reliable biomarkers have been identified for either phase [177].

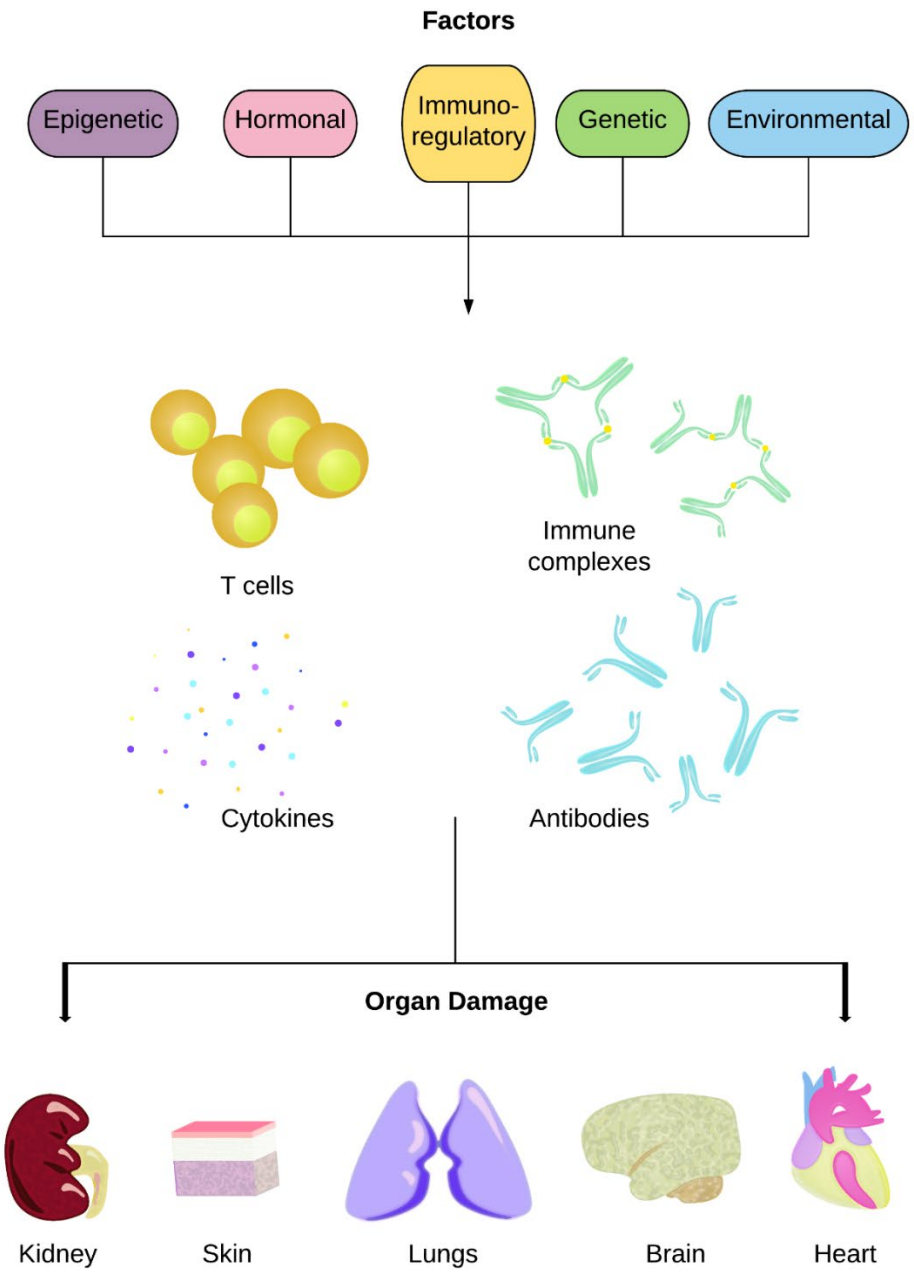

Figure 6. SLE triggers, immunological dysfunction, and systemic effects

SLE development is influenced by genetic, epigenetic, immunoregulatory, hormonal, and environmental factors working sequentially or simultaneously on the immune system. These factors affect T cell regulation leading to autoreactive or inflammatory T cells, inflammatory cytokine expression, and the generation of autoantibodies and immune complexes. This dysregulation, in addition to local factors, affect multiple organs, leading to systemic SLE damage [173].

1.2.1 T cell dysregulation in SLE

SLE is a complex autoimmune disorder involving the dysregulation of multiple classes of immune cells. However, this discussion will focus on T cells as an example of how small changes to regulation and homeostasis can result in widespread immune dysregulation and tissue damage. T cells have a critical role in SLE pathogenesis. T cells maintain SLE disease etiology through the accumulation of autoreactive memory T cells [177], helping activate autoreactive B cells [178], inappropriately homing lymphocytes to target tissues [173], and amplifying secretion of pro-inflammatory cytokines (IL-17 and IL-10) [177, 179] while repressing IL-2 production required to maintain normal T cell activation and proliferation (Figure 7) [180]. The causes of these activities are intertwined and attributed to altered T cell subset ratios and intensified T cell activation via aberrant TCR signaling, which will be discussed in depth in later sections.

SLE patient helper T cells fail to raise proper cytotoxic T cell responses to foreign antigen due to altered T cell subset ratios and function, yet provide excessive help to autoreactive B cells [177]. Helper T cells and double negative (DN) T cells ($CD4^-/CD8^-/CD3^+$) are expanded in SLE patients and provide costimulatory help to B cells that produce anti-dsDNA autoantibodies [181]. Autoreactive B cells receive excessive costimulatory help via the CD40 pathway as SLE T cells have increased CD40L expression [182, 183]. CD40L expression is upregulated by two mechanisms: 1) heightened TCR-CD3 engagement which increases intracytoplasmic Ca^{2+} levels and NFAT activation via calcineurin [184, 185], and 2) CD40L is hypomethylated in SLE T cells thereby increasing NFAT binding and CD40L transcription (Figure 8) [184, 185].

SLE T cells also direct inappropriate lymphocyte homing to tissues. SLE T cells have increased expression of CD44, a cell surface adhesion molecule which promotes cell migration

(Figure 8) [186]. In SLE patients, T cells are present in inflamed tissues including the skin and kidneys [173]. In the kidneys, T cell presence decreases the preservation of renal function [173] and contributes to tissue damage via inflammation. T cell homing is also affected by increased IL-17 production [179].

IL-17 is a potent pro-inflammatory cytokine that contributes to inflammation and autoimmunity [187]. SLE patients have an increased proportion of IL-17 producing CD4 T cells in their serum and peripheral blood [188]. IL-17 producing CD4 T cell subsets include T_h17 [189] and DN T cells [179]. IL-17 levels correlate with the development of lupus-like nephritis in several mouse models [190] and activate anti-dsDNA antibody production [191]. IL-17 increased transcription and IL-2 reduced transcription is regulated by the same transcription factor (cAMP responsive element modulator α (CREMα)) which has increased expression in SLE T cells [192]. CREMα transcription is influenced by hormones like estrogen, which provides an interesting link to the increased SLE incidence in females [193]. In addition to CREMα transcription regulation, IL-2 is also affected by transcription factors NF-κB and API, which have decreased expression in SLE T cells (Figure 8) [194, 195]. Consequently, SLE T cells have decreased IL-2 mRNA [180]. IL-2 is required for T cell activation, proliferation, and activation induced cell death (AICD) needed to remove autoreactive cells [196]. IL-2 is also critical for cell-mediated immunity against foreign agents, and promotes the survival and maintenance of regulatory T cells (T_{regs}) [197]. Thus, the dysregulation of both IL-17 and IL-2 explains several facets of SLE disease etiology (Figure 7).

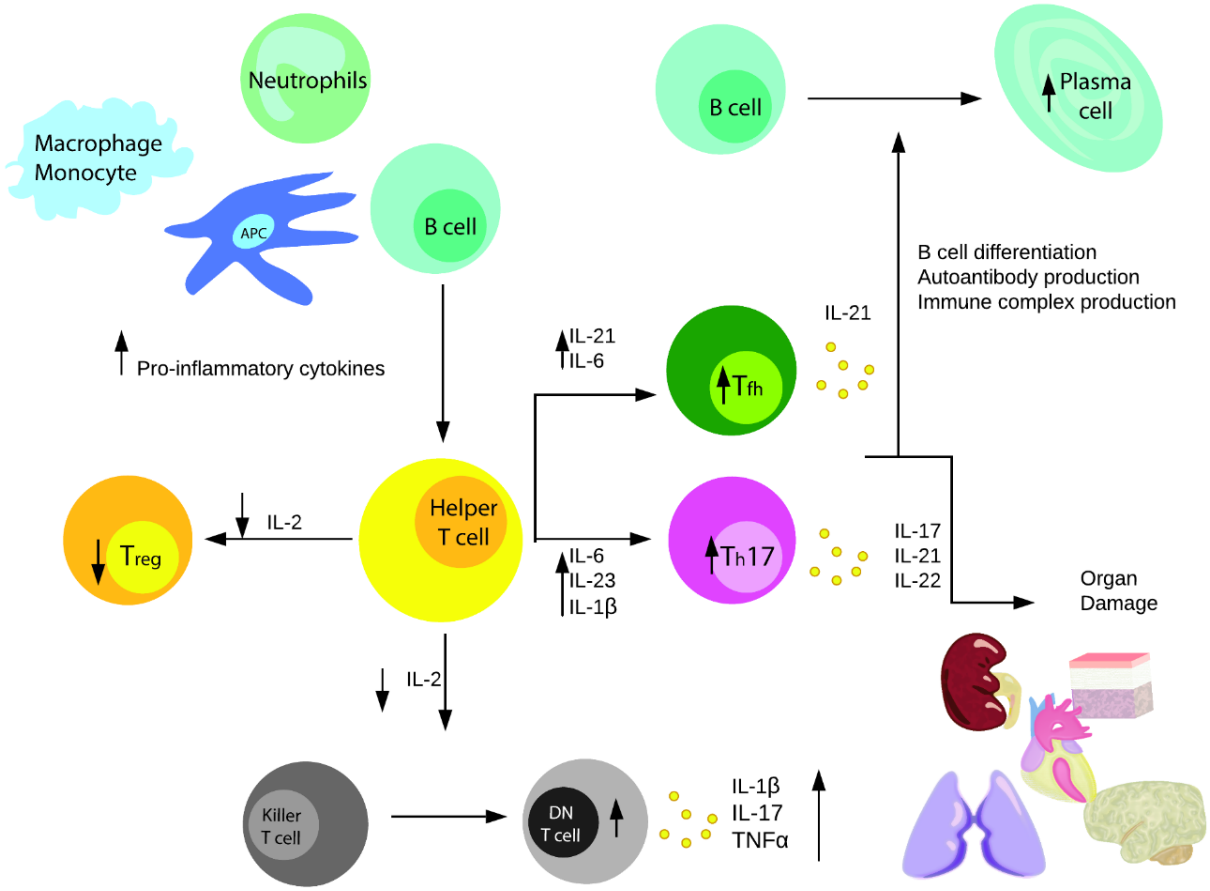

Figure 7. SLE dysregulates T cell differentiation and cytokine production

SLE naïve helper T cell development is affected by cytokines produced by macrophages, neutrophils, APCs, and B cells and decreased IL-2 production. This favors the development of T_{reg}, T_h17, T_{fh}, and DN T cells, and promotes the production of inflammatory cytokines such as IL-17 and TNFα. Taken together, these unbalanced signaling and cell subpopulations enhance B cell differentiation, autoantibody and immune complex production, promoting organ damage. Small arrows indicate increased or decreased cytokines or cell subsets in SLE compared to controls [198].

1.2.2 Altered T cell subsets in SLE

Peripheral blood T cell subpopulations are very complex and include effector, memory and regulatory subtypes (Figure 1) [199]. SLE patients not only have unique ratios of some T cell subsets, but the individual subsets function abnormally (Figure 2). These subsets include

DN T cells, cytotoxic T cells, invariant natural killer T (NKT) cells, and multiple helper T cell subsets including T_h17, extrafollicular (T_{fh}), and T_{regs}.

DN T cells have an αβ TCR but lack CD4 or CD8 and may derive from inactivated, exhausted, autoreactive or continuously stimulated $CD8^+$ killer T cells [200]. DN T cells are also seen in chronic infections [201, 202]. CD8 loss is mediated by CREMα, which also enhances IL-17 production [203, 204]. Normally, DN T cells are immunosuppressive by antigen competition and T cell killing by the Fas-FasL pathway or perforin/granzyme secretion [205, 206]. However, SLE DN T cells accumulate in the kidneys and produce IL-17, which enhances the pathogenesis of kidney disease via inflammation [179]. Like DN T cells, killer T cells kill problematic body cells by releasing perforin/granzymes to control infection, malignancy, and autoreactive immunity. SLE patient killer T cells have dampened cytotoxic function, which leads to an increased risk of infection and can trigger or exacerbate autoimmunity [207]. As result, SLE patients are more prone to viral infections, which is the highest cause of death in SLE patients [173].

Invariant NKT cells have an invariant TCR that recognizes certain glycolipid antigens presented on a non-classical MHC (CD1-d) [208]. Invariant NKT cells produce cytokines, are directly cytotoxic, and are implicated in autoimmune development [209]. SLE patients have reduced numbers of NKT cells in peripheral blood, and fewer the NKT cells are correlated with increased with disease activity [210]. These NKT cells also have impaired proliferation and cytokine production capabilities [210, 211].

SLE patients have increased accumulation of effector and memory helper T cells, which promote autoantibody production and tissue inflammation [212]. Aberrant expression of cell surface and signaling molecules leads to increased TCR stimulation and circumvents peripheral

tolerance mechanisms [177]. As previously stated, SLE effector helper T cells produce less IL-2 and more IL-17 than healthy controls [213]. This leads to a downregulation of T_h1 and T_{reg} cell cytokines (IFNγ and TGFβ) and skews the population towards a T_h17 subtype [214]. Increased numbers of T_h17 promote kidney disease and autoantibody production by kidney infiltration [215, 216] and loss of B cell tolerance [217]. B cell activation is also promoted by T_{fh} cells. SLE patients have significant increases of T_{fh}-like phenotype in their peripheral blood [218-221]. T_{fh} numbers correlate with plasmablast B cells and autoantibody titers, especially anti-DNA antibodies [222]. Additional evidence supporting T_{fh} involvement in SLE etiology is that SLE autoantibodies have undergone somatic hypermutation and are IgG isotype, indicating that the autoantibody producing B cells were activated in a T cell dependent manner [178]. Interestingly, T_{fh} differentiation is blocked by IL-2, which has reduced expression due to decreased T_h1 differentiation. Reduced IL-2 production also effects T_{reg} development [223]. Due to studies using different markers, there are conflicting reports about how T_{reg} numbers are affected by SLE [224]. However, it is well documented that SLE T_{reg} immune response control is dysregulated [225]. Loss of T_{reg} function exacerbates poor autoimmunity control in SLE patients.

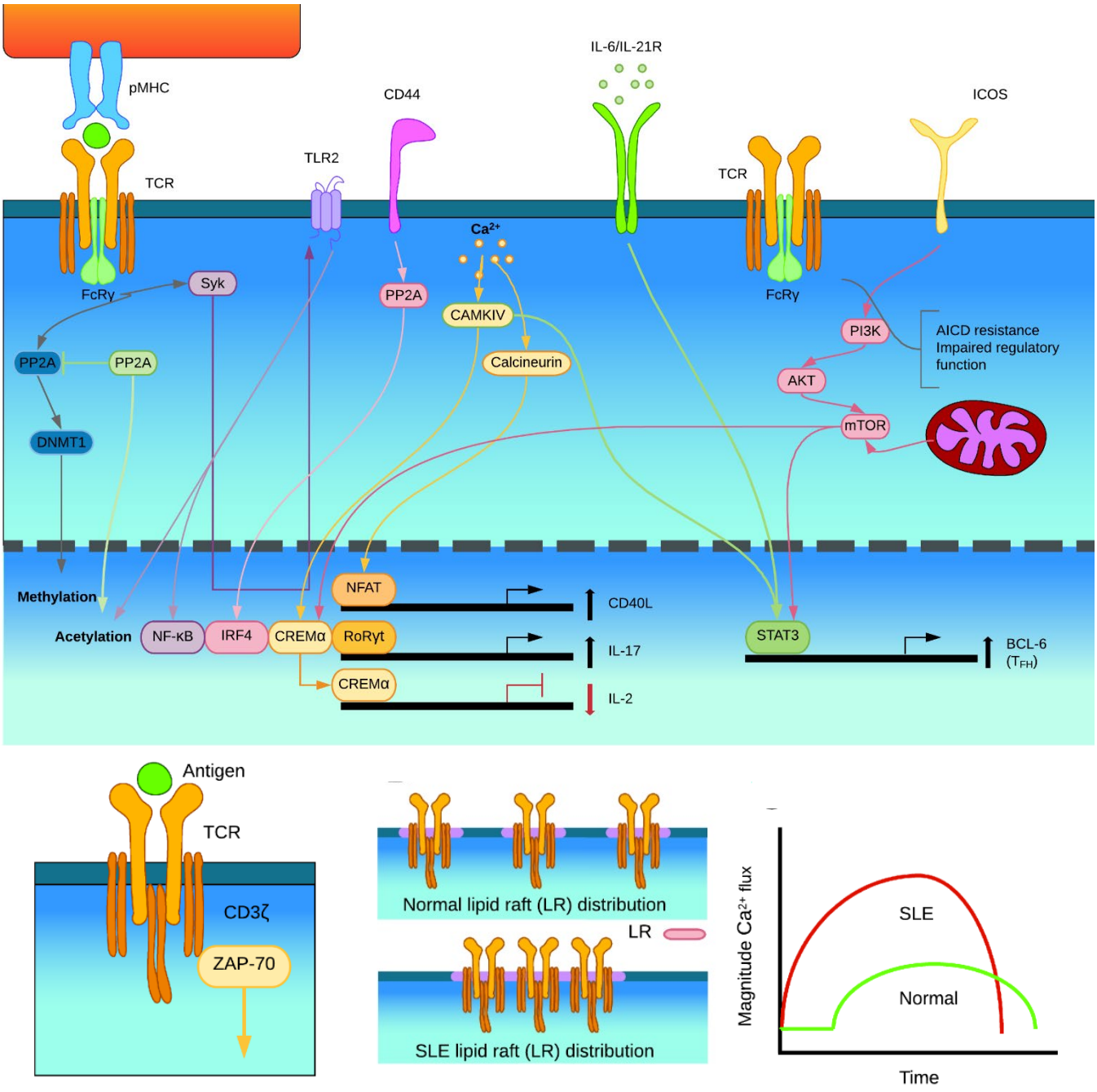

Figure 8. SLE T cells have altered TCR signaling apparatus and heightened signaling capabilities

Normal TCR signaling through CD3ζ and ZAP-70 [198]. In healthy naïve T cells, TCR are other costimulatory molecules are resting in separate lipid rafts on the cell membrane. However, in SLE naïve T cells, the lipid rafts are pre-aggregated leading to early and enhanced TCR signaling as measured by calcium flux [177]. Signaling is also enhanced by the replacement of CD3ζ with FcRγ, which signals through the Syk pathway. Other aberrant signaling pathways include activated pI3K-Akt-mTORC1 pathway, Rho associated protein kinase (ROCK), calcium/calmodulin kinase IC (CaMKIV), and protein

phosphatase 2a (PP2A). Dysregulated signaling leads to demethylation and acetylation of critical regulatory and cytokine genes, the increased production of B cell costimulation protein CD40L, enhanced production of pro-inflammatory cytokine IL-17, and increases T_{fh} cell fate. SLE cells also have repressed activation induced cell death (AICD), and impaired regulatory function. Pathways and arrows are arbitrarily assigned a color for clarity [190].

1.2.3 Altered TCR signaling in SLE

SLE T cells have a unique signaling apparatus compared to healthy T cells. Unlike healthy T cell where TCR/CD3ζ complex begins the activation cascade, in SLE, the CD3ζ is replaced by the homologous Fc receptor common g subunit (FcRγ) chain (Figure 8). FcRγ relays a many-fold stronger signal than CD3ζ/ZAP70 by recruiting spleen tyrosine kinase (Syk) [226]. This results in an early and heightened signaling event, which allows T cells to respond to low avidity autoantigens [226]. Syk signaling also promotes other downstream pathways that affect T cell signaling. For example, TLR2 expression is upregulated by the Syk pathway [227] leading to increased NF-κB activation [228] and chromatin opening at the promoters of IL-17 [229]. Inhibiting Syk in both murine models and SLE patients corrects aberrant TCR signaling by upregulating CD3ζ and normalizing IL-2 production, and may even reduce signs of autoimmunity and organ pathology [177, 230]. Additional TCR activation machinery is downregulated in SLE T cells including Lck [178, 222, 231, 232] and CD3ζ. CD3ζ expression is decreased through multiple mechanisms including transcription [233], mRNA [234], alternative splicing [235], proteasome degradation [236], caspase cleavage [237], and mTOR-dependent degradation [238]. CD3ζ expression is inhibited by protein phosphatase 2A (PP2A) which also increases FcRγ expression and CREMα [177], and hypomethylates genes associated with SLE pathogenesis [239], thereby increasing IL-17 production [240].

Physical T cell characteristics also affect TCR activation signaling. In normal T cells, TCR/CD3ζ complexes and associated signaling molecules are located in lipid rafts rich in

sphingolipids, cholesterol, and GM1, uniformly distributed across the cell membrane [241]. In SLE T cells, these lipid rafts are pre-clustered and aggregated, indicative of an activated state (Figure 8) [241]. Naïve T cells with clustered lipid rafts have early and enhanced calcium flux (Figure 8) [177]. Dissociating these rafts corrects TCR/CD3ζ mediated signaling [242, 243] and delays disease onset [242].

T cell activation, differentiation and function, and cell death are regulated by reactive oxygen intermediates (ROI) and ATP produced by the mitochondria. Mitochondrial transmembrane potential is a critical regulator of ROI and ATP. In SLE T cells, anomalous, persistent mitochondrial hyperpolarization, increases ROI production and depletes ATP causing spontaneous apoptosis but decreases activation induced apoptosis (Figure 8) [177]. mTOR, responsible for sensing mitochondrial membrane potential and activating downstream substrates, is abnormally increased in SLE patient T cells [238]. In healthy T cells, mTOR regulates a wide variety of cellular functions including growth, apoptosis, metabolism, actin reorganization and ribosome biogenesis by integrating cues from the environment [244, 245]. Without mTOR, helper T cells fail become effector T cells and skew towards T_{reg} development [246]. Targeting mTOR restores T cell activation, Ca^{2+} flux, and results in attenuated inflammation and clinical improvement [247, 248]. Interestingly, anomalous activation of the PI3K/AKT/mTOR [212, 249] and CaMKIV [250] pathways is also responsible for the dysregulated balance between effector cells and T_{reg} cells.

1.2.4 SLE and viruses

While the exact etiology of SLE is unknown, infections may act as environmental primers by inducing or promoting disease onset and exacerbating SLE in genetically predisposed individuals [251]. Etiology is especially difficult to sift through, as the causative agent is usually

gone when SLE is diagnosed [171]. The cumulative effects of repeated infections may trigger autoimmunity through a cross reactivity or bystander activation [252]. Cross-reactive T and B cells are activated through "molecular mimicry" when a pathogenic antigen closely resembles a human self-antigen [253]. Acute rheumatic fever is a common example of molecular mimicry-induced autoimmunity: immune responses against streptococcal M-protein inappropriately target cardiac myosin and heart-derived peptides, thereby damaging the heart [254]. In bystander activation, microbial products induce autoimmunity by "epitope spreading" or the enhanced presentation of autoantigens by activated APCs leading to the expansion of dormant autoreactive clones [171]. For example, elevated antibodies against Epstein-Barr virus (EBV) can lead to anti-Ro antibodies that cause skin and joint symptoms in SLE [255].

Due to the dysregulation of T cell activation and differentiation, lupus patients have increased susceptibility to certain viruses and higher viral loads [253, 256]. Specific viruses are implicated for certain SLE symptoms and include: Coxsakie virus (2B protein and Ro autoantigen) [257]; human parovirus B19 (induces short-lived low titers of autoantibodies) [258]; Cytomegalovirus (anti-CMV IgM antibodies and CMV DNA are sometimes detected at SLE onset) [259, 260]; human endogenous retroviruses (incite pathogenicity through molecular mimicry and immune dysregulation) [261]; and hepatitis C virus (infection higher in SLE and increases liver pathologies while decreasing cutaneous SLE features and anti-dsDNA antibodies) [262].

SLE is especially associated with EBV. 99% of young SLE patients have EBV infections compared to 70% of healthy controls [263]. EBV has a bidirectional effect: while SLE patients have a dysregulated anti-EBV response and abnormal viral latency period, EBV can trigger autoimmune processes [264]. EBV may cause defects in B cell tolerance checkpoints by

inducing B cell activating factor (BAFF), a homeostatic cytokine that regulates innate and adaptive immune responses [265]. As such, EBV infected B cells, acting as APCs, can proliferate and express viral anti-apoptotic molecules, and then provide T cells at target organs with survival signals via costimulation, thereby promoting chronic inflammation [266]. EBV can also lead to an increased antibody response against viral antigens EBNA, VCA, and EA [267]. Antibodies against EBV nuclear antigens may cross-react with host antigens Ro or Sm [268]; anti-Ro antibodies are detected in SLE preclinical period [268].

1.2.5 SLE and cancer risk

Abnormal immune cell signaling and viral dysregulation may contribute to SLE-specific cancer risk. However, there are conflicting reports whether malignancy is increased in SLE patients [269, 270]. This may be because a significant proportion of SLE patients develop cardiovascular or infectious disease complications at younger ages, thus do not reach an age where malignancy would have a greater impact on mortality [271, 272]. Several investigators, including large, multi-center studies demonstrate increased risk for specific types of cancer in lupus patients (Figure 9) [273, 274]. The highest increased risk is for non-Hodgkins lymphoma, where lupus patients have a 4.4-fold to 15-fold increased risk [275-278]. Lupus patients also had increased risk for leukemia and cancers of the cervix, vulva, lung, liver [276], thyroid [279, 280], and bladder [277]. Interestingly, breast and ovarian cancers seem to be less prevalent in lupus patients [276, 281, 282].

For cancers with increased prevalence, the relative contributions of SLE and its immunosuppressive treatments are unclear [283, 284]. For example, cyclophosphamide and long term steroid use have been associated with increased cancer risk [277, 283]. However, cancer diagnosis tend to occur shortly after SLE diagnosis, which suggests a strong role for disease

effects over medication [285]. Free radicals produced by SLE systemic inflammation may also promote cancer development [286]. Additionally, poorly controlled viral infections may contribute to increased cancer risk for SLE patients. For example, SLE T cells response is dysregulated by Epstein-Barr virus (EBV) [287, 288] and dysregulated immune response to EBV can lead to increased Burkitt's lymphoma [289]. EBV infection is also associated with non-Hodgkin's lymphoma and diffuse large B cell lymphoma [290, 291].

The increased risk for hematological cancer development in SLE has been associated variably with increased disease activity [283, 292], cyclophosphamide and glucocorticoid use, and EBV activity [274]. Lung malignancy risk is slightly elevated for SLE patients [276, 293, 294] and attributed to local inflammation of lung tissue [295]. In particular, SLE patients who smoke or have pulmonary manifestations [296] have increased rates of lung cancer. In contrast, SLE patients have a substantially decreased risk of breast cancer, which may be due to SLE autoantibodies [297-299]. Cell penetrating anti-dsDNA autoantibodies inhibit DNA repair, which is often mutated in BRCA2 breast cancers, thus may suppress breast cancer cells with intrinsic defects in DNA-repair [298]. Additionally, SLE patients have decreased expression of anti-apoptotic mediator heat shock protein 27 (HSP-27) that may suppress breast cancer incidence by causing cancer dormancy [300] and increasing susceptibility to treatments [301].

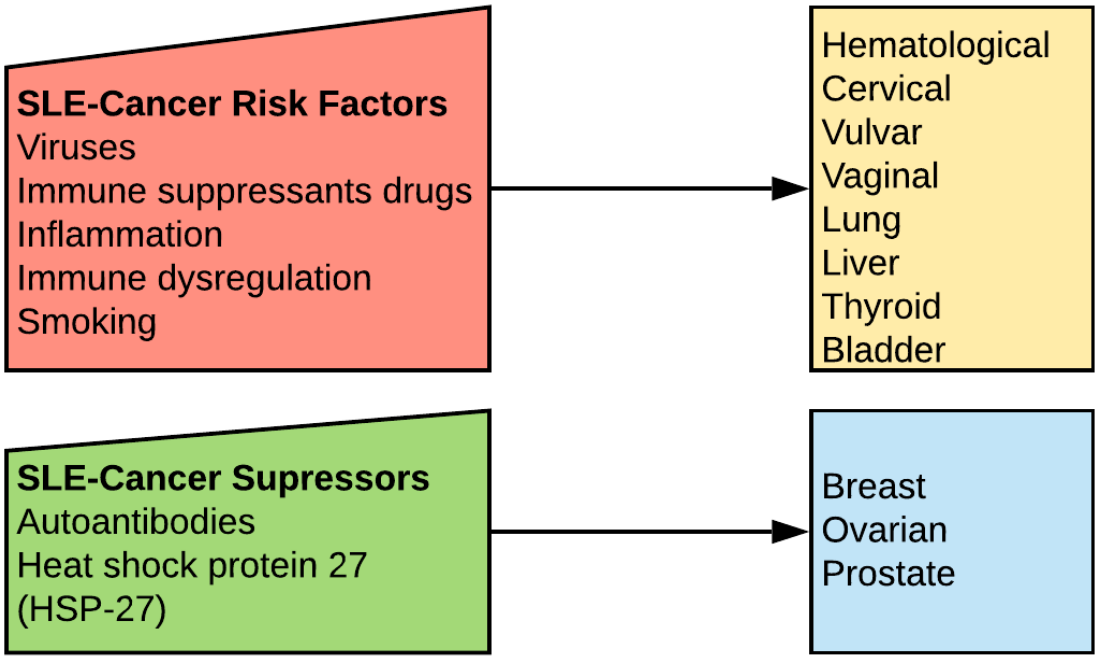

Figure 9. Factors that increase or decrease SLE patient cancer risk

Various environmental (including infectious disease), SLE immune dysregulation and treatment, and lifestyle choices increase (red box) or decrease (green box) SLE patient cancer risk. SLE patients have increased risk for some cancers (yellow box) and decreased risk for others (blue box) [302, 303].

1.2.6 Multiple sclerosis (MS) and cancer risk

EBV is also implicated in development of multiple sclerosis (MS) [304], a chronic autoimmune inflammatory disease affecting the central nervous system [305]. Like SLE, T and B cells generate autoantibodies that demyelinate nerve cells causing inflammation [306]. MS causes fatigue, pain, restricted movement, coordination, and cognitive and visual problems [307]. Also like SLE, MS immunosuppressive treatment may exacerbate cancer development [308]. It is unclear how MS affects overall cancer risk as studies have found MS patients have raised, lowered, and similar cancer risk compared to the general population [309]. This confusion

extends to breast cancer risk [309]. While MS patients have a decreased risk for ovarian and prostate cancers, hematological cancers are consistently increased again suggesting that poor viral control may lead to cancer development [310].

1.2.7 Lupus and cancer summary and questions

SLE is a complex systemic autoimmune disease created by inherent immune dysregulation. In addition to the morbidities caused directly by SLE, immune dysregulation promotes viral infections. It is unclear how these factors, together with SLE treatment, individually promote specific malignancies. To tease apart these contributions, MS patient cancer risk was also assessed.

Questions addressed include: do SLE patients have increased incidence of viruses, including EBV? Does SLE status, overall viral incidence, or both together best explain overall cancer incidence? Does SLE status, EBV or both together best explain increased SLE hematological cancer incidence? And how does MS compare to SLE for all of these factors? Can we tease apart SLE autoimmune contributions by looking at MS patients? Understanding the root of cancer incidence in SLE could inform preventative treatment and promote longer survival rates for SLE patients.

1.3 Vaccine hesitancy

Vaccines are one of the most successful infectious disease interventions [311, 312]. Vaccinations have led to the control or elimination of diseases that were once endemic, often called “childhood diseases” or vaccine preventable diseases (VPDs) [311]. VPDs include smallpox, polio, diphtheria, measles, and pertussis. Before vaccines, many more children became severely ill or died from these diseases. The use of vaccines has successfully eradicated smallpox worldwide and eliminated endemic poliomyelitis, measles, rubella, and congenital

rubella syndrome from the Americas [312, 313]. Most developed countries have high rates of childhood vaccination coverage, which suggests that vaccination is a widely accepted public health measure [313]. However, as recent outbreaks of VPDs such as measles [314, 315], poliomyelitis [316], and pertussis [317] suggest, national estimates of high uptake obscure clusters of under-vaccinated individuals [318]. These outbreaks are mainly linked to under-vaccinated or non-vaccinated communities [319].

In response to these outbreaks, in 2019, the World Health Organization (WHO) listed vaccine hesitancy (VH) as one of its top ten threats to global health along with items like climate change, limited primary care, and antibiotic resistance [320]. In 2012, the WHO's Strategic Advisory Group of Experts (SAGE) on Immunization defined VH as "delay in acceptance or refusal of vaccination despite availability of vaccination services. VH is complex and context specific, varying across time, place and vaccines. It is influenced by factors such as complacency, convenience and confidence" [321]. While vaccines prevent approximately 2-3 million deaths globally every year, another 1.5 million deaths could be prevented yearly if vaccines were administered in a timely manner to improve global coverage [320].

Many countries and communities have increasing numbers of individuals who are delaying or refusing vaccinations [322-324]. VH is driven by a complex milieu of factors that are specific to time, place, and vaccine. These issues include a host of sociodemographic factors, cultural values, poor public health literacy or communication, public perception of risk, easy access to conflicting information, and an increased distrust of experts [325-328]. Each country has unique challenges. Where one country may have a religious group that distrusts porcine components in vaccines, another country may associate immunizations with female infertility, and yet another country may observe VH in people of high socio-economic status living in urban

areas concerned about vaccine safety [324]. Public health officials are scrambling to respond effectively to VH, yet interventions are not a one-size-fits all solution and must be tailored to each situation.

1.3.1 What is vaccine hesitancy like in the United States?

To prevent disease outbreaks the WHO recommends at least 95% childhood vaccination rate to sustain herd immunity [329]. In 2017, (the most up-to-date statistics available), only 70.4% of children aged 19-35 months had complete vaccination coverage in the United States. Complete vaccination includes seven vaccines (11 diseases) and individual rates vary by vaccine: Diphtheria/tetanus/pertussis (83.2%), polio (92.7%), measles/mumps/rubella (91.5%), *Haemophilus influenza* type b (80.7%), hepatitis B (91.4%), chickenpox (91.0%), and pneumococcal conjugate vaccine (82.4%) [330]. Overall vaccination rates have improved from the early 2000s, however there are clear sociodemographic differences by economic and racial groupings. For example, 74.2% of Caucasians at or above the poverty level compared to 64.0% of Caucasians below the poverty level fully vaccinated their children [330]. This pattern was repeated for African American (73.8% vs 61.7%) and Hispanic (71.7% and 61.8%) communities, suggesting that availability, affordability, convenience or even education may delay vaccination in lower income populations [330]. Interestingly, for individuals above the poverty line, the racial divide noted in 2016 between Caucasians (74.6%), African Americans (64.4%) and Hispanics (68.9%) vastly improved in 2017 where Caucasians (74.2%), African Americans (73.8%), and Hispanics (71.7%) had roughly the same vaccine uptake [330]. Despite high vaccine rates for individual vaccines, up to 1.3% of children in the US have not received a single vaccine by 24 months [331]. This number is significantly higher than the 2001 rate of 0.3% [331]. Even among parents who vaccinate and do not pursue exemptions, VH ideology is more

common [331]. For example, in a survey of parents who vaccinated their children in Wisconsin, US, 23.4% of parents believed that children get more shots than is good for them, 33.7% believed that vaccines could overwhelm the immune system, and 34.2% supported non-medical exemption laws that would allow unvaccinated children to attend school [332].

Due to the success of vaccines, many parents of young children in the US do not have personal experiences with VPDs [333]. As such, it is easier for parents devalue the risk of VPDs and increasingly perceive the health and safety risks of vaccinations to be the greater threat [334-339]. This may partially explain the increase in the number of parents exempting children from one or more vaccines [340]. For example, while only 12% of parents of children 17 years old or younger reported refusing a vaccine, 54% of parents expressed concerns about vaccine safety [341]. The concerns raised by VH parents fall into four broad categories: 1) vaccination may not be effective, 2) safe, 3) or needed, or 4) that reasonable alternatives to vaccinations might be available. More specifically, parents worry about whether vaccines will work; that vaccines might cause permanent injury, including autism; whether loved ones are actually at risk for a VPD; whether VPDs are actually dangerous; whether vaccines pose religious or moral issues; and whether they can trust government officials and the pharmaceutical industry [331]. See Table 1 for more detailed examples.

Table 1. Detailed examples of concerns of VH parents from Smith, TC 2017 [342]

Examples of Specific VH Concerns
Vaccines are "toxic" and contain antifreeze, mercury, ether, aluminum, human aborted fetal tissue, antibiotics, and other dangerous chemicals that can lead to autism and an assortment of chronic health conditions. Slogan: "Green our Vaccines".
Vaccines are a tool of "Big Pharma;" individuals who promote them are merely profiting off of harm to children and/or paid off by pharmaceutical companies ("Pharma shills").
A child's immune system is too immature to handle vaccines; they are given "too many, too soon" and the immune system becomes "overwhelmed," leading to autism and an assortment of chronic health conditions.
"Natural immunity is better;" most vaccine-preventable diseases are harmless to most children, and natural exposure provides more long-lasting immunity. eg, "I had the chickenpox as a kid and I was just fine." Some individuals may also have the mistaken belief that all "natural" infections confer life-long immunity, whereas all vaccine-derived immunity is short-lived.
Vaccines have never been tested in a true "vaccinated versus unvaccinated" study; the vaccines in the current schedule have never been tested collectively.
Diseases declined on their own due to improved hygiene and sanitation; "vaccines didn't save us."
Vaccines "shed" (can be transmitted by vaccinated individuals to others); therefore, cases of vaccine-preventable diseases in the population are driven by the vaccinated, not the unvaccinated.

Researchers have grouped parents into three categories: vaccine acceptors who follow vaccination guidelines, fence sitters who have concerns about vaccines but give their children some vaccines, and rejecters who reject all vaccines [343]. Vaccine fence sitters and rejecters exhibit shared moral preferences for liberty (belief in individual rights) and harm (concern about the wellbeing of others) [343]. In addition to these qualities, vaccine rejecters have a diminished moral preference for authority (deference to those in positions of power) and a heightened moral preference for purity (abhorrence of body impurities) [343]. Furthermore, vaccine opposition, especially in vaccine rejection, may also stem from deep-rooted ideological beliefs [324, 344] or conspiracist ideational tendencies [345]. Correcting vaccine misinformation can cause some to become more entrenched in their position, resulting in conformational bias where parents hear correct information but draw incorrect conclusions supporting their own ideology [346, 347].

Thus, while corrections about vaccines can diminish, but not eliminate vaccine misinformation, they can also reduce intention to vaccinate among certain groups [343, 346, 347].

1.3.2 Measles: A case study of consequences of vaccine hesitancy

In 2019, measles resurged by 30% globally and while the reasons for the resurgence are complex, vaccine hesitancy is an important component [320]. In the US where measles was declared eradicated in 2000 [348], there were 1249 reported measles cases, the highest number of cases since 1992 [349]. These outbreaks often occurred in tight-knit communities with shared belief systems discouraging vaccination; 89% of reported cases were unvaccinated or had unknown vaccination status [349]. Every outbreak since 2000 was initiated by international travelers acquiring measles abroad [350]. Assessment of the 2013 measles outbreak in New York City, revealed that 78% of infected individuals were unvaccinated owing to parental refusal or intentional delay [351]. The direct costs of this outbreak for the New York City Department of Health and Mental Hygiene were high: $394,448 and 10,054 working hours [352]. This does not account for indirect costs experienced throughout the community such as lost wages to care for a sick child, individual family healthcare costs, and disrupted school attendance. Because measles is so infectious (9 in 10 unvaccinated individuals exposed to the measles will catch the virus), the latest studies suggest that effective herd immunity is only reached when >95% of the population is vaccinated; however most countries don't quite reach that level and are at or slightly above 90% coverage [353].

As demonstrated by the recent measles outbreaks, vaccine hesitancy poses health risks to the general public and immunocompromised individuals and causes economic disruption. The current SARS CoV2 outbreak has resulted in the worst level of unemployment since the great depression (a 14.8% unemployment rate at the time of writing) and there is no vaccine available

at this time. VPDs are controllable and therefore preventable disasters. Thus, it is of great importance to find interventions that effectively resolve vaccine concerns and improve vaccination rates in individual communities.

1.3.3 Vaccine hesitancy interventions

As VH is a serious threat to global health, research has focused on understanding the underlying causes and then creating effective interventions. There are two main types of interventions: 1) interventions that seek to correct erroneous misinformation, and 2) interventions that focus on directing attention to other issues. Often effective interventions have elements of both indirect and direct methods.

When deciding whether to vaccinate their children, parents deliberate between the perceived risks associated with vaccinating and the risks of not vaccinating [354]. Unfortunately, countering erroneous vaccine myths by relaying correct information risks repetition of the incorrect information leading to familiarization that may strengthen individuals' memories for the false information [355, 356]. Furthermore, it is difficult to provide convincing evidence of the absence of risk [357]. Thus, replacing an existing belief with an alternative belief (indirect method) may be more effective than attempting to counter it directly [355].

Direct interventions operate on the assumption that access to vaccination information will improve vaccination uptake. These interventions target improving access to vaccination services, enhancing demand for vaccinations, and health care provider-based or system-based interventions. There is strong evidence from numerous randomized controlled trials and quasi-experimental studies that show that these interventions have effectively improved vaccination rates [358]. These interventions include standing orders; vaccination requirements for child care, school, and college attendance; vaccination programs in schools or child care centers; reducing

patient out-of-pocket costs; healthcare provider reminders, assessment, and feedback; and patient reminder and recall systems. Health care provider recommendations have been shown to have a strong impact on improving vaccine uptake [359-368].

An example of a direct intervention includes strong recommendations from a health care provider can overcome VH more than soft recommendations [364, 369]. The type of conversations healthcare providers have with their patients can have a significant impact on the response of parents. In one study, parents of infants who were 19 months or younger had encounters with their physicians that either used conversational language ("What are you planning to do about the vaccines?") or presumptive language ("We have to do some shots.") [370]. Parents who heard presumptive language from their physician were 17.5 times more likely to accept vaccines than parents hearing conversational language [370]. Unfortunately, parents of adolescents report hearing presumptive language less than 15% of the time [371] while parents of infants report more than 70% of physician interactions involve presumptive language [370].

In this next example, direct methods competed against indirect methods. In one study, parents were randomly assigned to 1 of 4 web based interventions that ranged from direct information explaining the lack of evidence that MMR causes autism, to indirect textual information about the dangers of diseases prevented by MMR, to images of children who have the disease, and finally to a dramatic narrative about an infant that almost died from measles [347]. The material for each intervention was created by the Centers for Disease Control (CDC) and Prevention. However, none of these interventions increased parental intent to vaccinate. As suggested, directly confronting the MMR-autism link by citing an absence of data reduced the misperceptions of the myth yet decreased the parental intent to vaccinate. Counterintuitively,

images of sick children increased the belief of the vaccine-autism link while the narrative about the sick infant increased belief that vaccines have serious side effects [347]. However, another study refuted these findings using similar materials from the CDC. This study found that while direct confrontation of vaccines myths (such as vaccines cause autism) did not improve parental vaccine attitudes, focusing parental attention on the consequences of not vaccinating their children improved vaccination [354]. Thus, interventions seeking to inform parents of the dangers of VPDs have had mixed results. In these examples, direct interventions (confronting the false vaccine-autism link) rarely improves vaccine rates, and indirect interventions (education about VPDs) had opposing effects. The differences between these two studies may have been the timing or application of the materials used.

As parents seem to remain VH even after introduction to information designed to reduce vaccine misperception, children and adolescents may be important agents of change [347, 372]. As seen for other conditions such as asthma, hypertension, smoking, and weight/physical activity, children can be important behavior change agents relaying information learned at school to create child-led positive health changes in their homes [373-376]. Furthermore, teenagers and children will eventually become future influencers and parents and make decisions for their own families [377]. One way to educate children and parents is to involve teachers and doctors. Teachers and doctors are generally considered credible and respected sources of information and when they communicate directly with children and adolescents, they can enact highly effective change [378-380]. In one study, VH parents reported that doctors who took time to establish a trusting relationship and provide appropriate educational materials were the most effective at changing the parents' minds [381]. Thus, future VH interventions should consider including

children and teenagers as important vectors of change in their homes, especially in conjunction with healthcare providers and teachers who have creditability.

In conclusion, vaccines are the most effective public health intervention available for VPDs and yet VH threatens the efficacy of vaccine success. While VH interventions can be effective, they must be tailored to unique populations and beliefs. Reviews addressing the effectiveness of interventions concur that few interventions were directly targeted to vaccine hesitant individuals [382]. Furthermore, there is no strong evidence to recommend any specific interventions [382, 383]. There are hints that multicomponent and dialogue-based interventions were most effective, but that was compounded by the complexity of vaccine hesitancy and limited evidence about intervention effectiveness currently available [382, 383]. It is important to prioritize research to understand VH groups and to identify effective interventions that avoid confirmation bias or solidification of false concepts regarding vaccine risk and effectiveness.

1.4 Summary of research chapters

Chapter 2 is a recently submitted manuscript where we explore the effects of TCR-pMHCII affinity on T cell activation with and without CD4 signaling. We show that increased TCR affinity is correlated with increased production of IL-2. IL-2 production is dependent on the presence of CD4. While low affinity constructs are reliant on CD4-Lck signaling, high affinity construct signaling is abrogated by CD4 expression. This mechanism relies on the availability of Lck sequestered by the cytoplasmic tail of CD4. When CD4 is present for high affinity TCRs, CD4 is not efficiently recruited to the immunological synapse and thus sequestered Lck cannot begin the activation phosphorylation cascade. However, CD4 itself is inhibitory for high affinity constructs. If the CD4 cytoplasmic domain is truncated and Lck is free, there is still an inhibition signal from CD4 binding to MHCII. This pattern is shown for

both flTCRs and TCR-SCS CAR constructs. However, the affinity threshold for this effect is construct dependent: flTCRs demonstrate CD4 independent inhibition at intermediate affinity, while TCR-SCS CARs show CD4-independent inhibition at high affinity. Thus, CD4-Lck, TCR-pMHCII affinity, and construct design play crucial roles in helper TCR signaling and are important considerations when designing immunotherapies.

Chapter 3 is a recently submitted manuscript detailing the interaction of systemic lupus erythematosus (SLE) or multiple sclerosis (MS), viral infections and cancer. SLE and MS patients have dysregulated immune function that results in abnormal cancer risk and viral susceptibility. This is compounded by the fact that SLE and MS patients are generally treated with immune suppression drugs. It is unclear whether the dysregulated immune conditions found in SLE and MS patients independently lead to increased viral incidences and abnormal cancer risk, or if the increased viral incidence leads to increased cancer risk. Using electronic medical records from Vanderbilt University we used logistic regression to show that overall cancer risk for SLE patients is similar to control populations, but somewhat raised for MS patients, indicating that autoimmune disorders overall have unique underlying effects on cancer surveillance. SLE and MS patients both had increased risk for virus infections overall. This heightened risk extended to Epstein Barr virus (EBV) which is implicated in hematological cancers. As expected, hematological cancer risk was raised for SLE and MS patients in the presence of EBV, therefore hematological cancer increase is not solely due to the autoimmune disorders. SLE patients had decreased risk for hormone-based cancers, including breast and prostate cancers, which confirms previous findings. Overall, our results demonstrated that rather than autoimmune disorders independently increasing the risk of cancer and viral incidence, autoimmune disorders increased the risk of viral incidence and therefore viral-based cancer risk.

Chapter 4 is a published research paper describing an interview- and education-based intervention for vaccine hesitant college students. In 2019, the World Health Organization listed vaccine hesitancy as one of the top ten threats to global health. Urban centers with large clusters of vaccine-hesitant individuals are especially vulnerable to vaccine preventable disease (VPD) outbreaks. In 2016-2017, Utah County ranked sixth nationally for the total number of kindergartners that were under-vaccinated. To help combat this trend, we created an interview-based intervention deployed in three classes on BYU campus including Bio 100 (non-science students, vaccine discussion), MMBio 240 (science students, no vaccine discussion) and MMBio 261 (science students, vaccine discussion). Students were surveyed for their initial vaccine attitude score (VAS), asked to interview a member of the community that had had either a VPD or an autoimmune disorder, and surveyed again for their final VAS. We found that vaccine hesitant students who completed VPD interviews had significantly increased VAS. However, education had a greater effect when the course focused on diseases and immune function (MMBio 261), and a negative-neutral effect when the course focused on vaccine safety (Bio 100). Thus, educating the public about the risks of VPDs, the function of the immune system, and encouraging discussions with VPD-victims may promote vaccine uptake in the community.

Chapter 5 is the final chapter and summarizes the main findings for TCR-pMHCII affinity and T cell activation, autoimmune disorders-virus link for cancer, and vaccine hesitancy intervention. Future research plans to further investigate the mechanism for CD4-dependent inhibition is discussed.

1.5 Summary of appendices

Appendix I includes two abstracts published in *Journal of Immunology*. The first abstract covers work on high affinity T cells (chapter 2). The first abstract was created for a poster

presentation at the annual conference of *The American Association of Immunologists* presented by me in San Diego, CA in April 2019. The second abstract discusses work accomplished with Dr. Claudia M. Tellez Freitas on CD5 co-receptor in T cell metabolism and cognitive behavior. While I designed further behavioral, IgA, and metabolomics experiments for this project, another graduate student will complete the work. The abstract was created for an oral and poster presentation at the annual conference of *The American Association of Immunologists* presented by Claudia in Austin, TX in May 2018.

Appendix II is a list of presentations presented during my PhD.

Lastly, Appendix III contains a compilation of the work I published during my PhD/ dissertation as both a first author and co-author.

CHAPTER 2: CD4 Inhibits Helper T cell Activation at Lower Affinity Threshold for Full-Length T Cell Receptors Than Single Chain Signaling Constructs

The content of this chapter was submitted May 2020. It is currently under-review. Authors: Deborah K. Johnson, Wyatt Magoffin, Sheldon J. Myers, Jordan G. Finnell, John C. Hancock, Taylor S. Orton, Stephen P. Persaud, Kenneth A. Christensen and K. Scott Weber. It has been reformatted for this dissertation, but is otherwise unchanged.

Abstract

$CD4^+$ T cells are crucial for effective repression and elimination of cancer cells. Despite a paucity of $CD4^+$ T cell receptor (TCR) clinical studies, $CD4^+$ T cells are primed to become important therapeutics as they help circumvent tumor antigen escape and guide multifactorial immune responses. However, because $CD8^+$ T cells directly kill tumor cells, most research has focused on the attributes of $CD8^+$ TCRs. Less is known about how TCR affinity and CD4 expression affect $CD4^+$ T cell activation in full length TCR (flTCR) and TCR single chain signaling (TCR-SCS) formats. Here we generated an affinity panel of TCRs from $CD4^+$ T cells and expressed them in flTCR and three TCR-SCS formats modeled after chimeric antigen receptors (CARs) to understand the contributions of TCR-pMHCII affinity, TCR format, and coreceptor CD4 interactions on $CD4^+$ T cell activation. Strikingly, the coreceptor CD4 inhibited intermediate and high affinity TCR-construct activation by Lck-dependent and -independent mechanisms. These inhibition mechanisms had unique affinity thresholds dependent on the TCR format. Intracellular construct formats affected the tetramer staining for each TCR as well as IL-2 production. IL-2 production was promoted by increased TCR-pMHCII affinity and the flTCR format. Thus, $CD4^+$ T cell therapy development should consider TCR affinity, CD4 expression, and construct format.

2.1 Introduction

$CD4^+$ T cells are critical for tumor elimination through both indirect and direct mechanisms. Indirectly, $CD4^+$ T cells target tumor cells by activating tumor-killing cells such as $CD8^+$ T cells, macrophages, B cells and natural killer cells [143-145, 384]. $CD4^+$ T cells have direct cytotoxic effects against tumor cells that express major histocompatibility complex II (MHCII) [143-145, 384] and direct $CD4^+$ T cell responses are less toxic to the patient than a $CD8^+$ T cell response, especially when responding to overexpressed tumor associated antigens (TAA) [146]. The presence of tumor-specific $CD4^+$ T cells is correlated with improved patient survival following vaccination with cancer-associated peptides whether or not they are directly involved in tumor suppression [385-387]. Furthermore, $CD4^+$ T cells can sustain an immune response when $CD8^+$-specific antigens are lost which otherwise might result in tumor escape [388]. Despite these clear benefits, only one published clinical study [142] focuses on the immunotherapeutic benefits of $CD4^+$ T cell receptors (TCRs) [142, 389].

$CD4^+$ T cells are activated by interactions between the TCR and its cognate peptide presented on MHCII (pMHCII) [390]. TCRs can detect a single amino acid change and distinguish between self-proteins and mutated neoantigens [389], uniquely suiting TCR-based therapies for specific tumor targeting. Furthermore, unlike antibody-based chimeric antigen receptors (CARs), which are limited to extracellular targets, TCRs can target intracellular antigens presented by MHC molecules [389]. To rationally design optimal targeting strategies, it is essential to understand how the TCR:pMHC interaction impacts T cell responses. The relationship between TCR affinity and T cell activation is complex, but in general, T cell functional activity correlates with TCR binding affinity for pMHC [85, 99, 391-395]. However, there are important nuances to this general theme. For example, tumor-associated antigens may

be skewed towards lower-affinity clones due to thymic negative selection [83, 396], even the lowest-affinity TCRs can induce T cell proliferation, cytokine production and memory formation [84, 85]. On the other end of the spectrum, high affinity TCRs have been shown to enhance immune responses in some cases [104] and attenuate responses in others [111, 138, 397-400], with some reports showing evidence of an affinity threshold beyond which increased affinity does not impact the magnitude of the response [394, 395]. An additional consideration is that even when high-affinity TCRs are capable of heightened cytotoxicity and tumor control, these TCRs may be predisposed to autoimmunity [401]. Thus, the optimal affinities for TCRs engineered against tumor-specific peptides may lie within a low or intermediate affinity [91, 99, 397-399, 401-406]. As most affinity studies to date have focused on $CD8^+$ TCRs, $CD4^+$ T cell affinity thresholds are less well characterized.

The role of the CD4 coreceptor is an important consideration when associating TCR-pMHCII affinity to $CD4^+$ T cell activation. CD4 binds to MHCII as part of the TCR complex and contributes to proximal TCR signaling, proving especially critical for T cell function when cognate pMHC ligands are limiting (<30 complexes) [407]. TCR signaling dependence on CD4 is affected by the quality of TCR:pMHCII interaction and thus unnecessary upon stimulation with optimal ligands [408]. Thus, CD4 may be restricted to improving the TCR dwell time on pMHCII for lower affinity interactions [409]. As $CD4^+$ T cells can function without CD4 [147], CD4 may not have as great of an effect on T cell activation as CD8, particularly with high affinity TCRs.

To determine how TCR-pMHCII affinity and CD4 coreceptor interactions affect $CD4^+$ T cell activation, we examined activation of the CD4 transgenic murine T cells LLO118 and LLO56 that are stimulated by the same *Listeria monocytogenes* epitope. These TCRs differ by

15 amino acids and recognize the $LLO_{190-205}$ peptide presented by the MHCII molecule I-A^b with similar affinity [148, 410]. LLO118 has a more robust primary response and LLO56 has a more robust secondary response, indicating that TCR affinity is not the only parameter affecting activation in these cells. To examine the role of affinity in the activation responses of LLO56 and LLO118, we engineered an affinity panel of $CD4^+$ TCRs (ranging from 4 μM to 200 nM). After characterizing their affinity and avidity, the activation characteristics of two low affinity clones, two intermediate affinity clones, and one high affinity clone were examined in the full length TCR (flTCR) format or in three TCR-SCS CAR formats (CD28- and 4-1BB-based second generation CARs, and CD28/4-1BB third generation CAR). T cell receptor single-chain signaling chimeric antigen receptors (TCR-SCS CARs) are an exciting potential therapeutic option and as $CD4^+$ T cells are potent responders to cancer, we sought to understand how $CD4^+$ TCRs respond to a variety of affinities. TCR-SCSs constructs avoid mispairing with endogenous TCR chains, which is an inherent risk for engineered flTCRs [411]. CARs also produce more cytokines and are activated by higher antigen densities than flTCRs and may be more likely to ignore healthy cells with low amounts of TAAs, which may improve clinical outcomes [114, 412, 413].

We found that increased TCR affinity promotes production of IL-2 regardless of flTCR or TCR-SCS format. The flTCRs are more responsive to lower amounts of peptide stimulation, and contrary to $CD8^+$ TCR findings [62], produce more cytokine than TCR-SCSs. While there are some observable trends dependent on second and third generation TCR-SCS CAR format, IL-2 production varies depending on whether the TCRs were engineered from the LLO56 or LLO118 TCRs. CD4 promotes the activation of low affinity flTCRs and scTCRs, but CD4 is inhibitory in intermediate affinity flTCR and high affinity TCR-SCS CARs. The flTCR reaches

CD4 inhibition at a lower affinity than TCR-SCSs, suggesting that flTCRs perceive a stronger initial activation signal. These findings suggest that therapeutic CD4 TCR development should consider construct features, TCR affinity, and coreceptor activation contributions when choosing or engineering therapeutic TCRs and cell lines.

2.2 Materials and methods

2.2.1 Library construction

The scTCR constructs for LLO56 (residues 1-116) and LLO118 (residues 1-120) (Invitrogen) consist of the mature Vβ domain, a 13-aa linker (DAKKDAAKKDDAS) [414], followed by the mature Vα domain (LLO118 residues 1-112 or LLO56 residues 1-113), and an N-terminal HA tag (PYDVPDYA). To display scTCRs on yeast, the constructs were placed in pCT302 (NheI and BglII) (Addgene plasmid # 41845 ; http://n2t.net/addgene:41845 ; RRID:Addgene_41845) [415]. Stability clones were selected from scTCR transcripts replicated by error-prone PCR (Standard Taq, New England BioLabs, B9014S) [416]. Affinity libraries were generated using site directed mutagenesis of 5 amino acids in the CDR3β region and splicing by overlap extension (SOE) PCR [416, 417] using LLO118 and LLO56 specific primers (Q5 High Fidelity DNA Polymerase, New England BioLabs M0491) (Supplemental Materials).

To generate yeast libraries, 150 mL cultures of growth phase EBY100 yeast were collected and washed twice with 50 mL ice-cold water and once with ice-cold electroporation buffer (1 M Sorbitol/1 mM CaCl2) then resuspended in 0.1 M LiAc/10 mM DTT and incubated at 30°C and 225 rpm for 30 mins [418]. Cells were washed with 50 mL electroporation buffer, resuspended in 200 µL electroporation buffer and aliquoted with digested pCT302 backbone (NheI and BglII, 1,250 ng) and inserted (6,250 ng) into 0.2 mm gap cuvettes then electroporated (2.5 kV and 25 µF). Cells were allowed to recover for 1 hr in 4 mL 1 M sorbitol:YPD media

(1:1) and were resuspended in SD-CAA media and incubated for 2-3 days at 30°C before quantification. Stability and affinity library sizes ranged from 1.1 x 10^7 to 1.9 x 10^9.

2.2.2 Stability clone selection

Libraries calculated to have at least 10 copies of each clone were placed in 5 mL SG-CAA media for 36-48 hrs to induce scTCR expression [419]. To select stability clones, yeast libraries were incubated with either 2 µg/mL anti-mouse TCR Vα2 or anti-mouse TCR Vβ2 phycoerythrin-conjugated antibodies (BioLegend, clone B20.1 and B20.6, respectively) in 5 mL PBS 1% BSA for 2 hrs at 4°C, washed with 15 mL PBS 1% BSA and stained with 50 µL anti-PE MicroBeads in 2 mL PBS 1% BSA (Millitenyi 130-048-801) for 20 min at 4°C. Labeled clones expressing properly folded Vα or Vβ were positively selected in magnetic LS columns (Millitenyi 130-042-401). Selected cells were grown in 3 mL SD-CAA media (48 hrs) before induction in SG-CAA (36-48 hrs). Each library was subjected to three rounds of growth and sorting, and the most stable clone identified via flow cytometry (BD Accuri C6). Stability clones were used as templates for subsequent stability or affinity libraries.

2.2.3 Affinity clone selection

To select affinity clones, induced yeast libraries were incubated with tetramer ($LLO_{190-201}$/I-A^b) (I-A(b)CC (NEKYAQAYPNVS), NIH 22201), and sorted like stability clones. To isolate high affinity clones, libraries were exposed to an increasingly strict temperature and incubation regimen. Initially, libraries were subjected to high concentrations of tetramer (13.0 µg/mL), high temperatures (37°C), and long incubation times (3 hrs), and in later rounds, combinations of lower tetramer concentrations (3.25 µg/mL), lower temperatures (RT or 4°C), and shorter incubation times (1 hr) were used to isolate the clones with highest affinity. Each

library was column sorted three times. Isolated clones with increased tetramer binding were identified via flow cytometry (BD Accuri C6).

2.2.4 Tetramer dissociation

Each affinity and stability clone K_D was determined through tetramer dissociation [420]. Aliquots of $1x10^6$ induced cells were stained with 100 µL of various concentrations of $LLO_{190-201}$/I-A^b tetramer (0.152 nM to 12.16 nM) for 1.5 hrs at room temperature and quantified via flow cytometry. Tetramer binding was assessed as MFI of positive population and normalized to the highest recorded MFI using FlowJo. K_D was defined as 50% maximum binding concentration [420].

2.2.5 Tetramer decay

Half-life ($t_{1/2}$) was determined by staining $3x10^6$ cells of each affinity clone with 6.5 µg/mL of tetramer for 1.5 hrs at room temperature [103]. Samples were washed three times in PBS 1% BSA to remove excess tetramer. Following an initial timepoint measurement, 90 µL of 0.1 µg/mL or 1 µg/mL anti-mouse MHC class II (I-A/I-E) (clone: M5/114.15.2, eBioscience) was added and the decrease of tetramer binding was quantified at various time points (2, 5, 10, 15, 20, 30, 45, and 60 mins) by placing 10 µL of cells into 90 µL of buffer and running immediately on the flow cytometer.

2.2.6 scTCR expression, refolding and purification

The following protocol was modified from Garcia *et al*. Briefly, scTCR constructs were cloned into pET28a (Novagen) using NcoI and SacI restriction sites. Constructs were expressed in BL21 T7 Express *E. coli* (New England Biolabs) and protein expression was induced for 4 hrs (0.4 mM isopropyl β-D-thiogalactoside). Cells were lysed with 1 mg/mL lysozyme (ThermoFisher Scientific), 5 mM MgCl2, 1 µL/mL DNase I (Promega), 1% Triton-X 100, and

10 mM dithiothreitol followed by two rounds of sonification (Branson Digital Sonifer) for 1 min at 0.5 sec alternations at 40% power. 50-200 mg of inclusion body slurry was dissolved in 1 mL of 7M GnHCl and 10 mM beta-mercaptoethanol. 400 mL of 2 M GnHCl, 50 mM Tris-HCl, 2mM GSH, 0.2 mM GSSG and 0.1% NaAz were dripped into dissolved inclusion bodies for 2-4 hrs at 4°C. Then 2-2.5 L of 200 mM NaCl, 50 mM Tris-HCl, and 0.1% NaAz were dripped for 24 hrs (1.5 mL/min speed) at 4°C. Following an additional 24 hr spinning at 4°C, the refolded TCR solution was vacuum filtered with 0.22 µm PES membranes (Olympus Plastics), and then concentrated in an Amicon 8400 unit (Ultracel 10 kdal Ultrafiltration Discs) under 55psi N_2. Once the volume was reduced to 50-100 mL of refolded scTCRs, the samples were again filtered with 0.45 µm CA-membrane and GF prefilter syringe filter and purified by FPLC (AKTAstart) on a HisTrap column (GE Life Sciences). Purified scTCRs were concentrated using Amicon centrifugal filters (Ultra 4 10k) and quantified by Pierce BCA Protein Assay kit (Thermo Scientific).

2.2.7 Bio-layer interferometry (BLI)

BLI experiments were performed with an Octet RED96. Streptavidin (SA) biosensors (FortéBio) were hydrated and equilibrated in 1x HEPES buffered saline (HBS, 50 mM HEPES, 150 mM NalCl, pH 7.2), 2mM $CaCl_2$, 1 mM $MgCl_2$, 1 mg/ml milk, 0.1% Tween, and 0.02% NaN_3. SA sensors were loaded with 2.0 µg/mL biotinylated $LLO_{190\text{-}201}$/I-A^b monomer or $DQB_{187\text{-}101}$/I-A^b monomer to 1.0-2.0 nm. Loaded biosensors were equilibrated in assay buffer until baseline was achieved. scTCR association was probed in wells with assay buffer (stability clones 2 µM, 1 µM, 0.5 µM, 0.25 µM, 0.125 µM, 0.061 µM; affinity clones 800 nM, 400 nM, 200 nM, 100 nM, 50 nM, and 25 nM, or 20 nM, 10 nM, 5 nM, 2.5 nM, 1.25 nM, and 0.625 nM) with a blank reference-subtraction well for 400-600 sec. Ideal concentration range spanned one

log above and below the K_D where possible; however, this range had to be optimized depending on the sensitivity of the assay, and on the amount of protein available. Matching of sample and baseline imidazole and milk concentrations (through serial dilution of sample buffer into baseline wells) was critical for detection of scTCR binding. Blocking with bovine serum albumin increased non-specific binding while milk efficiently blocked NSB. Dissociation was observed in baseline assay buffer (600-1200 sec). Assays were run at 30°C with a plate shake speed of 1000 rpm.

Data was collected at 5 Hz, using 20-point signal averaging and analyzed using custom kinetic analysis. Due to non-specific binding at the later stages of the association and dissociation steps, K_D was calculated by extracting and selecting the data points from the initial association to determine k_{obs} (2-100 sec depending on the affinity of the constructs), plotting concentration vs rate, and then plotting those slopes against scTCR concentration and estimating k_{assoc} from the slope. k_{dissoc} is the slope of concentration vs rate of the dissociation step data (2-100 sec depending on the affinity of the constructs). K_D was determined by dividing k_{diss}/k_{assoc} and $t_{1/2}=\ln 2/k_D$.

2.2.8 Cell culturing

All $58^{-/-}$ T cell hybridoma cell lines were cultured in RPMI 1640, 10% FBS, 2g/L $NaHCO_3$ (23.8 mM), HEPES (4.2mM), L-glutamine (3.24 mM), 1% Penn-strep and split 1:5 or 1:10 every 2-3 days. Platinum Ecotrophic cells (Plat E) were cultured in DMEM, 10% FBS, 1% pen-strep, 1 µg/mL puromycin and 10 µg/mL blasticidin and split 1:4 every other day.

2.2.9 Retroviral transduction of T cell hybridomas

Affinity mutations were cloned into four possible constructs: full length TCRs (flTCRs), and three TCR-single chain signaling formats based on chimeric antigen receptor (CAR) formats

(second generation 4-1BB and CD28 CARs, and third generation 4-1BB/CD28 CAR). Inserts were cloned into pMSCV-IRES-GFP II (pMIGII) (Addgene plasmid # 52107 ; http://n2t.net/addgene:52107 ; RRID:Addgene_52107) using MfeI and XhoI (GenScript) [421]. All constructs were led by a Kozak sequence and either Vα2 signal peptide (MDKILTASFLLLGLHLAGVSGQ) and an additional Vβ2 signal peptide (MWQFCILCLCVLMASVATD) for flTCRs or high affinity M33 3rd gen CAR signal peptide (MLLALLPVLGIHFVLRDAQA) for all scTCR CAR constructs [422]. flTCR constructs have a P2A cleavage domain (GSGATNFSLLKQAGDVEENPG) [423] between Cα2 and Vβ2 domains.

Vectors were transfected into Plat E packaging cells grown overnight in 6-well plates with TransIT-VirusGEN (Mirus, MIR 6703). 48 h later, 1 mL of viral supernatant was mixed with 1 mL of 1x10^6 58$^{-/-}$ CD4$^-$ or 58$^{-/-}$ CD4$^+$ cells in a 6-well plate and spinfected for 2 h at 30°C at 1000 G (acceleration 6, brake 2). After 48h recovery, clones Vβ2, Vα2, and GFP$^+$ expression was checked by flow cytometry. Clones with under 85-90% GFP expression were sorted 1-3 times with magnetic LS columns (Miltenyi Biotec, 130-042-401) using 10 µL Vβ2-PE antibodies and 10 µL anti-PE MicroBeads (Miltenyi Biotec, 130-048-801) per manufacturer specifications. Clones were checked for TCR expression after each sort round. CD4T$^+$ and CD4T$^+$ Δbind [424] were cloned into pMIGII (MfeI/XhoI) and retrovirally transfected into existing 58$^{-/-}$ CD4$^-$ flTCR and TCR-SCS clones and sorted for >95% CD4 expression (CD4 PE-Cy7, GK1.5, Biolegend) by flow sorting (BD FACSAria II). 25,000 cells were stained with respective antibodies or tetramer for all affinity and stability measurements and measured with flow cytometry (BD Accuri).

2.2.10 T cell hybridoma peptide-specific activation and IL-2 measurement

2.8×10^4 T cell hybridoma clones were incubated with 2.8×10^5 splenocytes (1:10) isolated from BL6.C57 mice with varying amounts (10^{-8} M to 10^{-3} M) of peptide ($LLO_{190-205}$, GenScript) in 75 µL $58^{-/-}$ media in 96 well plate for 24 hrs. IL-2 production was measured using an IL-2 ELISA kit (KIT) and measured on a microplate reader. This study was approved and carried out in accordance with principles of the Basel Declaration and recommendations of Brigham Young University's Institutional Animal Care and Use Committee (IACUC protocol #18-0708).

2.2.11 Statistical analysis

Statistical analysis was performed via one-way ANOVA with Tukey's multiple comparison test ($p < 0.05$ was significant, no alpha adjustments required). Half-life ($t_{1/2}$) was determined by linear regression between time point 0 and the time point where no tetramer binding was detected [103]. To determine the K_D, we fit the data with a non-linear curve, based on one site-specific binding kinetics [420]. EC_{50} was determined with Sigmoidal, 4PL, X is log(concentration) least squares fit. Standard deviation is reported for each value. All analyses were conducted in GraphPad Prism.

2.3 Results

2.3.1 Yeast displayed TCR panel has varied affinities

Murine transgenic helper T cells LLO56 and LLO118 bear TCRs, which recognize the same naturally occurring *Listeria monocytogenes* peptide ($LLO_{190-205}$) presented on MHCII ($I\text{-}A^b$). The LLO56 and LLO118 TCR bind cognate pMHC with similar affinity (27.4 µM and 28.3 µM, respectively), yet have unique primary and secondary responses to TCR stimulus (summarized in Table 2) [148, 410]. To further elucidate the effects of TCR-pMHCII affinity on $CD4^+$ T cell activation, the variable regions of LLO56 and LLO118 (Figure 10A) were used as

templates for generating a panel of single-chain TCRs (scTCRs) with low (wild type), intermediate, and high affinities. scTCR libraries generated by random mutagenesis and expressed via yeast surface display were selected for protein folding stability through magnetic column sorting (Figure 10B). Vβ2 stability mutations were conserved between constructs while Vα2 stability mutations clustered in known stability hotspots (Figure 11). To generate affinity mutants, five amino acids in the stability mutants $LLO56_{low}$ and $LLO118_{low}$ complementarity determining region 3 of the β chain (CDR3β) were mutated by site directed mutagenesis and selected for improved binding affinity for $LLO_{190-201}$/I-A^b tetramers by magnetic column sorting. Increases in scTCR affinity cannot be explained by increases in scTCR expression, as HA and TCRα and TCRβ antibody binding remained the same across each experiment (Figure 10B). Additionally, none of the isolated stability or affinity mutants bound significantly to a non-target peptide tetramer ($DQB1_{87-101}$/I-A^b), indicating that the increase in tetramer binding is due to peptide-specific binding and not increased affinity for I-A^b alone (Figure 10C). Affinity mutant $LLO56_{int}$ with four CDR3β mutations (Figure 1D) bound $LLO_{190-201}$/I-A^b 1.5 log better than stability mutant $LLO56_{low}$ (Figure 10B). Affinity mutant $LLO118_{high}$ bound to the $LLO_{190-201}$/I-A^b tetramer 1.0-log better than affinity mutant $LLO118_{int}$ and 2.5-log better than stability mutant $LLO118_{low}$ (Figure 10B). $LLO118_{int}$ had three CDR3β mutations and $LLO118A_{high}$ had five additional CDR3β mutations (Figure 10D). While $LLO56_{int}$ and $LLO118_{high}$ mutants were used as templates for mutant libraries of the complementary determining region 3 of the α chain (CDR3α), no further clones with increased-affinity for $LLO_{190-201}$/I-A^b tetramer were isolated, suggesting that CDR3β is primarily responsible for $LLO_{190-201}$ peptide interactions for these specific TCRs. It is important to note that the stability clones initially isolated from yeast libraries relied on a frameshift mutation at the stop codon that added 19-amino acids from the

yeast expression vector to the carboxy end of Vα2 (RSDNNSVDVTKSTLFPPYF). While $LLO56_{low}$ and $LLO56_{int}$ successfully retained stability and affinity gains without the 19 amino acids, several attempts to create new LLO118 stability and affinity clones without the additional amino acids were unsuccessful. Therefore, the stabilizing 19 amino acids were maintained for LLO118 clones. Other studies have utilized TCR formats that express constant domains in order to maintain scTCR folding while adding additional affinity mutations to the TCRs, therefore this observation was not unexpected [425-427].

Table 2. Summary of T cell responses of LLO56 and LLO118 to antigen adapted from Persaud et al

Parameter	LLO56	LLO118
Primary response *in vivo*	+	+++
Secondary response *in vivo*	+++	+
Proliferation in response to peptide $LLO_{190-205}$ *in vitro*	+	+
Proliferation in response to *Listeria monocytogenes in vitro*	+	+
Rate of apoptosis *in vivo*	+++	+
IL-2 production *in vitro*	++	+
K_D (uM) $LLO_{190-205}$ (surface plasmon resonance)	+	+

LLO118 and LLO56 differentially respond to activation by peptide $LLO_{190-205}$. LLO118 and LLO56 have similar proliferation responses to $LLO_{190-205}$ *in vitro*, but *in vivo* LLO118 has a more robust primary response while LLO56 has a more robust secondary response. LLO56 produces more IL-2 *in vitro* and undergoes higher rates of apoptosis during the primary response than LLO118. Surface plasma resonance measurements of LLO56 and LLO118 dissociation constants are 27.4 μM and 28.3 μM, respectively [148, 410].

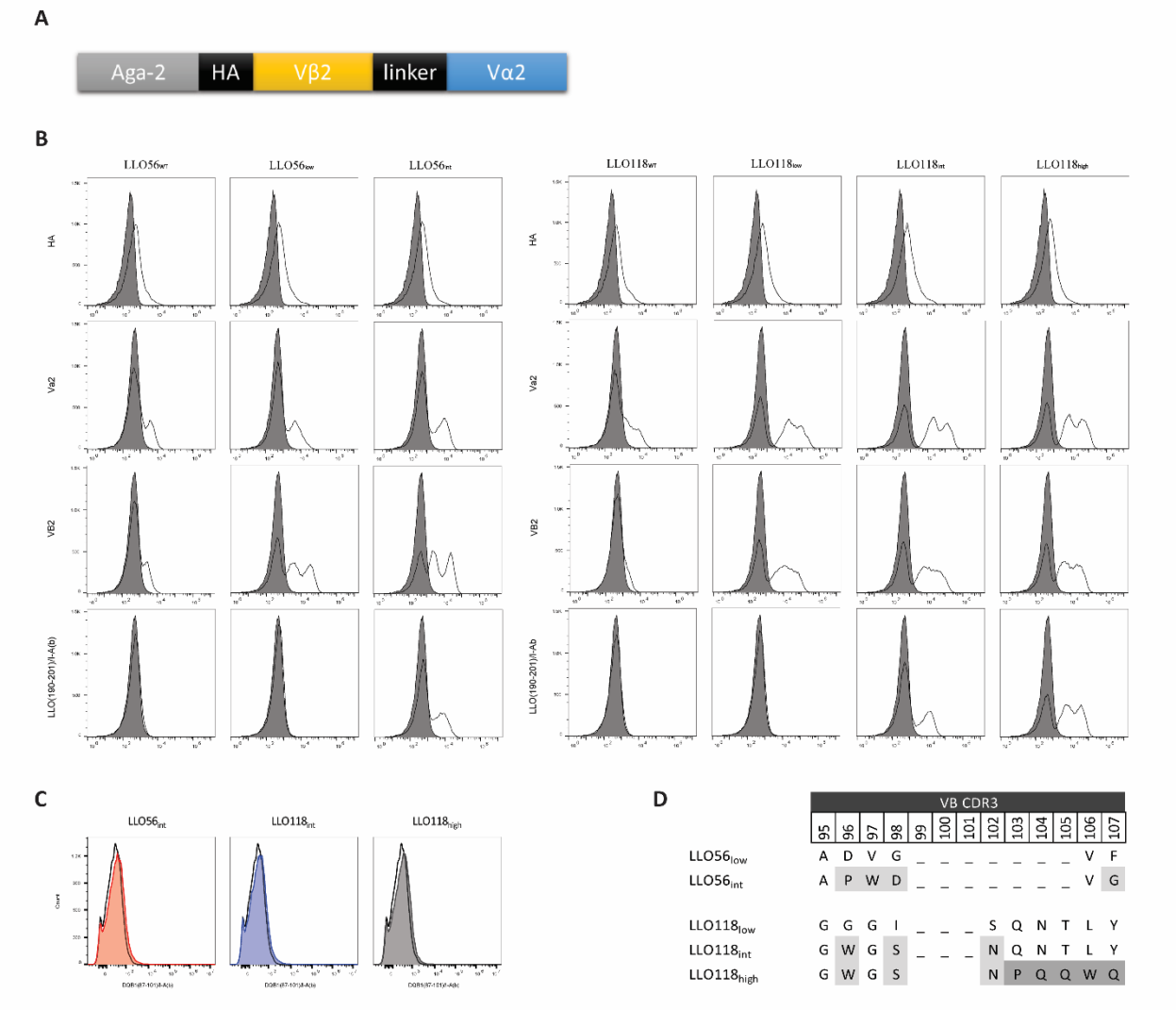

Figure 10. LLO118 and LLO56 stability and affinity maturation by yeast display

(A) Schematic of single-chain TCR (scTCR) which includes TCR α and β variable domains (Vα and Vβ) spliced from TCR constant domains and connected with a 13 amino acid linker. Aga-2 is the yeast mating protein that displays the scTCR on yeast cell membrane. Each construct contains an HA tag for antibody detection. **(B)** Wild type LLO118 and LLO56 were engineered for improved stability and higher affinity by yeast surface display. Clones with stability mutations were selected for using monoclonal anti-Vα or anti-Vβ antibodies. The surface displayed constructs were then selected for improved affinity using the peptide MHC tetramer $LLO_{190\text{-}201}/I\text{-}A^b$. Staining of wild type clones $LLO56_{WT}$ and $LLO118_{WT}$ (first and fourth columns), stability clones $LLO56_{low}$ and $LLO118_{low}$ (second and fifth columns), intermediate affinity clones $LLO56_{int}$ and $LLO118_{int}$ (third and sixth columns) and high affinity clone $LLO118_{high}$ (seventh column) is shown. Stains include antibodies against the HA epitope (first row), Vα2 (second row), and Vβ2 (third row), or $LLO_{190\text{-}201}/I\text{-}A^b$ pMHCII tetramer (fourth row). Gray-filled histogram represents cells-only control. Histograms are representative of n > 3

experiments. **(C)** Affinity clones were incubated with saturating amounts of non-target tetramer ($DQB1_{87\text{-}101}/I\text{-}A^b$). Histograms compare cells only (black clear) with affinity clones (colored, shaded). **(D)** Vβ2 CDR3 mutations that confer increases in affinity. CDR3β regions are hypervariable; therefore, gaps mark the length of other known Vβ2 CDR3β regions. First round of affinity selection (light gray) for all affinity clones while second round of affinity selection (dark gray) applies only to $LLO118_{high}$.

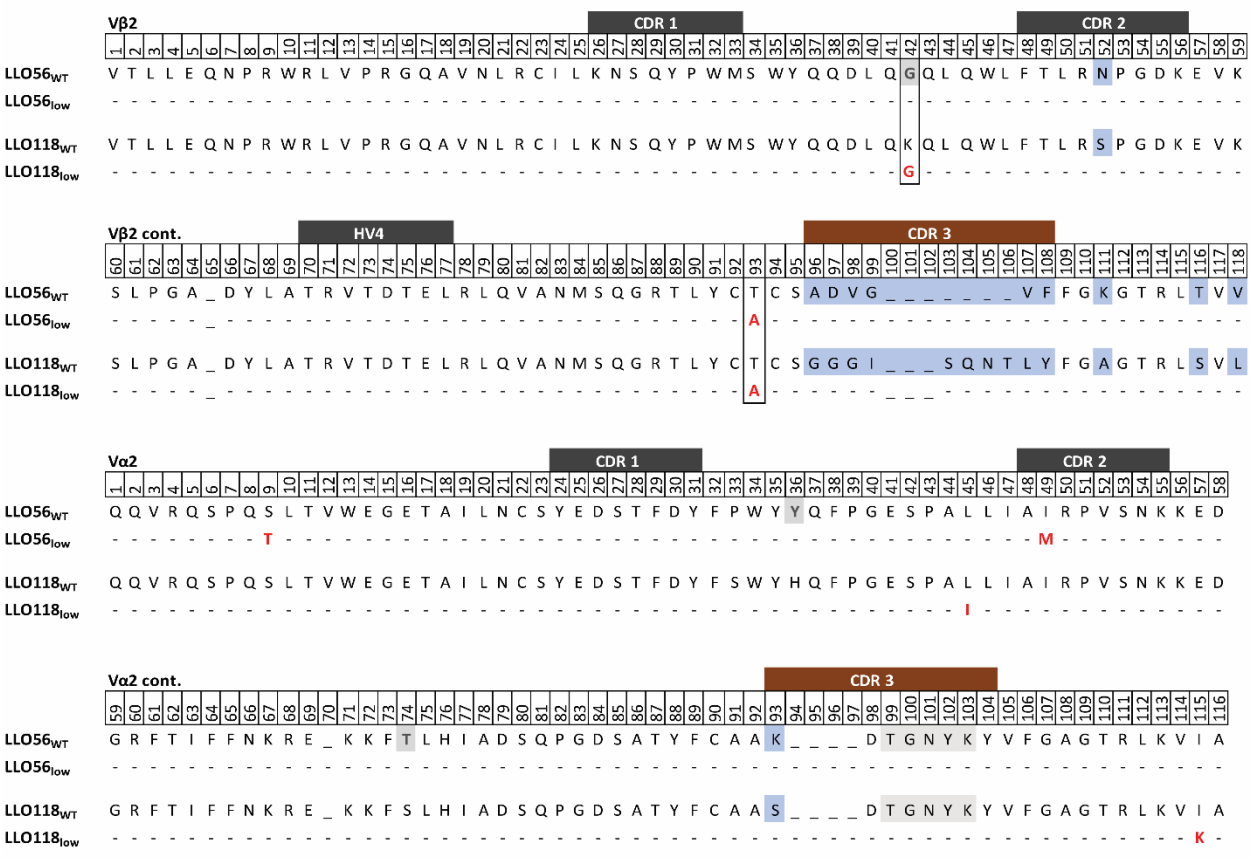

Figure 11. LLO118 and LLO56 single-chain TCRs stabilizing mutations

Wild type templates ($LLO118_{WT}$ and $LLO56_{WT}$) compared to stabilized single-chain TCR (scTCR) templates ($LLO118_{low}$ and $LLO56_{low}$). The original $LLO56_{WT}$ scTCR template included mutations of the amino acid K42βG, H36αY and S74αT (highlighted gray) known to enhance surface display levels in related TCRs [428]. Stability mutations selected by random mutagenesis and directed evolution are marked in red. Boxed amino acids show joint $LLO118_{low}$ and $LLO56_{low}$ selection (K42βG and T93βA), and mutations in another known stability hotspot (L45αI and I49αM) are unmarked. $LLO118_{low}$ independently selected I115αK, and $LLO56_{low}$ selected T93βA and S9αT.

The multivalent binding avidity of each clone was determined by $LLO_{190-201}$/I-A^b tetramer titration (150 pM-15.00 nM) (Figure 12A). Avidities ranged from the highest clone $LLO118_{high}$ (7.30 nM), to intermediate avidity clones $LLO56_{int}$ (39.20 nM) and $LLO118_{int}$ (44.80 nM) (Figures 12A and 2B and Table 3). Stability clones $LLO56_{low}$ and $LLO118_{low}$ were excluded from these analyses because binding was undetectable even at the highest concentrations of $LLO_{190-201}$/I-A^b tetramer (Figure 12B). Tetramer decay analysis determined that the multivalent half-life for $LLO118_{high}$ ($t_{½}$= 165 min, r^2= 0.76) was 165-times longer than $LLO118_{int}$ and $LLO56_{int}$ ($t_{½}$= ~1 min each, r^2= 0.97 each) suggesting that the increased avidity of $LLO118_{high}$ is predominantly due to a lengthened off-rate (Figure 12C). A second round of tetramer decay with lower levels of MHC inhibiting-antibody better resolved the half-lives of $LLO118_{int}$ ($t_{½}$=6.7 mins, r^2= 0.97) and $LLO56_{int}$ ($t_{½}$= 3.5 mins, r^2= 0.98) (Figure 12D), indicating that $LLO118_{int}$ has a longer dissociation rate than $LLO56_{int}$. The resulting panel of TCRs provides a range of tetramer avidities ranging from high to low (Figure 12E).

While tetramer avidity measurements may be more physiologically relevant as multiple TCR-pMHCs interact simultaneously during T cell activation, TCR-pMHC affinity measurements provide a standard measurement to compare between TCR systems. Therefore, TCR:pMHC affinity was measured by bio-layer interferometry. Due to non-specific binding at the later stages of the association and dissociation steps, the K_D was calculated manually by extracting the data from the early measurements; k_{obs} slopes (Figure 12F) were plotted against scTCR concentration (Figure 12G) and k_{assoc} estimated from the slope. k_{diss} is the slope of dissociation graphs (Figure 12H). K_D was determined by dividing k_{diss}/k_{assoc}. $LLO118_{high}$ (20.0 ± 13.9 nM) K_D was 215-fold higher than $LLO118_{low}$ (4.3 ± 0.7 μM) (Table 3). Intriguingly, while $LLO118_{int}$ and $LLO56_{int}$ avidity measurements were similar, their affinity measurements were

markedly different (20-fold). $LLO118_{int}$ (1.3 ± 0.3 μM) was only 3-fold higher affinity than $LLO118_{low}$ and $LLO56_{int}$ (66.2 ± 39.8 nM) was 43-fold higher than $LLO56_{low}$ (3.8 ± 1.3 μM) (Table 3).

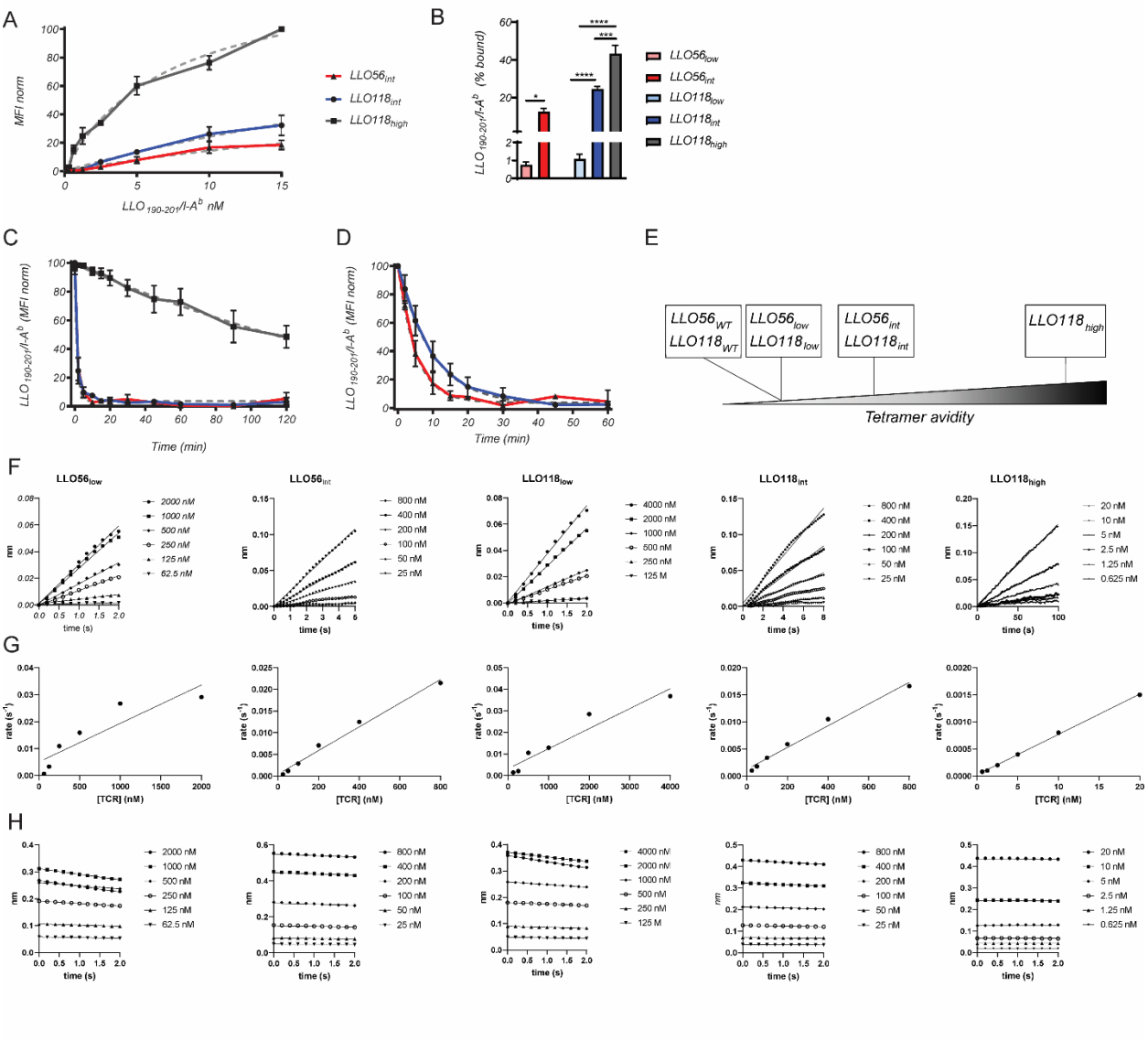

Figure 12. Avidity and affinity measurements of scTCRs

(A) To estimate avidity K_D, affinity clones presented by yeast were incubated with various concentrations of $LLO_{190-201}$/I-A^b tetramer (1.52 nM-50 nM) and 50,000 events were collected via flow cytometry. Grey dotted lines represent non-linear, one site-specific binding analysis of tetramer binding measurements used to estimate K_D for $LLO56_{int}$ (red), $LLO118_{int}$ (blue), and $LLO118_{high}$ (dark grey) (n=3 experiments). **(B)** Percent of cells bound by tetramer for each affinity clone. $LLO56_{low}$ had significantly lower binding

than LLO56$_{int}$ (0.75 ± 0.3% to 12.6 ± 3.4%, p = 0.0193) while LLO118$_{low}$ (1.1 ± 0.5%) was significantly lower than LLO118$_{int}$ (24.5 ± 2.9%) and LLO118$_{high}$ (43.3 ± 8.9%) (p < 0.0001 and p < 0.0001, respectively) and LLO118$_{int}$ was significantly lower than LLO118$_{high}$ (p = 0.0004). To determine $t_{1/2}$, yeast displayed affinity clones were incubated with **(C)** 1 M and **(D)** 0.1 M LLO$_{190\text{-}201}$/I-A^b tetramer. Following an initial measurement, 1.0 µg/mL anti-mouse MHC class II (I-A, I-E) monoclonal antibody was added and tetramer binding measured at each time point by flow cytometry. $t_{1/2}$ was estimated as the time it took to reach 50% MFI modeled by non-linear dissociation one phase exponential decay (dotted light grey lines) (n=3 experiments). **(E)** A graphical depiction of range of affinity clones. To estimate TCR affinity, scTCRs were secreted by *E. coli*, chemically refolded, and incubated with streptavidin sensors loaded with biotinylated LLO$_{190\text{-}201}$/I-A^b monomer using BLI. No binding was observed between scTCRs and streptavidin biosensors in the absence of LLO$_{190\text{-}201}$/I-A^b loading (n = 3-6 independent measurements). **(F)** k_{obs} is the linear slope of nm vs time. **(G)** k_{assoc} is the linear slope of k_{obs} vs scTCR concentration. **(H)** k_{diss} is the exponential slope of dissociation nm vs time.

Table 3. Summary of Figure 12

	Tetramer (Avidity)					Bio-layer Interferometry (Affinity)			
	$t_{1/2}$ (m)	r^2	K_D		r^2	k_{assoc} ($M^{-1}s^{-1}$)	k_{dissoc} (s^{-1})	$t_{1/2}$ (s)	K_D
LLO56 $_{low}$						18300 ± 7000	0.053 ± 0.020	14.5 ± 5.7	2.9 ± 0.7 µM
LLO56 $_{int}$	1	0.97	39.2 ± 46.7	nM	0.82	27600 ± 3000	0.018 ± 0.0007	422.0 ± 163.1	66.2 ± 39.8 nM
LLO118 $_{low}$						10200 ± 1500	0.043 ± 0.004	16.1 ± 1.3	4.3 ± 0.7 µM
LLO118 $_{int}$	1	0.97	44.8 ± 52.3	nM	0.96	20000 ± 1400	0.025 ± 0.007	28.8 ± 7.4	1.3 ± 0.3 µM
LLO118 $_{high}$	165	0.76	7.33 ± 1.37	nM	0.87	73000 ± 8600	0.002 ± 0.0007	460.0 ± 176.8	20.0 +/- 13.9 nM

IL-2 production data interpretation broken down into base TCR (LLO56 or LLO118), construct, and TCR-pMHCII affinity. "-" indicates that the condition inhibits or does not promote IL-2 production, "NA" indicates that effects on IL-2 production were "not appreciable", and "+" indicates that the condition promotes or least does not inhibit IL-2 production. Bolded interior boxes highlight the phenotype shared by intermediate affinity flTCR and high affinity TCR-SCS clones.

2.3.2 Construct format impacts surface expression and pMHCII-affinity independently

To quantitatively assess the effects of TCR-pMHC affinity, CD4, and construct format on helper T cell activation, TCR constructs were retrovirally transduced into murine T cell hybridomas, 58$^{-/-}$ CD4$^-$ (CD4$^-$) and 58$^{-/-}$ CD4$^+$ (CD4$^+$), which do not express endogenous TCRs. LLO56$_{low}$ and LLO56$_{int}$ were placed in the three TCR-SCSs formats, and LLO56$_{WT}$ and LLO56$_{int}$ were placed in flTCR constructs without stability mutations (Figures 13A and 13B).

Because of the necessity of the additional 19 amino acids, $LO118_{low}$, $LLO118_{int}$, and $LLO118_{high}$ affinity changes were not transferred to flTCR constructs. $LLO118_{low}$, $LLO118_{int}$, and $LLO118_{high}$ were placed in three TCR-SCSs formats based on commonly used second and third generation chimeric antigen receptor (CAR) formats (Figures 13A and 13B). The transduced cell lines were sorted with anti-Vβ2 antibodies via magnetic column selection for >85% GFP^+ and TCR expression (Figure 13C).

TCR stable surface expression varied by individual constructs. As assessed by Vβ2 expression, flTCR constructs were less stably expressed than all TCR-SCS constructs perhaps due to CD3 subunit availability, while TCR-SCS CD28 constructs were the most stably expressed format for both LLO56 and LLO118 constructs (Figure 13D). While most construct expression was equitable between $CD4^-$ and $CD4^+$ cell lines, TCR-SCS 4-1BB constructs had the most expression variability between constructs as CD4 expression destabilized $LLO118_{low}$ and $LLO118_{int}$ TCR-SCS 4-1BB expression ($p = 0.0018$ and $p = 0.0429$, respectively) (Figure 13D). $CD4^-$ $LLO118_{high}$ 4-1BB constructs were less stable than $CD4^-$ $LLO118_{low}$ 4-1BB and $LLO118_{int}$ 4-1BB ($p = 0.0030$ and $p = 0.0002$, respectively) (Figure 13D). $CD4^+$ $LLO118_{high}$ 4-1BB was also less stable than either $LLO118_{low}$ or $LLO118_{int}$ ($p = 0.0285$ and $p = 0.0493$) (Figure 13D). Overall CD4 expression did not significantly destabilize 3rd gen constructs except $LLO118_{low}$ ($p = 0.0401$) and $LLO56_{int}$ where CD4 stabilized VB2 expression ($p = 0.0109$) (Figure 13D).

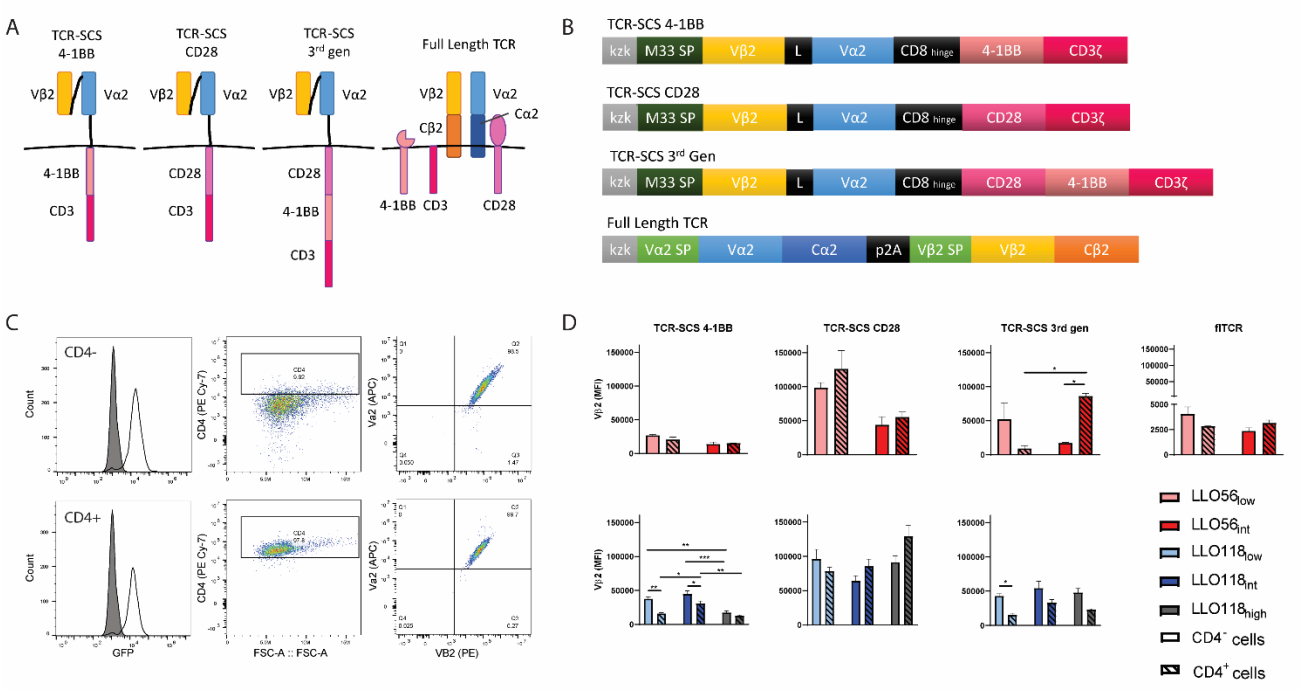

Figure 13. TCR-SCS and flTCRs are stably expressed in CD4$^-$ and CD4$^+$ T cell hybridomas

(A) Diagrams depicting the flTCR formats and three TCR-SCS formats in the cell membrane. All TCR-SCS constructs have signaling coreceptor CD3 in addition to the signaling domains of coreceptors CD28 and/or 4-1BB. **(B)** TCR constructs are produced from a bicistronic IRES-GFP vector. In addition to the P2A cleavage domain, TCR-SCS formats rely on TCR M33 signal peptide [429], whereas the flTCR has dedicated signal peptides for α2 and β2 to increase localization of both chains to the surface. The CD8 hinge acts as an intermembrane domain. **(C)** An example of TCR-SCS expression in CD4$^-$ (top) and CD4$^+$ (bottom) T cell hybridoma lines after magnetic column sorting with anti-Vβ2 monoclonal antibodies (LLO118$_{high}$ TCR-SCS CD28). Initial retroviral transfections ranged from 11% to 85% efficiency. Grey peaks in GFP histograms represent a GFP$^-$ cells-only control. GFP$^+$ cells were gated for CD4 expression and Vα2 and Vβ2 expression. Representative of n=3 measurements of 20,000 cells via flow cytometry. **(D)** Mean fluorescent intensity (MFI) of Vβ2 was used as a proxy for stable expression of constructs. Constructs were expressed in CD4$^-$ (solid bars) and CD4$^+$ (hatched bars) T cell hybridomas. Representative of n=3 measurements of 20,000 cells via flow cytometry.

Tetramer titrations were used to approximate the avidity of each flTCR or TCR-SCS construct. Intriguingly, the intracellular format strongly influenced the avidity of each intermediate and high affinity TCR construct (Figure 14A). There is no clear link across all clones between stable Vβ2 expression and construct avidity, although the most stable

constructs—TCR-SCS CD28—did have the highest apparent avidity ($LLO56_{int}$ and $LLO118_{high}$) (Figure 14A). Overall, CD4 expression (dotted lines) did not affect the avidity of the constructs, excepting $LLO118_{int}$ and $LLO118_{high}$ 3rd gen constructs where CD4 lessened and heightened avidity, respectively (Figure 14A). The MFI measured for each clone at 10^{-8} M (a non-saturated concentration) were used to compare avidity differences between affinity clones. LLO56 4-1BB, 3rd gen and flTCR constructs had no significant differences between $LLO56_{low}$ and $LLO56_{int}$ (Figure 14B). This may be due to the small affinity differences between $LLO56_{low}$ and $LLO56_{int}$ as measured in tetramer and bio-layer interferometry assays. However, LLO118 3rd gen constructs also did not show affinity-dependent avidity changes, thus intracellular signaling domains may also affect the avidity of extracellular scTCRs. There were significant avidity differences for LLO118 4-1BB clones; $CD4^-$ $LLO118_{high}$ 4-1BB had significantly better avidity than its cognate $CD4^+$ pairing ($p = 0.0004$), and was also significantly higher than $CD4^-$ $LLO118_{low}$ and $LLO118_{int}$ 4-1BB ($p = 0.0003$ and $p = 0.0092$, respectively) (Figure 14B). Additionally, TCR-SCS CD28 constructs for both LLO56 and LLO118, which are the most stably expressed constructs (Figure 13D), showed increased MFI by increasing TCR affinity (Figure 4B). $LLO56_{int}$ CD28 had significantly greater avidity than $LLO56_{low}$ CD28 ($CD4^-$ $p = 0.0122$ and $CD4^+$ $p= 0.0086$), as did $LLO118_{high}$ CD28 compared to $LLO118_{low}$ ($CD4^-$ $p = 0.0129$ and $CD4^+$ $p= 0.0113$) (Figure 14B). Taken together, while there is no systematic correlation, this data suggests that construct stability may influence avidity measurements, as CD28 clones had the highest stability and avidity, and confirms that generally, CD4 does not affect perceived avidity.

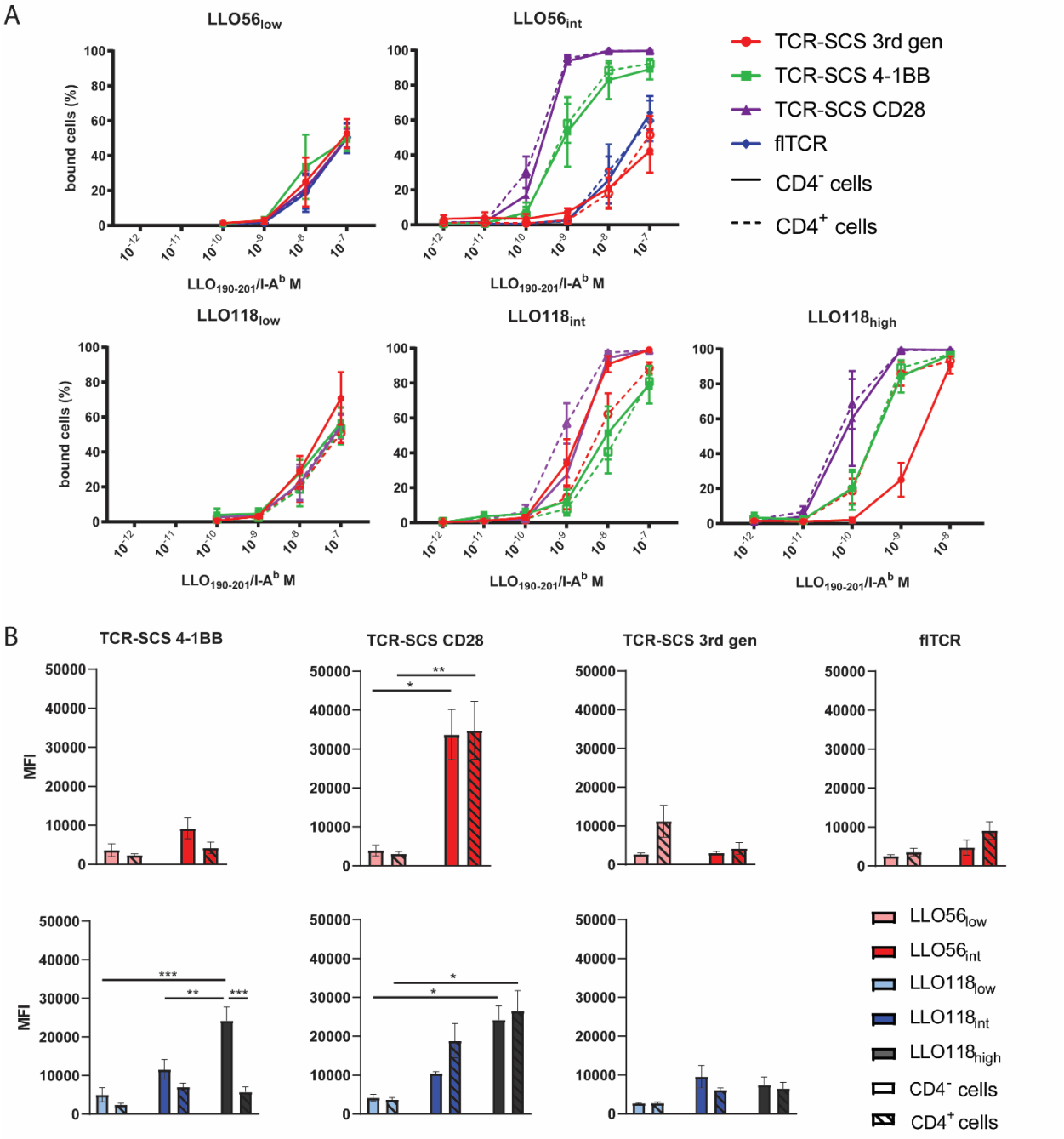

Figure 14. TCR-pMHCII avidity is affected by construct format

(A) flTCR or TCR-SCS expressing cell lines were incubated with varying amounts of $LLO_{190-201}$/I-A^b tetramer (10^{-7} M to 10^{-12} M) at room temperature for 2 hrs. Each TCR-format pair expressed in $CD4^-$ cell lines (solid lines) and $CD4^+$ cell lines (dotted lines) have similar affinities, whereas each unique construct format alters avidity of a single TCR. Representative of 3 independent measurements of 20,000 cells via flow cytometry. **(B)** Tetramer MFI measurements of 10^{-8} M separated by TCR and format where $CD4^-$ cell lines (solid bars) and $CD4^+$ cell lines (hashed bars) are paired.

2.3.3 CD4 inhibits high affinity TCR IL-2 production

To assess the effects of TCR-pMHCII affinity, CD4 expression, and format on T cell activation we measured IL-2 expression in response to increasing agonist peptide concentrations. As anticipated, $LLO56_{low}$ flTCR IL-2 production improved with CD4 expression, but CD4 expression unexpectedly reduced IL-2 production for $LLO56_{int}$ flTCR (Figure 15A). Despite the inconsistent role of CD4, flTCRs produced significantly more IL-2 at all affinity levels (Figure 15A) and were at least 1-log fold more sensitive to peptide than all TCR-SCSs (Figures 15B-D). IL-2 production for $CD4^-$ clones rose with increased TCR affinity for most constructs except 3rd gen constructs; $LLO56_{int}$ 3rd gen failed to produce more cytokines than $LLO56_{low}$ 3rd gen (Figure 15B) and $LLO118_{high}$ 3rd gen that produced less IL-2 than $LLO118_{int}$ 3rd gen (Figure 15C). This pattern of uneven gains across affinity and base TCR was also observed for 4-1BB constructs (Figures 15D and 15E); while LLO56 4-1BB did see gains across affinity (Figure 15D), LLO118 4-1BB constructs had limited affinity gains across the affinity gradient (Figure 15E). $CD4^-$ $LLO56_{int}$ CD28 and $CD4^-$ $LL0118_{high}$ CD28 produced more IL-2 than other TCR-SCS constructs which suggested that their heightened stable expression may promote IL-2 production (Figure 15F and 15G).

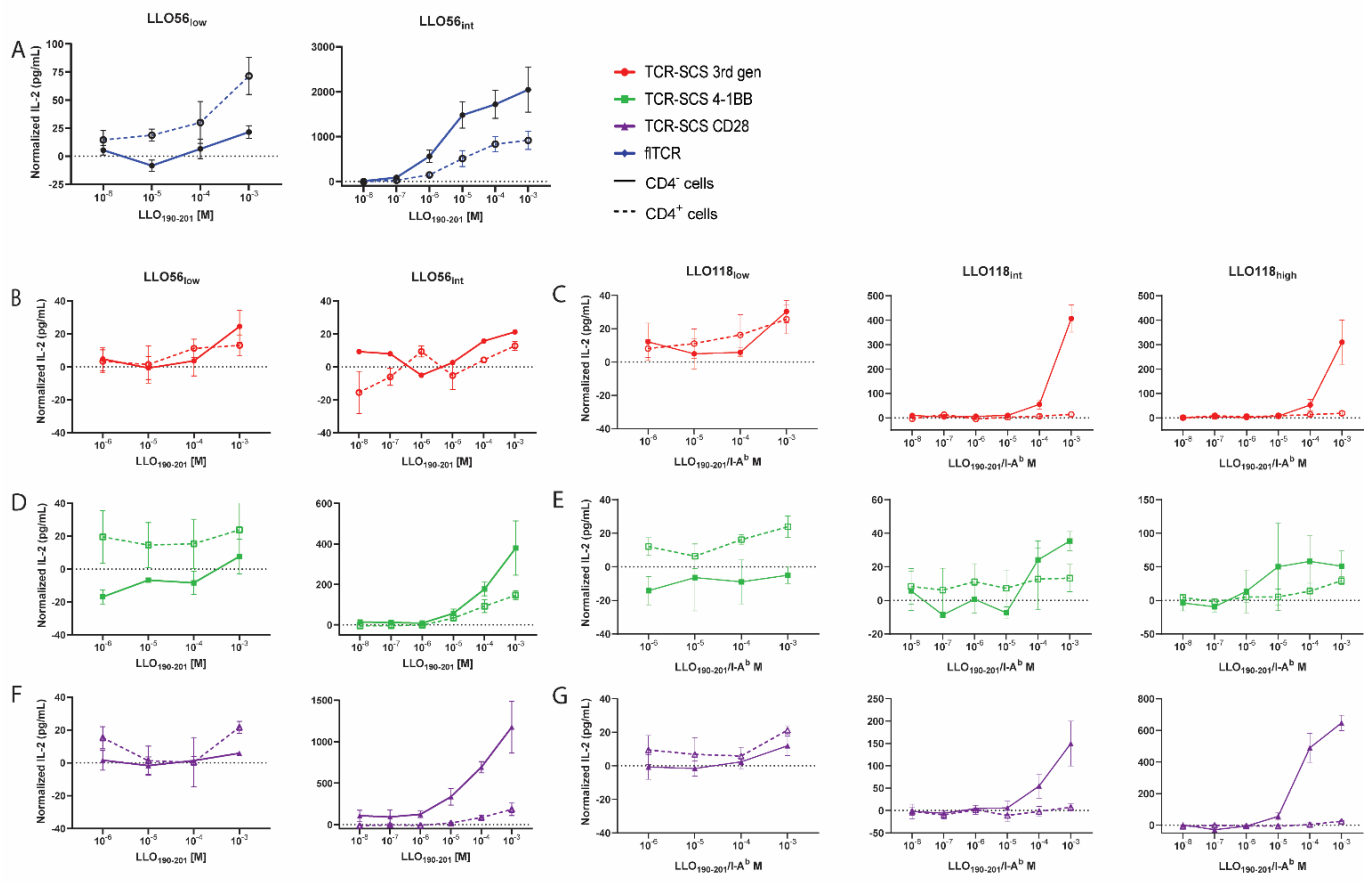

Figure 15. CD4 inhibits IL-2 production of intermediate and high affinity TCRs

CD4$^-$ (solid lines) and CD4$^+$ (dotted lines) T cell hybridoma cell lines were incubated with various concentrations of $LLO_{190\text{-}201}$ peptide (10^{-8} M to 10^{-3} M) presented by BL6/C57 splenocytes for 24 hrs. IL-2 production was measured by ELISA. Each sample was normalized by subtracting baseline IL-2 production from T cell hybridoma/splenocytes controls incubated without peptide. **(A)** LLO56 flTCRs, **(B)** LLO56 3rd gen SCS-TCRs, **(C)** LLO118 3rd gen SCS TCRs, **(D)** LLO56 4-1BB SCS-TCRs, **(E)** LLO118 4-1BB SCS-TCRs, **(F)** LLO56 CD28 SCS-TCRs, and **(G)** LLO118 CD28 SCS-TCRs. ELISAs run n=3 times.

While CD4 promoted the activation of all low affinity clones, it unexpectedly suppressed IL-2 production for all intermediate and high affinity constructs (Figure 15). The magnitude of IL-2 suppression is greatly dependent on whether the construct was a flTCR or TCR-SCS construct. For example, while $LLO56_{low}$ flTCR IL-2 production was assisted by CD4 expression, $LLO56_{int}$ flTCR IL-2 production was reduced 2.2-fold ($p = 0.0707$) at 10^{-3} M peptide

stimulation (Figure 16). In contrast, only one TCR-SCS had such a mild IL-2 reduction. $CD4^+$ $LL056_{int}$ 4-1BB IL-2 production was reduced by 2.5-fold ($p = 0.1551$) (Figure 16). The IL-2 production for the other intermediate and high affinity TCR-SCS constructs was intermediately reduced for $LLO56_{int}$ CD28 (6.4-fold, $p = 0.0104$), and severely reduced for $LLO118_{int}$ 3^{rd} gen (28.1-fold, $p = 0.0004$), $LLO118_{int}$ CD28 (21.2-fold, $p = 0.0400$), $LLO118_{high}$ 3^{rd} gen (16.5-fold, $p = 0.0051$) and $LLO118_{high}$ CD28 (25.9-fold, $p < 0.0001$) (Figure 16). Peptide sensitivity, defined as the lowest concentration where IL-2 response exceeds baseline IL-2 production, was equitable between $CD4^-$ and $CD4^+$ for constructs $LLO56_{int}$ flTCR and $LLO56_{int}$ 4-1BB (Figure 15A and 15D), but delayed 1-log fold for $LL056_{int}$ CD28 and 2-log fold for all $LLO118_{int}$ and $LLO118_{high}$ constructs (Figure 15C and 15E-G). This suggests that CD4 reduced peptide sensitivity for most TCR-SCS constructs, possibly in a TCR-dependent manner.

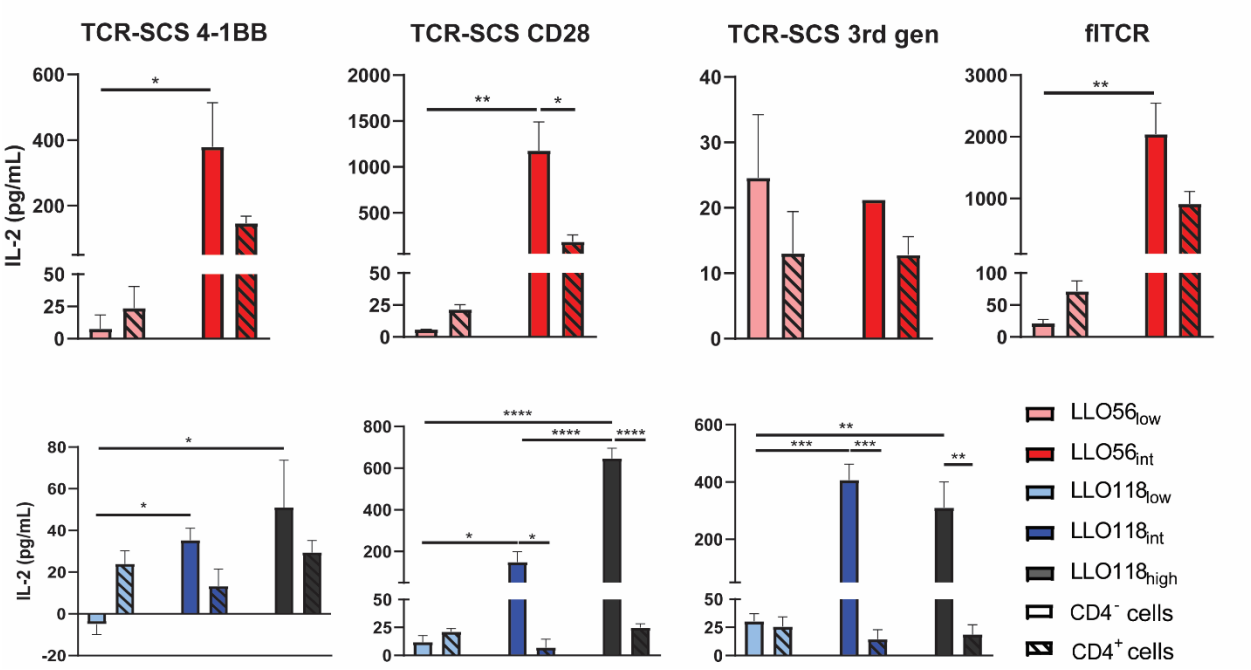

Figure 16. Comparison of IL-2 production at 10^{-3} M peptide stimulation

Analysis of levels of IL-2 production from Fig. 5 at 10^{-3} M peptide stimulation. IL-2 production between 58-/-CD4- affinity clones was significantly different for LLO56low and LLO56int 4-1BB ($p = 0.0211$), LLO56low and LLO56int CD28 ($p = 0.0039$) and LLO56low and LLO56int flTCR ($p = 0.0033$), LLO118low and LLO118int 3rd gen ($p = 0.0006$), LLO118low and LLO118high 3rd gen ($p = 0.0070$), LLO118low and LLO118int 4-1BB ($p = 0.0441$), LLO118low and LLO118high 4-1BB ($p = 0.0115$), LLO118low and LLO118int CD28 ($p = 0.0489$), LLO118low and LLO118high CD28 ($p < 0.0001$), and LLO118int and LLO118high CD28 ($p < 0.0001$).

2.3.4 Lck sequestration by CD4 inhibits some TCR IL-2 production

Lck is an early proximal signaling kinase that colocalizes to the cytoplasmic domain of CD4 [51, 52]. If Lck is poorly recruited to the TCR-pMHCII synapse, then T cell activation may be diminished [62]. We hypothesized that our high affinity clones may poorly recruit CD4-Lck to the immunological synapse, decreasing activation, and therefore reducing IL-2 production as observed in $CD4^+$ intermediate and high affinity clones. To parse out the potential contributions of CD4-Lck sequestration, CD4-MHCII interactions, and any CD4-dependent inhibition, we expressed a selection of our flTCR and TCR-SCS clones in four $58^{-/-}$ T cell hybridoma lines [62,

424]. LLO56 TCR-SCS 3rd gen and LLO118 TCR SCS 4-1BB clones were dropped due to their poor performance in the first IL-2 tests. The $58^{-/-}$ $CD4^-$ T cell hybridoma cell line ($CD4^-$) lack CD4, which allows Lck to interact freely with the TCR-pMHCII complex and nullifies CD4-MHCII interactions (Figure 17A). The $58^{-/-}$ $CD4^+$ T cell hybridoma cell line ($CD4^+$) has wild type CD4 which sequesters Lck to its cytoplasmic tail and binds to MHCII (Figure 17B). $58^{-/-}$ $CD4T^+$ T cell hybridoma line ($CD4T^+$) is truncated C-terminally (maintains amino acids 1-421) which allow Lck to colocalize but not bind to CD4 while CD4 still binds to MHCII (Figure 17C) [424, 430]. Finally, $58^{-/-}$ $CD4T^+$ Δbind ($CD4T^+$ Δbind) frees Lck and is mutated to prevent CD4 binding to MHCII by altering residues 68–73 from KGVLIR to DGDSDS (Figure 17D) [424].

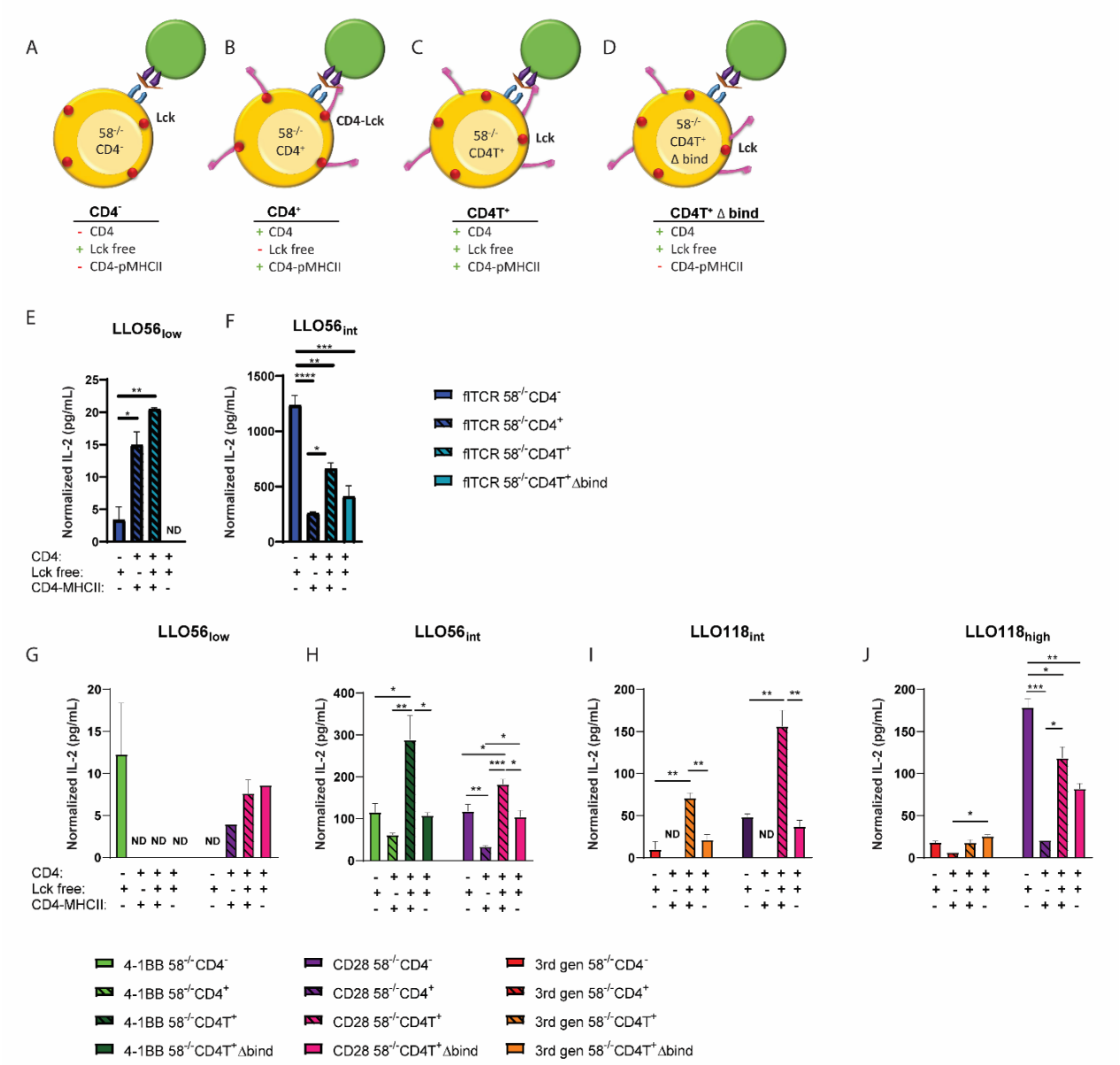

Figure 17. IL-2 production of intermediate and high affinity clones is influenced by Lck sequestration, CD4-MHCII interaction and CD4 presence

(A) $CD4^-$ has no CD4 and Lck is spread ubiquitously along the inner membrane. **(B)** $CD4^+$: wild type CD4 interacts with MHCII and the majority of Lck is sequestered to the cytoplasmic tail of CD4. **(C)** $CD4T^+$: mutant CD4T interacts with MHCII but does not sequester Lck which is spread ubiquitously along the inner membrane. **(D)** $CD4T^+$ Δ bind: mutant CD4T Δ bind does not bind to MHCII nor Lck (Lck is not sequestered to the cytoplasmic tail of CD4). LLO56 low **(E)** or intermediate **(F)** flTCRs expressed in various T cell hybridoma clones [$CD4^-$ (dark blue), $CD4^+$ (dark blue hatched), $CD4T^+$ (light blue), $CD4T^+$ Δ bind (light blue hatched)] were incubated with 10^{-3} M $LLO_{190\text{-}205}$ presented on Bl6.C57 splenocytes for 24 hrs. IL-2 production was measured by ELISA. LLO56 low **(G),** LLO56 intermediate **(H),** LLO118 intermediate **(I)**, and LLO118 high **(J)** TCR-SCS (41BB, CD28, or 3rd gen) were expressed

in various T cell hybridoma clones ($CD4^-$, $CD4^+$, $CD4T^+$, $CD4T^+$ Δ bind) that were incubated with 10^{-3} M $LLO_{190-205}$ presented on Bl6.C57 splenocytes for 24 hrs. IL-2 production was measured by ELISA (n=3). TCR-SCS format is defined by the color [TCR-SCS 4-1BB (light green, dark green), TCR-SCS CD28 (purple, pink), and TCR-SCS 3rd gen (red, orange)]. The combination of color shade and presence or absence of hatched lines identifies the specific hybridoma. p-values were determined by multiple comparison one-way ANOVA for each graph are from left to right $LLO56_{int}$ 4-1BB $CD4^-$ to $CD4T^+$ ($p = 0.0176$), $CD4^+$ to $CD4T^+$ ($p = 0.0036$), and $CD4T^+$ to $CD4T^+$ Δ bind ($p = 0.0138$); $LLO56_{int}$ CD28 $CD4^-$ to $CD4^+$ ($p = 0.0054$), $CD4^-$ to $CD4T^+$ ($p = 0.0232$), $CD4^+$ to $CD4T^+$ ($p = 0.0002$), $CD4^+$ to $CD4T^+$ Δ bind ($p = 0.0245$), and $CD4T^+$ to $CD4T^+$ Δ bind ($p = 0.0153$); $LLO118_{int}$ 3rd gen $CD4^-$ to $CD4T^+$ ($p = 0.0027$), and $CD4T^+$ to $CD4T^+$ Δ bind ($p = 0.0077$); $LLO118_{int}$ CD28 $CD4^-$ to $CD4T^+$ ($p = 0.0018$), and $CD4T^+$ to $CD4T^+$ Δ bind ($p = 0.0010$); $LLO118_{high}$ 3rd gen $CD4^+$ to $CD4T^+$ Δ bind ($p = 0.0366$); and $LLO118_{high}$ CD28 $CD4^-$ to $CD4^+$ ($p = 0.0010$), $CD4^-$ to $CD4T^+$ ($p = 0.0221$), $CD4T^-$ to $CD4T^+$ Δ bind ($p = 0.0021$), and $CD4^+$ to $CD4T^+$ ($p = 0.0117$).

$CD4T^+$ and $CD4T^+$ Δbind constructs were retrovirally transduced into existing $CD4^-$ T cell hybridomas containing TCR-SCS or flTCR constructs and the clones were sorted for GFP, TCR, and CD4 expression by flow sorting. $CD4^-$ hybridomas did not express CD4, and there was consistent CD4 expression between the various $CD4^+$ clones (Figure 18A). TCR surface expression was consistent across cell lines for most TCR constructs, except $LLO56_{low}$ and $LLO56_{int}$ CD28 clones which were most stably expressed in $CD4^+$ cells ($p < 0.0001$ and $p < 0.0001$, respectively) (Figure 18B). Similarly, avidity measured by tetramer was mainly consistent between clones except for $LLO56_{low}$ 4-1BB where $CD4^-$ had higher avidity than all $CD4^+$ clones ($p = 0.0014$), and $LLO118_{int}$ CD28 where $CD4^+$ clone had the highest avidity ($p = 0.0063$) (Figure 18C).

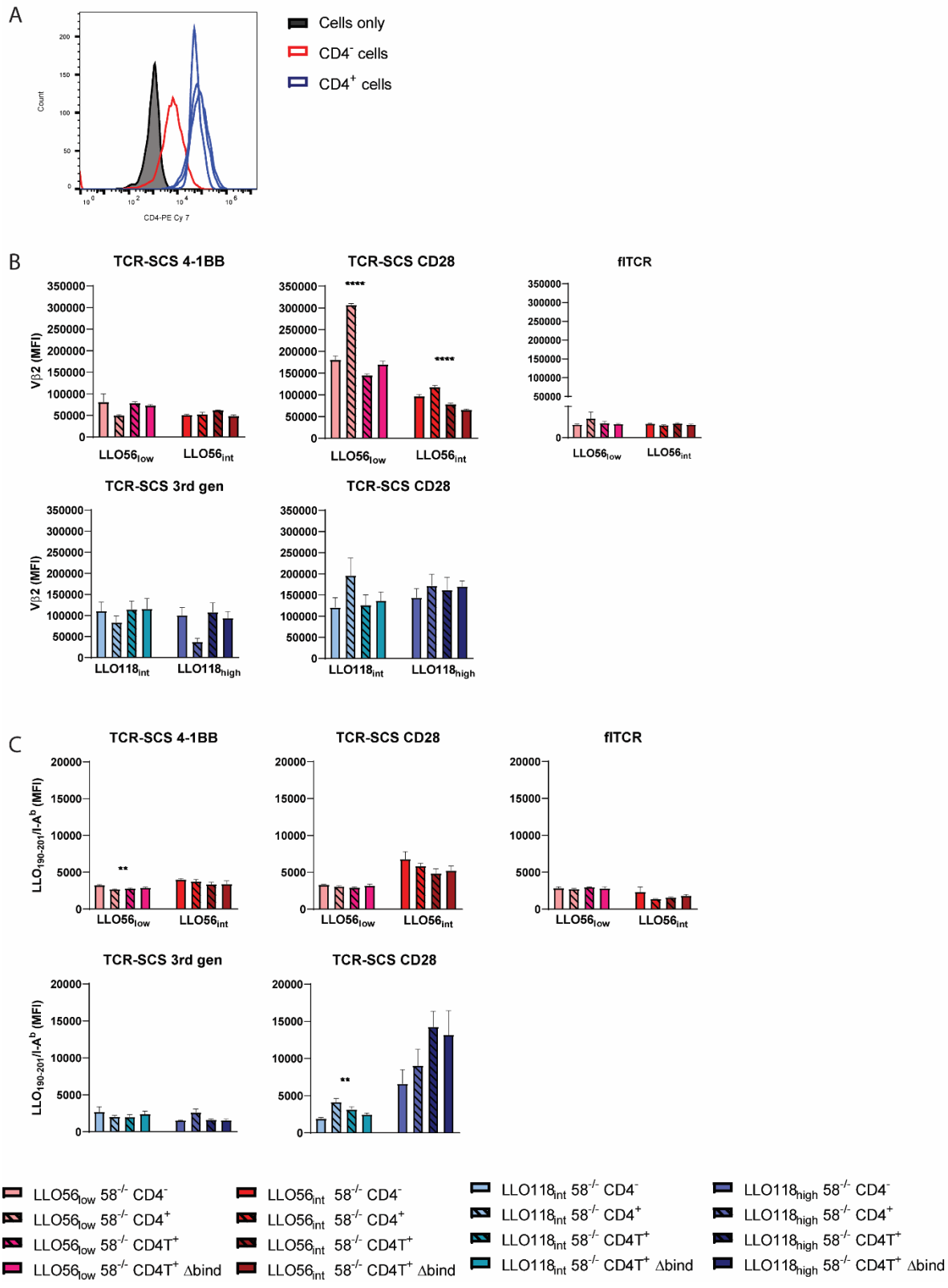

Figure 18. CD4 variant-expression does not affect construct stable expression or avidity

(A) Representative histogram of post-sort CD4 expression between T cell hybridoma cell lines for a single TCR construct. $58^{-/-}$ $CD4^-$ hybridoma (red peak) is shifted above the cells only control (grey peak) due to background fluorescence from the antibody master mix, and the $58^{-/-}$ $CD4^+$ clones (blue peaks) are expressed similarly. **(B)** Stable expression of Vβ2 by construct in order of $58^{-/-}$ $CD4^-$, $58^{-/-}$ $CD4^+$, $58^{-/-}$ $CD4T^+$, $58^{-/-}$ $CD4T^+$ Δbind T cell hybridomas. $LLO56_{low}$ (pink colors), $LLO56_{int}$ (red colors), $LLO118_{int}$ (light blue colors), $LLO118_{high}$ (dark blue colors). Hatched lines indicate CD4-MHC interactions. $LLO56_{low}$ CD28 ($p < 0.0001$) and $LLO56_{int}$ CD28 ($p < 0.0001$) **(C)** To ascertain whether avidity was maintained within each TCR-construct despite CD4 variations, each cell line was tested at sub-saturating tetramer concentrations: $LLO56_{low}$ (10^{-8} M), $LLO56_{int}$ (5×10^{-10} M), $LL0118_{int}$ (10^{-9} M), and $LL0118_{high}$ (5×10^{-10} M). Colors and order as found in (B). $LLO118_{int}$ CD28 ($p = 0.033$). Groups analyzed for differences with one way ANOVA.

As expected, $LLO56_{low}$ flTCR IL-2 production was promoted by the presence of CD4-MHCII interactions ($CD4^+$ $p = 0.0105$ and $CD4T^+$ $p = 0.0014$) and the absence of CD4-MHCII interaction in the $CD4^-$ and $CD4T^+$ Δbind abrogated $LLO56_{low}$ flTCR IL-2 production (Figure 17E). CD4 Lck-sequestration did not affect $LLO56_{low}$ flTCR IL-2 production since the $CD4^+$ and $CD4T^+$ clones responded the similarly to antigen (Figure 17E). In contrast, intermediate affinity $LLO56_{int}$ flTCR IL-2 production is inhibited by CD4 Lck-sequestration, as $CD4^+$ produced significantly less IL-2 than $CD4T^+$ ($p = 0.0162$). However, the more striking phenotype is $LLO56_{int}$ flTCR CD4-dependent inhibition, as IL-2 production is significantly reduced by the presence of CD4 in any form. $CD4^-$ $LLO56_{int}$ flTCR cells produced significantly more IL-2 than any clone expressing CD4 ($CD4^+$ $p < 0.0001$, $CD4T^+$ $p = 0.0019$, and $CD4T^+$ Δbind $p = 0.0002$) (Figure 17F). Furthermore, CD4-MHCII interaction was not a significant contributor to intermediate affinity flTCR IL-2 production as there was no significant change in IL-2 production between the $CD4T^+$ and $CD4T^+$ Δbind clones (Figure 17F).

Low affinity TCR-SCS clones $LLO56_{low}$ 4-1BB and $LLO56_{low}$ CD28 were low IL-2 producers and the role of CD4 was conflicting as all CD4 iterations inhibited IL-2 production for

4-1BB but promoted IL-2 production for CD28 whether or not CD4 binds to MHCII (Figure 17G). It was also difficult to draw conclusions about Lck-sequestration for low affinity TCR-SCS constructs due to low levels of IL2 production. Intermediate TCR-SCS clones $LLO56_{int}$ 4-1BB, $LLO56_{int}$ CD28, $LLO118_{int}$ 3rd gen and $LLO118_{int}$ CD28 had a unique ubiquitous phenotype comparable to the phenotype described for intermediate affinity flTCR clones. IL-2 production was most reduced when CD4 sequestered Lck in the $CD4^+$ clones (Figure 17H and 17I). However, intermediate TCR-SCS $CD4T^+$ constructs produced the most IL-2, indicating unrestricted Lck promotes the greatest T cell activation (Figure 17H and 17I). $CD4T^+$ Δbind compared to $CD4T^+$ significantly reduced intermediate TCR-SCS construct IL-2 production to $CD4^-$ levels suggesting that CD4-MHCII binding supports IL-2 production for intermediate TCR-SCS affinity (Figure 17H and 17I). Noticeably, high affinity LLO118 TCR-SCSs followed the same inhibition patterns seen for $LLO56_{int}$ flTCR where inhibition by Lck sequestration and CD4 presence was not significantly affected by MHCII-CD4 binding (Figure 17J). Taken together, these data indicate that flTCRs and TCR-SCS have independent affinity thresholds for the inhibitory effects of Lck-sequestration and CD4-dependent inhibition, and the activation promoting effects of CD4-MHCII interactions (summarized in Table 4). Thus, IL-2 inhibition is affected by CD4-Lck sequestration, CD4-pMHCII interaction, and by a CD4-dependent mechanism in an affinity- and format-dependent manner.

Table 4. Summary of Figure 17

TCR	Construct	Affinity: low		Affinity: int		Affinity: high	
LLO56	*flTCR*	CD4	NA	**CD4**	**-**		
		Lck free	NA	**Lck free**	**+**		
		CD4-MHCII	+	**CD4-MHCII**	**NA**		
	4-1BB	CD4	-	CD4	NA		
		Lck free	+	Lck free	+		
		CD4-MHCII	NA	CD4-MHCII	+		
	CD28	CD4	+	CD4	NA		
		Lck free	NA	Lck free	+		
		CD4-MHCII	NA	CD4-MHCII	+		
LLO118	*CD28*			CD4	NA	**CD4**	**-**
				Lck free	+	**Lck free**	**+**
				CD4-MHCII	+	**CD4-MHCII**	**NA**
	3rd gen			CD4	NA	**CD4**	**NA**
				Lck free	+	**Lck free**	**+**
				CD4-MHCII	+	**CD4-MHCII**	**NA**

IL-2 production data interpretation broken down into base TCR (LLO56 or LLO118), construct, and TCR-pMHCII affinity. "-" indicates that the condition inhibits or does not promote IL-2 production, "NA" indicates that effects on IL-2 production were "not appreciable", and "+" indicates that the condition promotes or least does not inhibit IL-2 production. Bolded interior boxes highlight the phenotype shared by intermediate affinity flTCR and high affinity TCR-SCS clones.

2.4 Discussion

Here we generated a panel of MHCII-specific TCRs with increasing affinity in order to interrogate the relationships between TCR format, TCR-pMHCII affinity, and the coreceptor CD4 on $CD4^+$ T cell activation. The contributions of these factors were assessed using IL-2 production; flTCRs produced more IL-2 than all TCR-SCS constructs at each affinity level and IL-2 production generally increased with rising TCR affinity for all constructs. In low affinity TCRs, CD4 enhanced IL-2 production for both flTCR and TCR-SCS formats. For intermediate

or high affinity TCR clones, IL-2 production was abrogated by CD4-Lck sequestration and an unknown CD4-dependent mechanism. These effects, activation promotion by increased affinity and CD4-MHCII, or activation suppression by Lck-sequestration and CD4 itself, had unique affinity thresholds that are dependent on construct type (flTCR or TCR-SCS). Lck sequestration affected activation for all intermediate and high affinity constructs, while CD4-MHCII ceased to promote activation and CD4-dependent inhibition repressed IL-2 production at unique affinity thresholds for flTCR constructs (intermediate affinity) and TCR-SCS constructs (high affinity).

Following colocalization to the TCR, CD4 signals via Lck bound to its cytoplasmic tail [51, 52]. Lck phosphorylates immune-receptor tyrosine-based activating motifs (ITAMs) of the CD3 subunits of the TCR complex, which then initiates other early signaling machinery of the T cell [51, 53, 431]. CD4-bound Lck activation may be reliant on a mechanism distinct from CD4-free Lck activation, which is likely mediated by tyrosine-protein kinase Fyn, thus may obscure mechanism comparison [407, 432, 433]. However, despite this complication, CD4-Lck-dependent inhibition could occur in two fashions. First, optimal TCR affinity-mediated signaling is dependent on fine-tuning the intensity and duration of the Lck phosphorylation cascade and high affinity TCRs may have early intense Lck phosphorylation resulting in acute transient activation [434]. Conversely, if CD4 is not recruited to the TCR, it could sequester Lck away from the activation complex, which prevents the activation phosphorylation cascade thereby attenuating T cell activation [62]. The first option suggests that all high-affinity TCR signaling would be attenuated regardless of whether Lck was interacting with CD4; however, IL-2 output reduction in the presence of CD4-Lck sequestration is clearly demonstrated by our intermediate and high affinity CD4+ T cell hybridoma clones. It is also possible that with an increase in affinity and the subsequent decrease in off-rate or increase in half-life, CD4-Lck fails to cycle

through the TCR-pMHC synapse, thereby decreasing CD3 phosphorylation and thus downstream activation. Signaling activation is affected by both TCR-pMHCII dwell time and CD4-Lck interactions [430, 435, 436]. CD4 increases TCR signaling on low-affinity pMHCII by increasing TCR-CD3 dwell time [409]. CD4 dwell time on pMHCII is proportional, yet faster, to TCR dwell time, suggesting that TCR:pMHCII interaction kinetics would directly affect the duration that CD4 molecules cycle through the immunological synapse in a processive-like manner [435]. TCR-pMHCII interactions are highly ordered and uniform, increasing the likelihood that the spatial relationship between Lck and the ITAMs of the TCR-SCS or flTCRs are consistent. Thus, kinetic factors, such as TCR-pMHCII affinity would greatly influence the stability of the macrocomplex and consequently the duration of Lck interactions with the ITAMs [409, 437]. These kinetics alone could explain the drop in activation observed for our high-affinity, slow off-rate TCR clones. To support this idea, CD8 also acts as a dominant negative inhibitor for ligands that do not recruit fresh CD8 to the TCR-CD3 complex [62].

Previous research suggests that CD4 can send an inhibitory signal independent of Lck via post activation antibody-mediated ligation, which attenuates IL-2 production and ongoing activated T cell response [64]. This response was also observed in a clonal variant expressing a form of CD4 unable to associate with Lck, suggesting that CD4 has independent inhibitory or regulatory function [64]. Furthermore, CD4-mediated inhibition has also been observed during CD4-MHCII interactions leading to a decrease in IL-2 mRNA [64]. While we did not seek the source for our Lck-independent CD4 inhibition nor acquire IL-2 mRNA levels, we noted that there was an affinity threshold for this behavior that was independent of MHC interaction, and therefore may be a unique mechanism to that reported in Chervin et al. 2009. The affinity threshold for this Lck-independent CD4 inhibition was lower for flTCR (intermediate affinity)

than TCR-SCS (high affinity). This may be due to the signaling power of each construct: flTCR-CD3 complexes have 10 ITAMs with 20 tyrosine residues available for phosphorylation, whereas TCR-SCS domains have only 3 ITAMs and 6 tyrosine residues [413, 438, 439]. The increased availability of ITAMs per activated Lck may also explain why LLO56$_{int}$ flTCR experienced less IL-2 production inhibition in the presence of CD4—more signal per Lck molecule despite CD4-Lck movement restriction. It is also curious that the CD4-MHCII interaction supports activation in intermediate affinity TCR-SCS clones, suggesting that while CD4 may not contribute to the overall affinity of TCR-SCS constructs, it may stabilize the interaction between TCR-pMHCII or provide an additional Lck-independent activation signal. The increased interaction stability is more likely as high affinity TCR-SCS IL-2 production is not significantly improved when CD4 interacts with MHCII, suggesting high affinity constructs likely have stable interactions independent of CD4 contributions. Taken together this data suggests an affinity threshold where, up to a point, increased time for CD4-MHCII interactions improves TCR-dependent signaling when it is not Lck-limited, but after a certain affinity point, increased dwell time slows TCR-dependent signaling and positive benefits of CD4-MHCII interactions become redundant.

In addition to the stability challenges presented by scTCR format, the TCR-SCS intracellular format also affected the stability of each TCR. TCR-SCS CD28 format was more stably expressed than other TCR-SCS or flTCR formats, and as noted in other studies, the enhanced surface expression of TCR-SCS CD28 formats via increased stability may explain their improved avidity and T cell activation [440-442]. However, it is difficult to ascertain whether the increased IL-2 production of TCR-SCS CD28 is due to enhanced stable surface expression or the innate characteristics of CD28-intracellular signaling domains. As observed in numerous

antibody-based CAR studies comparing CD28 domains to 4-1BB domains, intracellular signaling domains differentially impact multifactorial T cell response characteristics, including cytokine production [443]. For example, CD28-CAR constructs are well known for their increased IL-2 production and subsequent T cell exhaustion compared to 4-1BB CARs [444, 445]. Thus, the observed increase in IL-2 production for TCR-SCS CD28 constructs may be attributable to the innate characteristics of CD28-intracellular signaling domains rather than increased stable surface expression. As CD28-CARs phosphorylate CD3 more quickly yet do not exceed the levels of CD3 phosphorylation exhibited by 4-1BB CARs, this may be due to signaling intensity [445]. Additionally, because CD28 recruits Lck to lipid rafts where it associates with CD4, CD28 may be better able to recruit Lck [446, 447]. While TCR-SCS 3rd generation constructs had mixed activation success and overall reduced cytokine production compared to TCR-SCS CD28 constructs, this may be attributable to 3rd generation CAR T cells improved expansion and persistence and may mimic some characteristics of 4-1BB CAR T cells, like reduced cytokine production [448, 449].

CD4^{+} T cells are promising newcomers to immunotherapy. CD4^{+} TCRs convey exquisite target specificity and direct robust immune responses through indirect mechanisms that avoid tumor antigen escape. While much development and thought has been devoted to the activation benefits and off-target effects of increased TCR-pMHC affinity, especially for CD8^{+} TCRs, further TCR-therapeutic development should give consideration to the unique affinity thresholds of TCR-SCS and flTCR formats and the potential inhibitory effects of CD4.

2.5 Acknowledgements

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CHAPTER 3: Contributions of Viral Infections to Risk for Cancer in Systemic Lupus Erythematosus and Multiple Sclerosis

The content of this chapter was submitted in April 2020. It is currently under review. Authors: Deborah K. Johnson, Kaylia M. Reynolds, Matthew D. Montierth, Vera M. Todd, Joshua C. Denny, April Barnado, Brian D. Poole, and Mary F. Davis. It has been reformatted for this dissertation, but is otherwise unchanged.

Abstract

Patients with systemic lupus erythematosus (SLE) have a heightened risk for hematological, lung, liver, vulvar/vaginal, cervical, and thyroid malignancies and decreased risk for breast, ovarian, and prostate malignancies. We examined the effects of viral infections in SLE patients on this pattern of susceptibility to cancer, focusing especially on hematological cancers. A multiple sclerosis (MS) cohort control was included to assess the contribution of a systemic autoimmune disease that also predominantly affects women of childbearing ages and lupus-specific factors on virus susceptibility and cancer risk.

Materials and methods

Electronic medical records were extracted from Vanderbilt University. ICD-9/10 codes and laboratory values were collected for carcinogenic viruses including Epstein Barr virus (EBV), Herpesviruses (HPV), and Hepatitis A/B/C (Hep), and SLE-associated cancers including hematological, lung, anal-vaginal, thyroid, hepatobiliary, bladder cancers, prostate, and breast cancers. The case matched SLE cohort (SLE-cases n=2,313, and SLE-controls n=5,702) and MS cohort (MS-case n=7,277, MS-control n=7,277) were examined by multilinear logistic regression.

Results

Viral infection was strongly associated with increased risk for cancer, especially hematological cancers. SLE and MS patients were more susceptible to all viral infections, including EBV. Confirming previous findings, patients with SLE were more susceptible to hematological cancers when viruses were present and less susceptible to breast and prostate cancers. Patients with SLE did not demonstrate altered risk for overall incidences of malignancy compared to the control population. In comparison, MS patients were slightly more prone to overall malignancies than control patients and had increased risk for hematological cancers when viral infection was also present.

Conclusions

The increased prevalence of viral infection in SLE patients heightens their risk for hematological cancers. The increased risk for virus-associated cancer is strong enough that it counterbalances the lower risk for other types of cancer observed in SLE patients when considering overall cancer risk. Both SLE and MS patients had increase viral infection and subsequent hematological cancer risk. MS patients, however, had increased risk for cancers in general, and did not show the decreased risk for hormonally-affected cancers seen in SLE. Preventing viral infections by vaccination may be especially helpful in controlling risk for cancer in SLE and MS patients.

3.1 Introduction

Systemic lupus erythematosus (SLE) is a common, debilitating, and complex systemic autoimmune disease primarily affecting women of childbearing age. Its diverse symptoms include arthritis, fatigue, rash, sensitivity to sunlight, and in severe cases, kidney damage, blood disorders, neurological damage, and death. While SLE survival has improved, disease- and

treatment-related morbidity remain substantial. SLE patients have an increased risk for certain cancers [302] despite having a lower risk for hormonally-based cancers such as breast, ovarian and prostate cancers [450, 451]. Several studies suggest that SLE patients are also more susceptible to viral infections, including viruses associated with cancer such as Epstein-Barr virus or Human Papilloma virus [253, 256].

It is unclear how the overall cancer risk of SLE patients is affected by the relative contributions of SLE itself, its treatments, and other environmental factors such as increased viral infection [283, 284]. Compared to the general population, SLE patients experience increased incidence of hematological, hepatobiliary, vulvar, vaginal, and cervical cancers, all of which have conspicuous links to viruses [274, 452]. SLE patients may lack viral control due to immune dysfunction. For example, SLE T cells are dysregulated in response to Epstein-Barr virus (EBV) [287, 288] and dysregulated immune response to EBV can lead to increased Burkitt's lymphoma [289]. EBV infection is also associated with non-Hodgkin's lymphoma and diffuse large B cell lymphoma [290, 291]. The increased EBV viral loads in SLE patients [453] may help explain the increased incidence of hematological cancers.

EBV is also implicated in the development of multiple sclerosis (MS) [304], a chronic autoimmune inflammatory disease affecting the central nervous system that, like SLE, primarily affects women of childbearing ages [305]. Furthermore, similar to SLE, immunosuppressive treatment in MS may alter the risk of cancer development compared to the general public [308, 309]. While MS patients have a decreased risk for ovarian and prostate cancers, hematological cancers are consistently increased, again suggesting that poor viral control may lead to cancer development [310]. Thus, MS patients are a good autoimmune disease control to identify the relative contributions of SLE and viruses to cancer risk development.

This study seeks to understand the contributions of SLE and viruses to cancer development in SLE patients. The risk of viral infections and cancers were compared using electronic health records by logistic regression examining ICD-9 and -10 billing codes and laboratory values from Vanderbilt University Medical Center's electronic health record database. Our data demonstrates that viral status within an autoimmune disorder more fully predicts cancer risk than autoimmune-induced immune dysfunction alone.

3.2 Materials and methods

3.2.1 Study population

Patient demographic information, virus laboratory results, and ICD-9/10 billing codes were extracted from Vanderbilt University Medical Center Synthetic Derivative (VUMC SD) database, which contains de-identified electronic health records for over 3 million patients seen at Vanderbilt University Medical Center. We used a previously identified systemic lupus erythematosus (SLE) cohort with matched controls by age, race and gender (Figure 19) [454]. These SLE cases were identified using previously validated and published algorithms with positive predictive values (PPVs) ≥ 90% [455]. Controls were subjects who had ≥ 3 outpatient visits in the past 5 years at VUMC. Multiple sclerosis (MS) cases were identified with a computer algorithm as previously described [456]. Briefly, selected MS control patients did not have any ICD codes for other autoimmune diseases and were matched by age in 2018, race, and gender (Table 5). Patients missing values for their sex, who were older than 95 years in 2018, or who had both SLE and MS were excluded. Furthermore, MS case and control patients were removed if they overlapped with the SLE controls. The final MS study cohort consisted of 7,277 cases and 7,277 controls, and the SLE final study groups was composed of 2,313 cases and 5,702 controls (Figure 19).

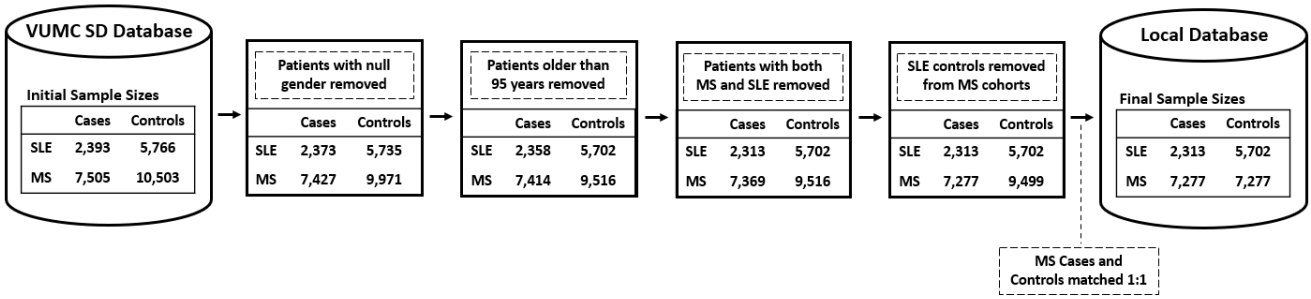


Figure 19. Filtering procedure for MS and SLE cohorts

Table 5. Demographic information, viral incidence and cancer incidence for SLE and MS cohorts by case and control

		SLE Case	*SLE Control*	*MS Case*	*MS Control*
	Total (n)	**2313**	**5702**	**7277**	**7277**
Demographic Information	*Female*	2058 (89%)	5132 (90%)	5603 (77%)	5167 (71%)
	Age	55	52.3	56.7	54.4
	Asian	36 (2%)	123 (2%)	20 (0%)	88 (1%)
	African American	535 (23%)	1419 (25%)	659 (9%)	606 (8%)
	Hispanic	57 (2%)	166 (3%)	66 (1%)	111 (2%)
	Native American	2 (0%)	5 (0%)	6 (0%)	2 (0%)
	Caucasian	1552 (67%)	3729 (65%)	5461 (75%)	4503 (62%)
	Other	6 (0%)	16 (0%)	7 (0%)	43 (1%)
	2+ Ethnicities	5 (0%)	7 (0%)	8 (0%)	6 (0%)
Viral Status	*Unknown*	120 (5%)	237 (4%)	1050 (14%)	1918 (26%)
	None	2016 (87%)	5194 (91%)	7116 (98%)	7221 (99%)
	Any	297 (13%)	508 (9%)	161 (2%)	56 (0.8%)
	Hep A/B/C +	198 (9%)	326 (6%)	20 (0.3%)	9 (0.1%)
	EBV+	163 (7%)	196 (3%)	115 (2%)	33 (0.5%)
	HPV+	30 (1%)	62 (1%)	28 (0.4%)	15 (0.2%)
Cancer Status	*All*	232 (10%)	571 (10%)	297 (4%)	236 (3%)
	Breast	39 (2%)	175 (3%)	89 (1%)	68 (0.9%)
	Prostate	8 (0.3%)	48 (0.8%)	32 (0.4%)	37 (0.5%)
	Hematological	163 (7%)	203 (4%)	124 (2%)	65 (0.9%)
	Lung	17 (0.7%)	63 (1%)	44 (0.6%)	36 (0.5%)
	Thyroid	12 (0.5%)	52 (0.9%)	24 (0.3%)	12 (0.2%)
	Anal/Vagina/Cervical	10 (0.4%)	46 (0.8%)	11 (0.2%)	13 (0.2%)
	Hepatobiliary	4 (0.2%)	31 (0.5%)	3 (0.04%)	9 (0.1%)
	Bladder	3 (0.1%)	24 (0.4%)	7 (0.1%)	8 (0.1%)

3.2.2 Identifying malignancies and viral infections

Patients were classified as having a malignancy (hematological, lung, vaginal, anal, hepatobiliary, bladder, thyroid, breast, or prostate) if their records contained at least two ICD-

9/10 billing codes for a specific cancer (Table 5). Anal and vaginal cancers are both associated with HPV infection, and thus were grouped to achieve sufficient patient numbers for analysis. To confirm viral infection history, patients were assigned positive viral status if they had a minimum of two instances of a positive laboratory value and/or ICD-9/10 billing code in their records (Table 6). More viral infections were detected in the SLE cohort than the MS cohort (Table 5). Hepatitis (hep) includes both hepatitis B and hepatitis C infections.

Table 6. ICD-9 /10 and lab codes used to identify patient cancer and viral incidence

Cancer Codes	**ICD-10**	**ICD-9**
Hematological	C81, C82, C83, C84, C85, C86, C87, C88, C89, C90, C91, C92, C93, C94, C95, C96, D46, D47, Z85.79	200-207 by 0.01, 273, 287.30, 287.3
Lung	C34, Z85.11	162-163 by 0.01
Anal/Vaginal/Cervical	C51, C52, C53, C21	180-181 by 0.01, 184.0, 184.4, 154.2, 154.3, 154.8
Hepatobiliary	C22, C23, Z85.05	155-157 by 0.01
Bladder	C67, Z85.51	188-189 by 0.01
Thyroid	C73, Z85.85	193-194 by 0.01
Breast	C50, Z85.3	174-176 by 0.01
Prostate	C61, D07.5, Z85.46	185-186 by 0.01

Viral Codes	**ICD-10**	**ICD-9**
EBV	B27	75-76 by 0.01
HPV	B97.7, B85.81, B85.82, R87.81, R87.82, R85.81, R85.82	079.4, 78.11, 795.05, 795.15, 796.75, 795.79, 795.099, 795.19
Hep	B15, B16, B17, B18, B19	70-71 by 0.01

Lab Viral Names	
EBV	EBV
HPV	HPV
Hep	HCV, HBV

3.2.3 Statistical analysis

Multilinear logistic regression models were used to predict overall viral incidence and cancer risk, and calculate risk for individual viruses and cancers (Table 7). To account for multiple testing, p-values were adjusted via a Bonferroni correction by multiplying the p-value by the number of regressions run for each autoimmunity group (SLE = 8, MS = 4). All filtering and statistical tests were completed in R 3.6.2.

Table 7. Covariate and cohort specifications for each regression performed

Response Variable	Disease Status	Gender	Age	Viral Infections	Interaction
Viral Status	SLE/MS	M/F	Age	None	None
Malignancy Status	SLE/MS	M/F	Age	EBV, HPV, HEP	(SLE/MS)*Viral Status
Hematological Status	SLE/MS	M/F	Age	EBV	(SLE/MS)*EBV
Lung Status	SLE	M/F	Age	EBV, HPV, HEP	SLE*Viral Status
Anal/Vaginal Status	SLE	F	Age	HPV	SLE*HPV
Breast Status	SLE	F	Age	EBV, HPV, HEP	SLE*Viral Status
Prostate Status	SLE	M	Age	EBV, HPV, HEP	SLE*Viral Status

3.3 Results

3.3.1 Overall viral incidence and cancer incidence

To uncover the link between autoimmune disorders, viral incidence and cancer incidence, we determined whether patients with systemic lupus erythematosus (SLE) and multiple sclerosis (MS) had higher incidence of positive viral status for Epstein Barr virus (EBV), Human Papilloma virus (HPV), and Hepatitis B or C (Hep) compared to control patients. Only infections that led to a physician visit or laboratory test would be entered into the medical records; most viral infections do not meet these criteria. Positive viral status is therefore a better

proxy for serious infections than for all infections. SLE patients had an increased overall viral incidence (odds ratio (OR) 1.60, 95% CI 1.37, 1.87, $p = 1.58 \times 10^{-6}$) than controls. Males (considering both the SLE patients and controls) were more likely to have higher viral incidence (OR 1.58, 95% CI 1.27, 1.95, $p = 2.16 \times 10^{-4}$) (Fig. 20a). MS patients had an even larger increased viral incidence compared to controls (OR 3.31, 95% CI 2.44, 4.56, $p = 2.15 \times 10^{-13}$), however, there was no increased risk for men compared to the MS cohort as a whole (OR 1.10, 95% CI 0.80, 1.49, p = 1.00) (Fig. 20b). This may be due to a larger number of men included in the MS cohort (n=1674) compared to the male SLE cohort (n=255), or because men diagnosed with SLE tend to have severe disease and may be prone to heightened viral incidence. For all logistic regressions, while age was statistically significant, its meaningful significance is unclear as the OR was close to 1.00 for both SLE and MS cohorts.

SLE and MS patients have increased viral incidence as well as an autoimmune disorder. Therefore, we examined whether the autoimmune disorder or viral incidence better predicted overall cancer risk by looking at overall cancer risk in both SLE patients and MS patients. SLE patients have increased risk for some cancers and decreased risk for others; therefore it was unsurprising that SLE alone did not significantly predict overall cancer incidence (OR 0.81, 95% CI 0.67, 0.96, p = 0.17) (Fig. 20c). Furthermore, the interaction between SLE and overall viral incidence did not predict overall cancer incidence (OR 1.75, 95% CI 1.12, 2.72, p = 0.11) (Fig. 20c). However, SLE patients with a positive viral test (OR 1.76, 95% CI 1.33, 2.31, $p = 4.12 \times 10^{-4}$) and male SLE patients (OR 1.65, 95% CI 1.33, 2.03, $p = 2.72 \times 10^{-5}$) had increased cancer risk (Fig. 20c). The interaction between MS and viral status did not predict overall cancer incidence (OR 1.41, 95% CI 0.56, 4.11, p = 1.00) (Fig. 2d). While not significant, MS alone showed a trend for increased in risk of overall cancer incidence (OR 1.25, 95% CI 1.05, 1.50, p =

0.059) (Fig. 20d). Additionally, gender was not a meaningfully significant contributor for cancer risk in the MS cohort (OR 1.20, 95% CI 0.98, 1.45, p = 0.27) (Fig. 2d). As in the SLE patients, viral status significantly predicted overall cancer risk for the MS cohort (OR 4.64, 95% CI 1.74, 10.4, p = 0.0025) (Fig. 20d). This suggests that viral status is a more important predictor for cancer development than either autoimmune disorder. Since autoimmune patients have an increased risk for viral incidence, this may explain the heightened risk in SLE patients for virus-associated cancers documented in other studies [274, 283, 452].

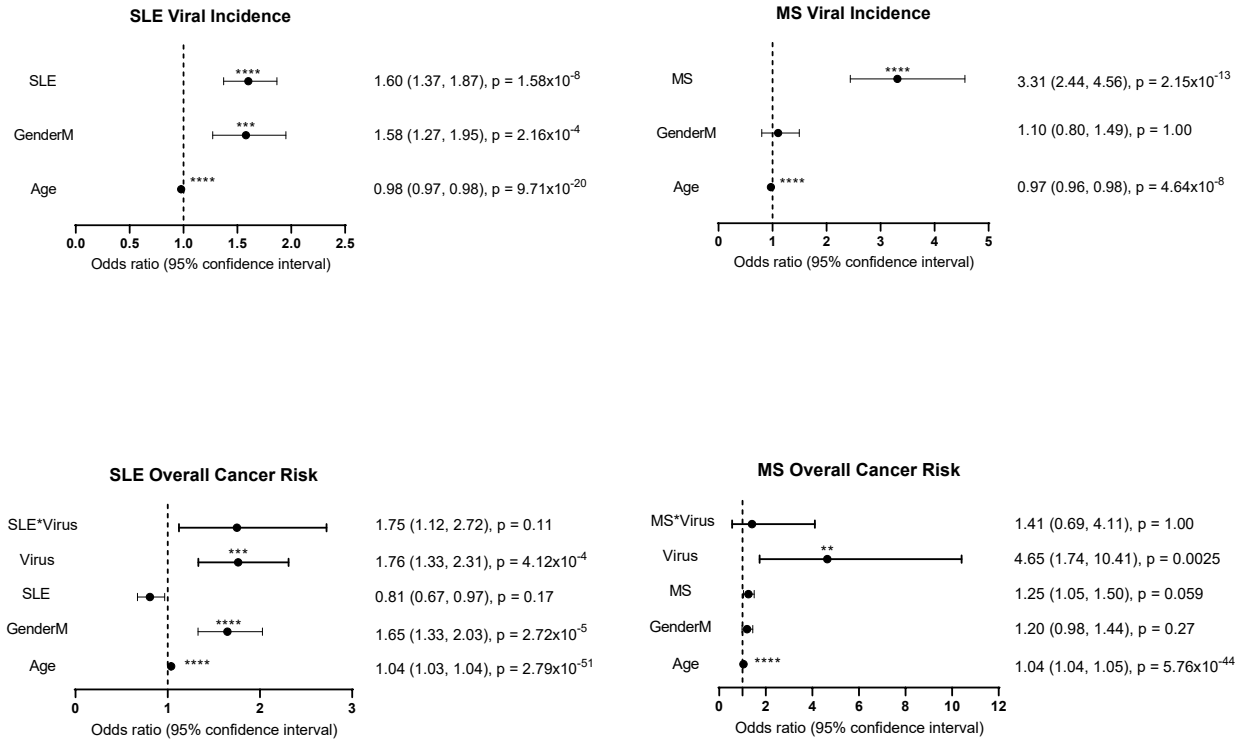


Figure 20. Autoimmune disorders increase viral incidence and viral incidence increases overall cancer risk

Odds ratios (OR) and 95% confidence intervals of SLE and MS cohorts. For all graphs, the dotted line represents an OR of 1.0. An OR to the right of the dotted line represents an increase in risk. An OR to the left of the solid line represents a reduction in risk. (a) Overall viral incidence for SLE cohort is significantly predicted by SLE (OR 1.60, 95% CI 1.37, 1.87, $p = 1.58x10^{-6}$) and male (OR 1.58, 95% CI 1.27, 1.95, $p = 2.16x10^{-4}$). (b) Overall viral incidence for MS cohort is significantly predicted by MS (OR 3.31, 95% CI 2.44, 4.56, $p = 2.15x10^{-13}$). (c) Overall cancer incidence for SLE cohort is significantly predicted by the viral status (OR 1.76, 95% CI 1.33, 2.31, $p = 4.12x10^{-4}$) and male (OR 1.65, 95% CI 1.33, 2.03, $p = 2.72x10^{-5}$). (d) Overall cancer incidence for MS cohort is significantly predicted by viral status (OR 4.65, 95% CI 1.74, 10.41, $p = 0.0025$).

3.3.2 Hematological cancers

To more specifically examine this heightened risk for viral-influenced cancers, we next investigated if viral incidence and SLE or MS increased the risk for hematological cancers. SLE and MS patients were more likely to have tested positive for EBV infection (OR 2.27, 95% CI 1.83, 2.81, $p = 6.99 \times 10^{-13}$; and OR 3.99, 95% CI 2.72, 6.00, $p = 2.49 \times 10^{-11}$) (Fig. 21a and b).

Male participants in both the SLE and MS cohorts may have a slight, though not significant, increase in EBV incidence (OR 1.50, 95% CI 1.09, 2.02, p = 0.079; and OR 1.47, 95% CI 1.03, 2.08, p = 0.13) (Fig. 21a and b).

The best predictor for hematological cancer risk was the interaction of SLE or MS with EBV (OR 1.92, 95% CI 1.53, 2.41, $p = 1.38 \times 10^{-7}$; and OR 1.83, 95% CI 1.33, 2.52, $p = 7.85 \times 10^{-4}$) (Fig. 21c and d). Men were also significantly more likely to develop hematological cancer in both cohorts (OR 3.69, 95% CI 2.22, 5.84, $p = 7.56 \times 10^{-7}$; and OR 17.12, 95% CI 4.90, 46.20, $p = 1.43 \times 10^{-6}$) (Fig. 21c and d). Taken together, these data suggest that patients with SLE or MS are more likely to have clinically relevant EBV infections and therefore more likely to develop hematological cancer compared to healthy controls.

We sought to confirm this finding with other SLE-increased cancers including hepatobiliary, anal/vaginal, bladder and thyroid cancers. The MS cohort had surprisingly few tests for these viruses overall (Hep C (n=29, 0.2%), HPV (n=43, 0.3%)) compared to the SLE cohort (Hep C (n=524, 6.5%), HPV (n=92, 1.1%)) (Methods: Table 5). To compound this problem, the number of patients in both the SLE and MS cohort with cancer and positive viral ICD-9/ICD-10 and lab codes were too low for statistical power to see correlations for these cancers (Methods: Table 5). Therefore, we could not run logistical regression on these cancer and viral combinations.

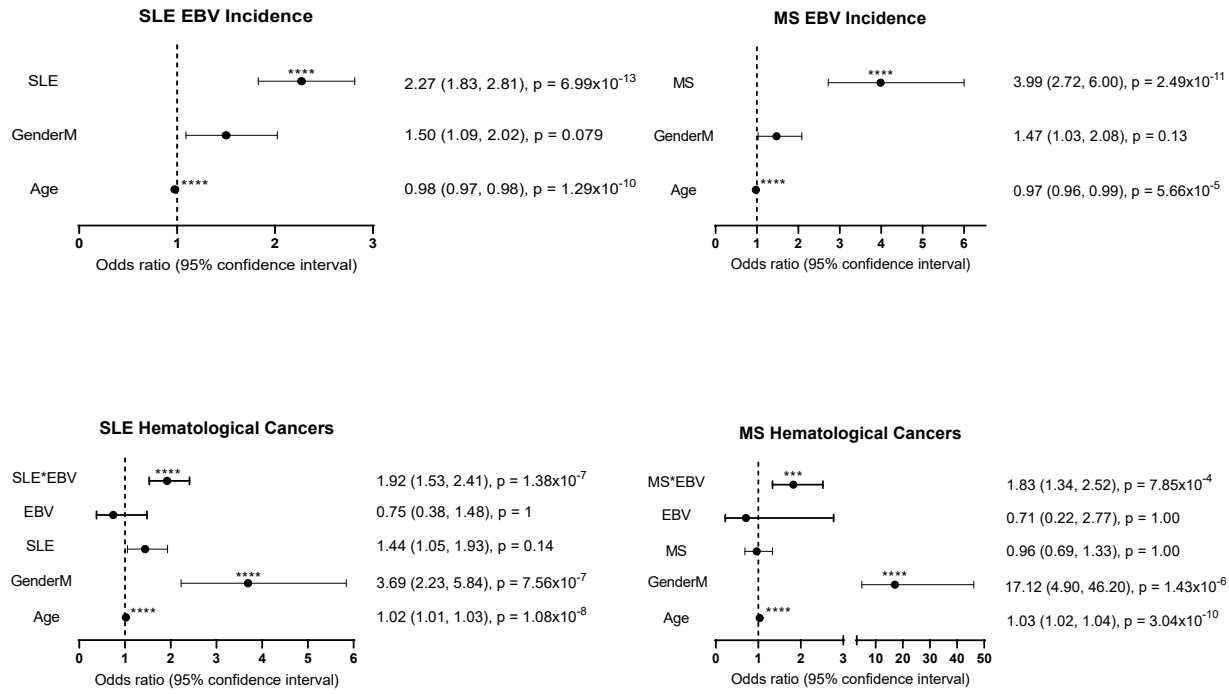


Figure 21. Autoimmune disorders increases risk of EBV infection and autoimmune disorder with EBV increases risk for hematological cancers

Odds ratios (OR) and 95% confidence intervals of SLE and MS cohorts. For all graphs, the dotted line represents an OR of 1.0. An OR to the right of the dotted line represents an increase in risk. An OR to the left of the solid line represents a reduction in risk. (a) EBV viral incidence for SLE cohort is significantly predicted by SLE (OR 2.27, 95% CI 1.83, 2.81, $p = 6.99 \times 10^{-13}$). (b) EBV incidence for MS cohort is significantly predicted by MS (OR 3.99, 95% CI 2.72, 6.00, $p = 2.49 \times 10^{-11}$). (c) Hematological cancer incidence for SLE cohort is significantly predicted by the interaction of SLE with EBV (OR 1.92, 95% CI 1.53, 2.41, $p = 1.28 \times 10^{-7}$) and male (OR 3.69, 95% CI 2.23, 5.84, $p = 7.56 \times 10^{-7}$). (d) Hematological cancer incidence for MS cohort is predicted by interaction of MS with EBV (OR 1.83, 95% CI 1.34, 2.52, $p = 7.85 \times 10^{-4}$) and male (OR 17.12, 95% CI 4.90, 46.20, $p = 1.43 \times 10^{-6}$).

3.3.3 Cancers for which SLE and MS patients are at decreased risk

SLE patients have decreased risk for hormonally-influenced cancers, including breast and prostate cancers. To confirm that our SLE cohort was properly curated and that viruses are not involved in these cancers we examined how SLE and viral status (HPV, EBV, Hep) affected

breast and prostate cancer incidence for women and men, respectively. Breast cancer risk was halved for patients with SLE (OR 0.517, 95% CI 0.35, 0.74, p = 0.0036) (Fig. 22a). Neither viral infection alone (OR 1.35, 95% CI 0.77, 2.23, p = 1.00) nor SLE and virus interaction (OR 0.60, 95% CI 0.13, 1.96, p = 1.00) predicted breast cancer incidence. Prostate cancer risk was also greatly reduced for SLE patients (OR 0.22, 95% CI 0.089, 0.50, p = 0.0053) (Fig. 22b). Again, neither viruses alone (OR 0.31, 95% CI 0.050, 1.10, p = 0.99) nor SLE and viruses together (OR 5.63, 95% CI 0.23, 74.69, p = 1.00) predicted prostate cancer incidence for the SLE cohort (Fig. 22b). In contrast, MS patients had similar breast (n = 89, 1%) and prostate cancer (n = 32, 0.4%) risk compared to control populations (n = 68, 0.9% and n = 37, 0.5% respectively). Logistic regression was not run for MS samples as there was a lack of viral medical records for these patients. This evidence demonstrates that not all cancers are increased in SLE patients and that not all cancer risk is heightened by viral infections.

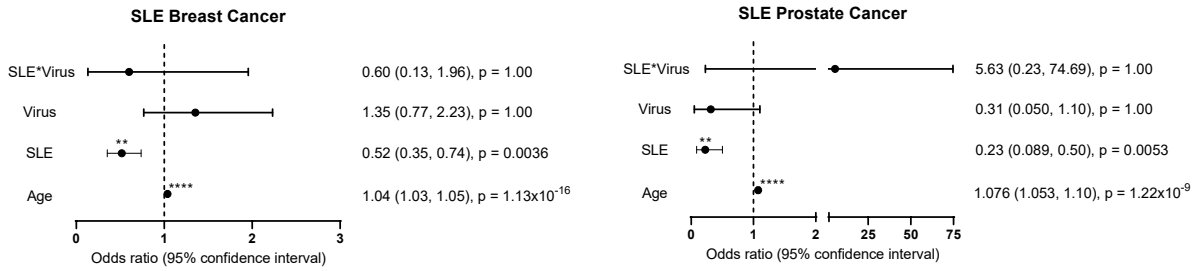


Figure 22. SLE decreases risk for hormonal cancers

Odds ratios (OR) and 95% confidence intervals of SLE and MS cohorts. For all graphs, the dotted line represents an OR of 1.0. An OR to the right of the dotted line represents an increase in risk. An OR to the left of the solid line represents a reduction in risk. (a) Breast cancer incidence in female SLE cohort is significantly decreased by SLE status (OR 0.52, 95% CI 0.35, 0.74, p = 0.0036). (b) Prostate cancer incidence in male SLE cohort is significantly decreased by SLE status (OR 0.23, 95% CI 0.089, 0.50, p = 0.0053).

3.4 Conclusions and Discussion

Systemic lupus erythematosus (SLE) patients experience increased incidences of some types of cancers including hematological, lung, vulvar, vaginal, and cervical malignancies. It is difficult to differentiate whether the disease, medication side effects, or the effects of autoimmune disease on virus infections are the underlying cause of elevated cancer risk in SLE patients. For example, increased SLE disease activity often leads to intensified drug treatments, making it difficult to tease out the relative contributions of immunosuppressive drugs and SLE itself on cancer risk [277, 283]. Cancer diagnosis tends to occur early after SLE diagnosis, often within the first year, suggesting SLE itself influences cancer risk more strongly than drug exposure given the limited time for drug exposure [276].

In this study, we found that viral infection status is a powerful risk factor for malignancies in general and hematological malignancies in two autoimmune diseases. SLE and multiple sclerosis (MS) patients have a heightened risk of positive viral infection status as noted by laboratory results and ICD-9/10 codes, indicative of increased viral infection incidence or heightened intensity of viral infections compared to controls. SLE patients are at a higher risk than controls for cancers associated with viral infection, but lower or equal risk for other types of malignancies. SLE patients overall cancer risk was not significantly different from controls. MS patients showed a different pattern; MS status itself conferred a slightly higher, though not significant, risk for malignancy overall, as well as a higher risk for viral infections. Viral infections continued to have the strongest influence on risk for cancer in the MS patients. Thus, we can conclude that increased susceptibility to more severe viral infection substantially contributes to overall cancer risk in both SLE patients and MS patients.

The contributions of autoimmune disease and viral infections to cancer development are particular to the type of cancer. SLE and MS patients are greatly more susceptible to clinically relevant EBV infection, and hematological cancers are also associated with SLE or MS and EBV infection status. Thus, the risk for hematological cancers is likely largely due to EBV infection, which is associated with Non-Hodgkin's lymphoma, Burkitt's lymphoma, and other B cell malignancies [290, 291, 457]. Control of EBV infection is known to be dysregulated in both SLE and MS, and there is weaker immunity to this virus in these diseases [264, 291, 458-463]. Therefore, it is likely that this dysregulation leads to increased risk for hematological cancers.

We confirmed previous findings that suggest that SLE itself confers a lower risk for breast and prostate cancer [282, 464, 465]. It is likely that SLE affects hormones or hormone regulation important for the development or proliferation of these cancers [466]. As expected by general population studies, viral infection did not affect the rate of these types of malignancies in the SLE population.

The use of an EHR system allowed access to thousands of records, and made this project possible. However, it also has limitations. Determining what is a "positive viral status" is challenging, and we certainly missed many viral infections. Only infections that led to a physician visit or laboratory test were entered into the medical records; most viral infections do not meet these criteria. Positive viral status is therefore a better proxy for serious infections than for all infections. Some viral infections, such as EBV, may not be commonly noted in the medical records. For example, although nearly all SLE patients are infected with EBV, increased viral replication or viral load, which is common in SLE patients [274, 452], would not necessarily be tested for unless it lead to mononucleosis or other symptoms, and would therefore not contribute to a positive viral status for this work. This may have resulted in an

underestimation of EBV cases in both SLE and healthy patients and may have affected the results regarding hematological cancers and HPV-associated cancers. Furthermore, SLE and MS patients are commonly screened for hepatitis B/C before beginning immunosuppressive medications, therefore these viruses may have been oversampled compared to the control population. As we relied on clinical notes from the HER, we do not have SLE disease activity measures recorded, so we cannot assess the impact of lupus activity on risk for viral infection or malignancy.

Finally, due to the success of early screening programs in the United States, cervical cancer is relatively rare, as are the other HPV-associated cancers. Instead of cervical cancer, therefore, we used cervical changes as an indicator for cervical malignancy. This likely altered the final HPV incidences since this cancer indicator would have been used to denote positive viral status, and HPV infections that do not lead to abnormalities are less likely to be noted.

Our findings indicate that increased viral infection in SLE and MS patients better explains the elevated risk of certain cancers than SLE and MS disease alone. However, patients with MS had a slight increased risk of malignancies separate from viral risk factors, highlighting that different autoimmune diseases may uniquely affect cancer risks. Both SLE and MS patients had increased risk for hematologic cancers, which were also affected by risk for viral infection. The increased rate of viral infection seems to overcome a general decrease in risk for cancers in SLE patients, leading to an equivalent overall malignancy risk compared to controls. Therefore, special care should be taken with viral infections in SLE patients. Although EBV infection interventions are currently limited to monitoring patients with active infections, there are vaccines for hepatitis B and HPV and treatment for hepatitis C. It is likely that these vaccines

and treatments would especially benefit SLE patients by reducing the associated risk for malignancy.

3.5 Acknowledgments

Much thanks to the Vanderbilt University Synthetic Derivative Database team and to Dr. Scott Weber for critical review.

CHAPTER 4: Combating Vaccine Hesitancy with Vaccine-Preventable Disease Familiarization: An Interview and Curriculum Intervention for College Students

The content in this chapter was previously published in *Vaccines.* Johnson, D. K. et al. "Combating Vaccine Hesitancy with Vaccine-Preventable Disease Familiarization: An Interview and Curriculum Intervention for College Students." *Vaccines,* 2019 May; 12:7(2). doi: 10.3390/vaccines7020039. I hereby confirm that the use of this article is compliant with all publishing agreements. The content is unchanged but has been formatted for this dissertation.

Abstract

In 2019, the World Health Organization (WHO) listed vaccine hesitancy in its top ten threats to global health. Vaccine hesitancy is a "delay in acceptance or refusal to vaccinate despite availability of vaccination services". Urban areas with large amounts of vaccine hesitancy are at risk for the resurgence of vaccine-preventable diseases (VPDs). Many vaccine-hesitant (VH) parents may be unfamiliar with the consequences of VPDs, and thus might be swayed when confronted with the symptoms and dangers of VPDs. As such, we sought to educate college students (future parents) in an urban vaccine-hesitant hotspot by assigning them to interview family or community members who had experienced a VPD. Student vaccine attitudes were assessed by surveys before and after the interviews. Vaccine-hesitant students who conducted a VPD interview but received no additional vaccine educational materials were significantly more likely (interaction term $p < 0.001$) to become pro-vaccine (PV) (68%) than students who conducted an autoimmune interview and received no additional educational materials. Additionally, students whose interviewees experienced intense physical suffering or physical limitations or students who were enrolled in a course with intensive VPD and vaccine

curriculum had significantly increased vaccine attitudes. This suggests that introducing students to VPDs can decrease vaccine hesitancy.

4.1 Introduction

Vaccines are victims of their own success. Due to the effectiveness of vaccination programs, many people have limited or no experience with vaccine-preventable diseases (VPDs) [333]. Parents increasingly assume that the risks associated with VPDs are minimal compared to potential health and safety risks of vaccinations themselves [335-338]. This has led to a rise in vaccine hesitancy by parents that results in a "delay in acceptance or refusal to vaccinate despite availability of vaccination services" [326]. Urban centers with large clusters of vaccine-hesitant individuals are particularly vulnerable to VPD outbreaks among exposed, unimmunized children. In the 2016–2017 school year, Utah County (Provo) in Utah, USA ranked sixth nationally for the total number of entering kindergartners that were under-vaccinated as measured by non-medical exemption (NME) waivers (n = 662 NME) [467]. As many of these parents may have never experienced VPDs, we hypothesized that designing an intervention for college students (future parents) in Provo, Utah might help improve vaccine attitudes and future vaccine uptake for themselves and their families.

Influencing students before they become parents will likely encourage pro-vaccination behaviors for their future and current families, as children and adolescents who participate in health education activities in school can positively influence family health management [373-375]. However, there is evidence that correcting erroneous assumptions about potential health and safety risks may be ineffective, causing a "backfire effect" and further entrenching vaccine-hesitant individuals' beliefs [346, 347]. Vaccine hesitancy often arises from deep-rooted ideological beliefs and conspiracist ideational tendencies. As this kind of thinking has been

shown to become further entrenched when those holding it are presented with contradictory information, correcting misinformation is often counterproductive [344, 345, 468]. Further, it is difficult to provide convincing data for the absence of risk; consequently, vaccine-hesitant parents may be recalcitrant to messages aimed at alleviating concerns about vaccine safety and side effects [354, 469]. Rather, pro-vaccine interventions may be more effective if they warn of health dangers to individuals from VPDs [354]. Additionally, the vaccine-hesitant movement uses emotionally charged stories with dire long-term consequences to effectively convey anti-vaccine ideology. Combating this rhetoric with a similarly emotional appeal may be an effective preventative strategy [470]. Therefore, we predicted that hearing about the effects of VPDs from family and community members who suffered from VPDs would improve the students' attitudes towards vaccination. We further predicted that classroom education could improve attitudes towards vaccines.

In this study, we analyzed three courses with different vaccine instructional approaches varying from none to intensive. Enrolled students were also assigned to interview a family or community member about their personal experience with either an autoimmune disease (control) or a VPD. Students exposed to either intensive VPD-focused vaccine instruction or who interviewed individuals who had had a VPD had statistically significant and meaningful gains in vaccine attitude.

4.2 Materials and methods

4.2.1 Study population

Students at a large private institution in the Western United States were enrolled in a quasi-experimental survey-based intervention study. Students were eligible if they attended one

of three courses in the Winter 2018 semester: general education non-science major BIO 100 (*Principles of Biology*-one section), and microbiology and molecular biology major-specific courses MMBio 240 (*Molecular Biology*-two sections), and MMBio 261 (*Infection and Immunity*-one section). Vaccination principles were taught in BIO 100 and MMBio 261, but not in MMBio 240. All students enrolled in the courses were encouraged to participate and offered extra credit for their time and efforts. Our study sample consisted of 425 students who completed the study requirements (574 began the study). The study was conducted in accordance with the Declaration of Helsinki, and study procedures were approved by the Institutional Review Board at the institution (study # E17263). All participants received and signed a consent form that included a description of the study and were assigned a random number to protect their identities. Students were informed that their instructors would never see their names associated with any of the survey results, and steps were taken to avoid such instructor access.

4.2.2 Assigning vaccine attitude groups and randomization process

To determine initial vaccine attitudes, students took a pre-interview survey (see Table 8) and were asked to rate each question from 1–5 where 1 is "strongly disagree" and 5 is "strongly agree". Each question concerning vaccine attitude was chosen to cover a specific aspect of vaccine hesitancy. Pre-intervention vaccine attitude scores (VASs) were tallied from questions 1, 4, 9, 11, and 13 (Table 8). Question 1 is a test of general attitude towards vaccines. Question 4 addresses side effects, relevant since many vaccine-hesitant individuals are afraid of these side effects. Question 9 is about the common belief that vaccines cause autism, a major concern for many vaccine-hesitant people. Question 11 gives an opportunity for the participants to opine on the positive aspects of vaccination in terms of how well they work. Question 13 is a question

about how the vaccine attitude would affect action and give it a more real-world, rather than theoretical, effect. To avoid answer bias, students were not informed that the study was about vaccination opinions and additional questions about autoimmune diseases and depression were included in the survey. Scores from questions 4 and 9 were reverse coded to account for the negative nature of the question. These questions were written in a negative way to avoid biasing the study by presenting vaccines in only a positive light in the questions. Students with VASs between 20 and 25 points were categorized as "Pro-Vaccine" (PV) and students that had a VAS less than 20 points were categorized as "Vaccine-Hesitant" (VH). A cutoff value of 20 was chosen because it meant, on average, that the student at least "agreed" (score or converted score of 4) with all of the vaccine attitude questions. A score of less than 20 would mean that the student on average either did not at least "agree" with the pro-vaccine statements, or that they had a serious disagreement with at least one statement about vaccines. Students were assigned to interview groups by alternating autoimmune (negative control for survey and interview effects) and vaccine-preventable disease (VPD) interview assignments alphabetically within PV and VH categories so that equal numbers of students were assigned to each interview intervention.

Table 8. Pre-Interview Survey (VPD)

Pre-Interview Survey: **Table 8**

Rate each question from 1 to 5 where 1 is "strongly disagree" and 5 is "strongly agree"

1. **Vaccines are more helpful than harmful**
2. Treatment for autoimmune diseases is more helpful than harmful
3. Medications for depression are more helpful than harmful
4. **Vaccines often have severe side effects**
5. People with autoimmune diseases suffer considerably
6. Medication for depression is effective at treating depression
7. There are effective treatments for autoimmune diseases
8. Depression can be overcome using willpower
9. **Vaccines cause autism**
10. Exercise is the best treatment for autoimmune diseases
11. **Vaccines are effective at preventing disease**
12. Medications for depression have severe side effects
13. **I am likely to fully vaccinate my children/I have fully vaccinated my children**

Students were emailed their survey assignments and related paperwork, and based on their group assignment, were asked to interview members of the community who had experienced either a VPD or an autoimmune disease before the end of the semester using the interview questions shown in Table 9. To encourage study completion, students received full points for extra credit with completed survey submission (Bio 100 10 points, 1% of grade; MMBio 240 20 points, 2.3% of grade; MMBio 261 20 points, 2.3% of grade). At the end of the semester, students were administered a post-interview survey (see Table 10) that reiterated the pre-interview survey questions and included follow-up questions about the survey itself. These questions (14–18 in the post-interview survey) were written to identify the aspects of the interview that had the most significant impact on vaccine attitudes. They provided both an opportunity to rank several factors and the ability to explain in their own words how the various aspects of the interview affected them. We then assigned students a post-intervention VAS and

assessed for changes in overall vaccine scores between the pre-and post-intervention surveys. Included in this analysis was determining whether students moved from the vaccine-hesitant to pro-vaccine group, or vice-versa.

Table 9. Interview Questions (VPD)

Interview Questions	**Table 9**
1. What is your relationship to the person who had the disease?	
2. When did they develop the disease?	
3. Which disease was involved?	
4. What type of physical suffering did the disease cause? How bad was it?	
5. How did the disease limit the person's ability to do normal activities?	
6. How did the disease affect the person's interaction with other people?	
7. How did the disease affect the person's friends, family, or loved ones?	
8. How did the disease affect the person financially?	
9. Were there any other effects of the disease?	

Table 10. Post-Interview Survey (VPD)

Post-Interview Survey: **Table 10**

1. Vaccines are more helpful than harmful
2. Treatment for autoimmune diseases is more helpful than harmful
3. Medications for depression are more helpful than harmful
4. Vaccines often have severe side effects
5. People with autoimmune diseases suffer considerably
6. Medication for depression is effective at treating depression
7. There are effective treatments for autoimmune diseases
8. Depression can be overcome using willpower
9. Vaccines cause autism
10. Exercise is the best treatment for autoimmune diseases
11. Vaccines are effective at preventing disease
12. Medications for depression have severe side effects
13. I am likely to fully vaccinate my children/I have fully vaccinated my children

Complete next section only if you interviewed a VPD-subject

Circle an answer: much more opposed, slightly more opposed, no effect, slightly more in favor, much more in favor

14. How did hearing about the subject's physical suffering affect your opinion of vaccines?
15. How did hearing about how the disease limited normal activity affect your opinion of vaccines?
16. How did hearing about how the disease affected the subjects' interactions with other people affect your opinion of vaccines?
17. How did hearing about how the disease affected the subject's family, friends or loved ones affect your opinion of vaccines?
18. How did hearing about the disease's financial impact on the subject affect your opinion of vaccines?

Rank the following:

___ Physical suffering

___ Limitation of activities

___ Interactions with other people

___ Effect on family, friends or loved ones

___ Financial impact

19. Please explain briefly, what effect, if any, the project had on your attitude towards vaccination and why it has that effect.
20. If this interview did NOT affect your attitude towards vaccination, why not?

4.2.3 Analyses

Changes between groups' pre- and post-intervention VASs were assessed with factorial ANOVAs. Individual group changes over time were assessed by paired sample *t*-tests, and differences between two groups at specific time points were assessed by independent sample *t*-tests. Bonferroni corrections were applied to any multiple comparisons to account for alpha inflation. Standard deviations were reported for statistics less than 5 points. All other statistics reported 95% confidence intervals (CI). All analyses were performed using SPSS Statistics 25 (IBM). Figures were generated in Prism 8 (GraphPad) and tables were generated in Excel 2016 (Microsoft).

4.3 Results

4.3.1 Overview and pre-interview intervention vaccine attitudes

A total of 574 students volunteered to take the pre-interview survey during the Winter 2018 semester. Based on their pre-intervention vaccination attitude scores (VASs), students were designated either pro-vaccine (PV) (87%) or vaccine-hesitant (VH) (13%) and assigned to the control group (autoimmune survey, $n = 286$) or the intervention group (vaccine-preventable disease (VPD) survey, $n = 288$). Of the students, 74% ($n = 425$) completed all requisite parts of the study (pre-interview survey, community/family interview, post-interview survey) and were included in the final analysis (Figure 23). The VH group was defined as VAS < 20 and the PV group was defined as VAS ≥ 20 based on the pre-interview survey responses. There were no statistically significant differences in the sociodemographic characteristics among the classes nor did the course they were enrolled in significantly affect the assignment to VH and PV groups (Table 11). Course year explains the age difference between the courses: Bio 100 is a general education course for first year students, MMBio 240 is a second-year major-specific course, and MMBio 261 is a second to third year major-specific course. Furthermore, sex, race, and age were

not significantly correlated with pre-intervention vaccine attitudes (Table 12). Student willingness to vaccinate current/future children was significantly different between VH and PV groups (scale of 1–5 from strongly disagree to strongly agree) with means of 3.84 and 4.92, respectively (independent t-test CI 95% 0.814–1.355; $p < 0.001$).

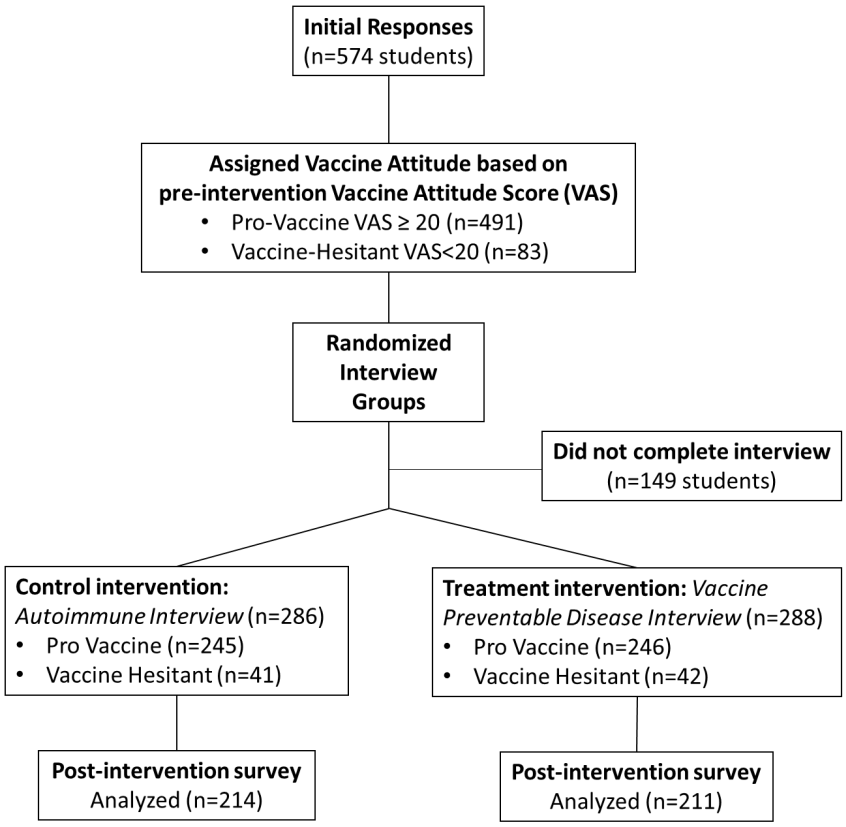


Figure 23. Participant flow through the randomized treatment

Table 11. Baseline characteristics of the participating classes (n=425)

Class Demographics	Total % (n)	BIO 100 % (n)	MMBio 240 % (n)	MMBio 261 % (n)
Total	100% (425)	13% (56)	70% (298)	17% (71)
Gender				
Male	62% (263)	70% (39)	61% (182)	59% (42)
Female	38% (162)	30% (17)	39% (116)	41% (29)
Age	21.2 ± 0.21	19.7 ± 0.50	21.3 ± 0.24	22.1 ± 0.40
Pre-Vaccine Attitude Score				
Vaccine Hesitant	13% (56)	18% (10)	14% (41)	7% (5)
Pro Vaccine	87% (369)	82% (46)	86% (257)	93% (66)

There are no statistically significant differences among the classes for gender distribution, age, or Pro-Vaccine or Vaccine-Hesitant group assignment.

Table 12. Baseline characteristics of Vaccine Hesitant and Pro-Vaccine groups (n=425)

Socio-Demographic Characteristic	Total % (n)	Vaccine Hesitant % (n)	Pro-Vaccine % (n)
Total	100% (425)	13% (56)	87% (369)
Gender			
Male	62% (263)	14% (36)	86% (227)
Female	38% (162)	12% (20)	88% (142)
Age	21.2 ± 0.21	21.0 ± 0.74	21.3 ± 0.21
Race/Ethnicity			
African American	1% (3)	-	100% (3)
Asian	3% (11)	27% (3)	73% (8)
Caucasian	87% (370)	13% (48)	87% (322)
Hispanic	3% (12)	17% (2)	83% (10)
Native American	0.2% (1)	100% (1)	-
Other	6% (26)	8% (2)	92% (24)

There were no statistically significant differences in ethnicity, gender, or age between vaccine opinion groups.

4.3.2 Interview intervention improves student vaccine attitude scores

Vaccine attitudes improved when the participants gained a personal understanding of how vaccine-preventable diseases affect individuals and communities. Vaccine-hesitant students enrolled in MMBio 240 (no vaccine curriculum) who were part of the intervention group (n = 19) showed a significant increase in VAS; average VAS shifted from 17.58 ± 0.84 to 20.53 ±

0.94 (paired t-test CI difference (diff) 95% 4.077–0.817; $p < 0.001$), an average increase of 2.95 ± 2.34 points (Figure 24). Of these students, 68% (n = 13) had sufficient increases in their VASs to move from the vaccine-hesitant group to the pro-vaccine group. Conversely, vaccine-hesitant students who were part of the control group (n = 22) had no significant increase in VAS (paired t-test CI diff 95% 1.856–0.038; $p = 0.059$) which shifted only 17.27 ± 0.87 to 18.18 ± 1.31, an average increase of 1 ± 2.05 point ($p = 0.059$). Only 27% (n = 6) of students in the control group increased their scores sufficiently to move from the vaccine-hesitant group to the pro-vaccine group. Post-intervention VASs are significantly different between control and intervention VH groups (independent t-test CI diff 95% 4.066–0.623; $p = 0.009$), whereas post-intervention VASs are still significantly different between VH and PV students in the intervention group (independent t-test CI diff 95% 3.702–1.733; $p < 0.001$). $\alpha = 0.0125$.

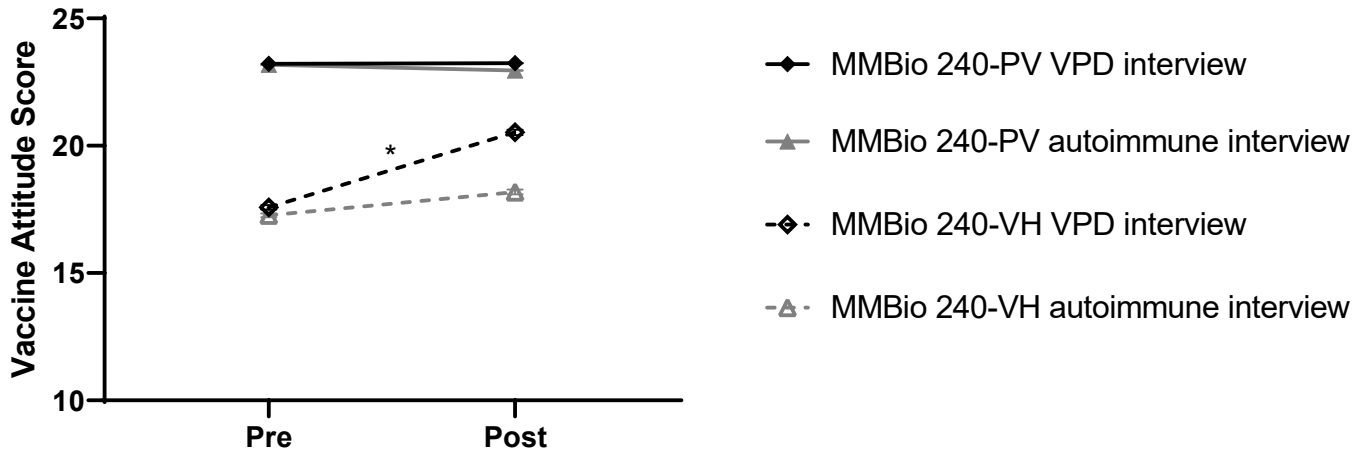


Figure 24. Vaccine-preventable disease interview significantly improves attitudes towards vaccines

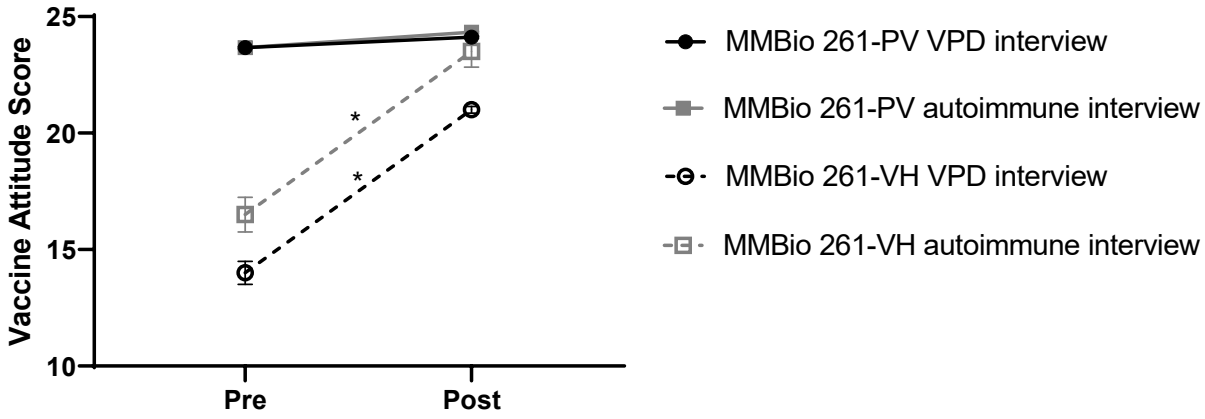
A significant difference (interaction term $p < 0.001$) for vaccine-hesitant (VH) students in MMBio 240.

4.3.3 Interview intervention improves student vaccine attitude scores

Intensive vaccine education may be even more effective at improving vaccine attitudes than interviewing individuals who have had a VPD. All vaccine-hesitant students ($n = 5$) enrolled in MMBio 261 (intensive immune, VPD, and vaccine education) significantly increased their VASs by 7.00 ± 1.41 points on average regardless of survey intervention ($p < 0.001$), (pre-control group MMBio 261 VH mean 16.50, CI 95% 14.41–18.59; post-control group VH mean 23.500, CI 95% 12.616–25.384; pre-intervention group VH mean 14.000, CI 95% 12.29–15.71; post-intervention group VH mean 21.00, CI 95% 19.46–22.54) (Figure 25a). For all VH students, including intervention and control groups, the pre-intervention VAS mean was 15.00 ± 2.06 and the post-intervention VAS mean was 22.00 ± 2.23, decidedly in the pro-vaccine range. Four out of five VH students increased their VASs sufficiently to move from the vaccine-hesitant category to the pro-vaccine category with an average increase of 7.50 ± 1.00 points. The final student, who participated in the intervention group, increased their VAS from 13 to 18 points, an increase of 5 points. There is a significant difference between pre- and post-intervention group MMBio 261 VH students ($p = 0.026$, $n = 3$ paired t-test CI diff 95% 11.97–2.03). Statistics cannot be run across time between pre- and post-control group VH students since there are a low number of respondents ($n = 2$). Although these results are promising, large in magnitude, and statistically significant, they are based on a small number of vaccine-hesitant students in the class ($n = 5$). Furthermore, students in MMBio 261 are majoring in a life sciences degree and may be more prone towards persuasion by scientific reasoning than the non-major students in the general education Bio 100 course.

To highlight the need for tailored and intensive vaccine education, vaccine-hesitant students in Bio 100 had a non-significant yet distinct upward trend over time regardless of survey intervention (Figure 25b). Overall, VASs do significantly change across time ($p = 0.036$)

and vaccine attitude/survey groups ($p < 0.001$). All students in Bio 100 received brief instruction on how vaccines work, the rarity of vaccine side effects, the benefits of herd immunity to society, and no specific conversation about VPDs. The average VH student increased 1.9 ± 2.37 points between pre- and post-intervention VASs. There is no significant difference after the survey intervention between post-control group and post-intervention group VH students (independent *t*-test CI diff 95% 3.47–7.47; $p = 0.42$), or between post-control group PV and post-control group VH students (independent *t*-test CI diff 95% 8.32–1.57; $p = 0.136$). There is a significant difference after survey treatment between post-intervention group PV and post-intervention group VH students (independent *t*-test CI diff 95% 8.24–3.38; $p < 0.001$).



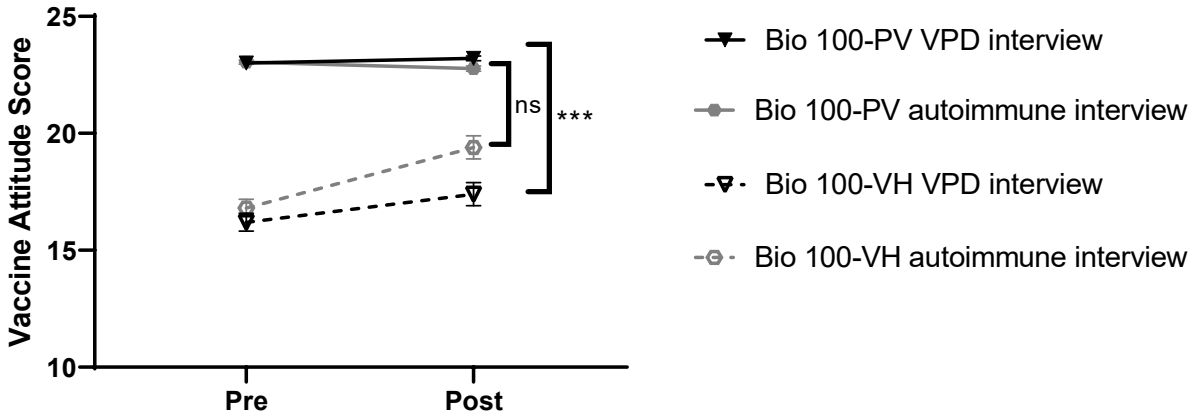


Figure 25. Education can significantly increase vaccine attitude

(**a**) Vaccine attitude scores (VASs) of MMBio 261 vaccine-hesitant students significantly increased regardless of survey intervention ($p < 0.001$), Difference between pre-control group VH and pre-intervention group MMBio 261 VH students is not significant (CI diff 95% 4.72–9.71; $p = 0.35$). (**b**) While there is an upward VAS trend for all Bio 100 VH students, it is not significant, suggesting that education has more influence than intervention.

4.3.4 Vaccine-hesitant students' VAS change dependent on pre-intervention VASs and class

This intervention focuses on the vaccine attitudes and responses of vaccine-hesitant students to an interview intervention. To better understand what aspects of the interview intervention positively influenced VH students, we focused on analyzing the scores of VH students by comparing pre- and post-intervention VASs. Overall, most VH students (75%) have increased VASs, while 50% of all VH students advance to PV scores by the end of the study (Table 13). This gain, however, depends on class or interview group as previously described. For example, interview group determines the fate of MMBio 240 VH students but not Bio 100 VH students (Table 13). Class enrollment predicts pre- to post-intervention VAS changes (Figure

26a). MMBio 261 students have the greatest increase (7.50 ± 1.00), while students in Bio 100 and MMBio 240 had similar gains (3.40 ± 1.50 and 3.50 ± 2.00 points, respectively).

Yet, once students are broken into groups based on pre-intervention VASs, it becomes clear that not all VH students are alike (Figure 26b, Table 14). Students with the lowest pre-intervention VASs (11–15 points) are unlikely to become PV (n = 2.18%) and only gain an average of 2.91 ± 2.74 points ($p < 0.001$). This average is clearly defined by survey groups: intervention group students gain an average of 4.67 ± 2.65 points, whereas control group students gain an average of 0.8 ± 1.92 points. Students in this low score category who gained 5+ points (n = 4) were all part of the intervention group. This suggests that the most vaccine-hesitant students are swayed by VPD interviews. Students with middle VH pre-intervention VASs (16 or 17 points) gain an average of 4.00 ± 3.07 ($p = 0.0095$) and are more likely to become PV (n = 9, 60%). Overwhelmingly, students in this middle category who gained 5+ points were either in MMBio 261 (n = 3) or had conducted a VPD survey in Bio 100 or MMBio 240 (n = 4). Two students in this category were not in MMBio 261 and conducted autoimmune surveys, thus their reasons for change are not predictable. The final group of VH students with the highest pre-intervention VAS (18 or 19 points) gained the least, an average of 1.27 ± 2.02 points ($p = 0.0018$, n = 17.57%). As these students are near the highest range already, it is not surprising that no students gained more than 5 points as a 6 point gain places them at the top of the VAS range.

Table 13. Survey intervention and education significantly improves vaccine hesitant student VAS

Change post-treatment	Overall VH % (n)	BIO 100 % (n)	MMBio 240 % (n)	MMBio 261 % (n)
Total	**56**	**10**	**41**	**5**
VAS Increased	75% (42)	80% (8)	71% (29)	100% (5)
VAS No Change	11% (6)	0% (0)	17% (7)	0% (0)

VAS Decreased	14% (8)	20% (2)	12% (5)	0% (0)
Pro Vaccine VAS (20+ pts)	50% (28)	50% (5)	46% (19)	80% (4)

Breakdown of all vaccine hesitant (VH) post-intervention VAS regardless of survey-treatment. All students who reached a VAS of 20+ were reassigned pro vaccine (PV). "VAS Increased" includes students who became PV.

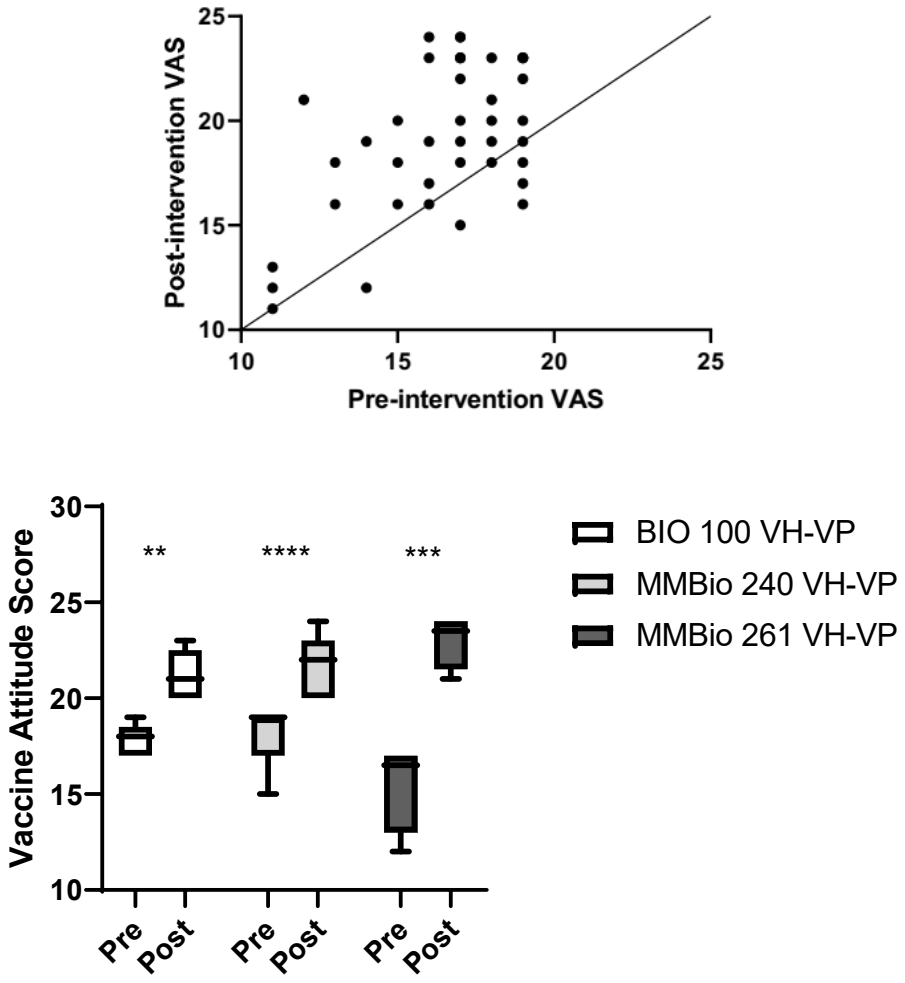


Figure 26. Vaccine-hesitant students make varying gains based on starting score and class attended

(**a**) VAS changes for VH students with PV post-intervention VASs. VH to PV students in MMBio 261 had an average VAS increase of 7.5 ± 1.0 points, whereas students in Bio 100 and MMBio 240 gained an average of 3.4 ± 1.5 and 3.5 ± 2.0 points, respectively. (**b**) VH students' gains are determined by pre-intervention VASs. Plotting pre-intervention VASs against post-intervention VASs for VH students shows student responsiveness is dependent on pre-intervention VASs. The line indicates no change between pre-and post-intervention scores, so the farther away from the line the larger the change.

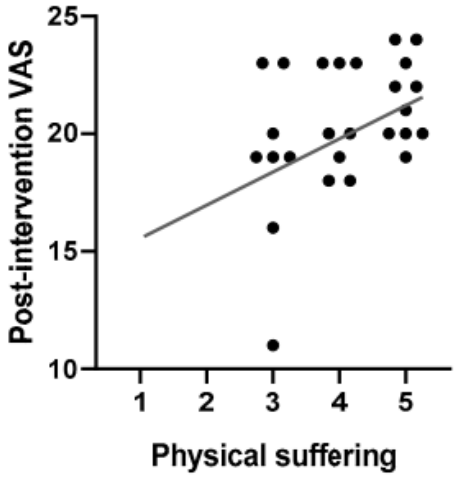
Table 14. Numerical breakdown of all VH students by pre-intervention VAS (figure 26b)

Pre-intervention VAS	**11 to 15 pts**	**16-17 pts**	**18-19 pts**
total	11	15	30
Post-intervention VAS			
VH	82% (9)	40% (6)	43% (13)
VP	18% (2)	60% (9)	57%(17)
Change (avg)	2.91 ± 2.74	4.00 ± 3.07	1.27 ± 2.02
VAS Decreased	1	1	6
Age (avg)	20.9 ± 0.9	21.0 ± 0.2	21.1 ± 1.3

4.3.5 Vaccine-hesitant student post-intervention VAS increase correlated with perceived physical suffering and physical limitations

In the post-survey interview, students in the intervention group were asked to assess how much each of the following characteristics affected their opinion of vaccines: physical suffering, limited normal activity, limited interaction with others, impact on family and friends, and financial costs (Methods, Box 3). These attributes were assessed from "strongly more opposed to vaccination" to "strongly more in favor of vaccination" and assigned the values of 1–5 points. VH students' post-intervention VASs are significantly and moderately correlated with physical suffering (4.08 ± 0.845, r2 = 0.405, p = 0.04) (Figure 27a) and limitation on normal activities (3.88 ± 0.653, r2 = 0.518, p = 0.007) (Figure 27b). VH students with a positive pre- to post-intervention VAS change agree or strongly agree that physical suffering is of major importance;

although the amount change in VAS compared to strength of agreement is not significant, there is a visible upward trend (one-way ANOVA, p = 0.3089) (Figure 27c). Even more strikingly, VH students with the greatest VAS change (6–9 points, n = 5) strongly agree that normal activity limitations affect their vaccine opinions (one-way ANOVA, p = 0.0206) (Figure 27d). This suggests that VH students are more influenced by stories from VPD victims that include physical suffering and activity limitation.



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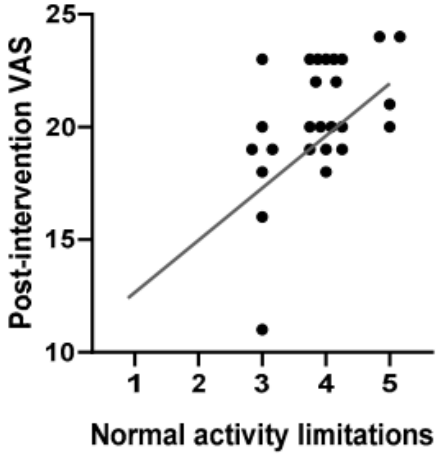

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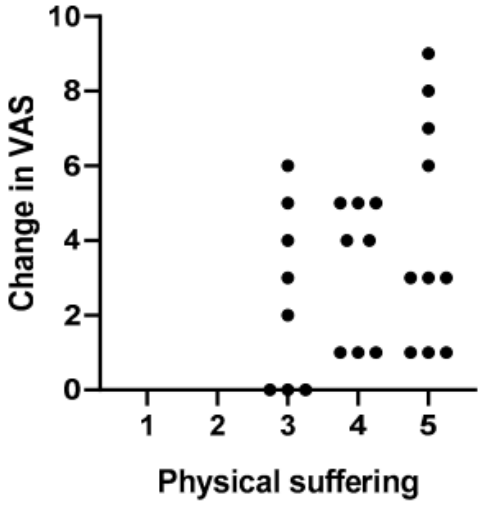

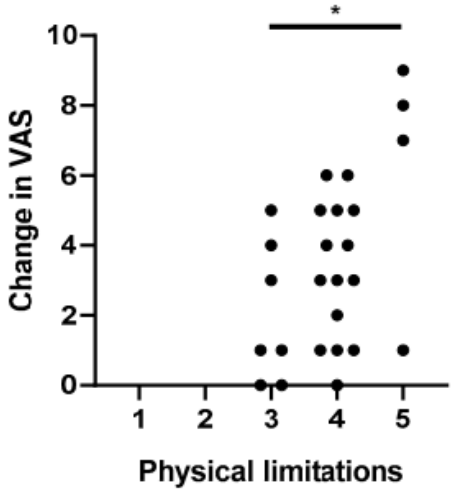

Figure 27. Post-intervention VAS and positive pre- to post-intervention VAS changes are influenced by (a,c) physical suffering and (b,d) physical activity limitations

(**a**) Post-intervention VAS is predicted by physical suffering (r2 = 0.405, $p = 0.04$) and (**b**) physical limitations (r2 = 0.518, $p = 0.007$). (**c**) While the student's perception of physical suffering did not predict the amount of VAS change ($p = 0.3089$), (**d**) the student's perception of normal activity limitations is significantly predicted ($p = 0.0206$). * $p < 0.05$.

4.3.5.1 Interview examples correspond to student perceptions of physical suffering and physical limitations

Examples of interview responses for physical suffering and physical limitations from students with the greatest VAS change (6–9 points) suggest that extreme cases enhance student response. One student interviewed a member of their church congregation who had shingles: "The pain was so bad that she ended up at a pain management clinic where they did steroid shots into her spine. The pain meds didn't even touch [reduce] her pain, even the heavy ones. For months she couldn't leave the house." This interview led the student to explain (Methods, Table 10, question 25) that "The project showed how the lack of vaccination is essentially accepting the pain and suffering that comes with disease." Another student interviewed his or her grandmother about tuberculosis: "Before getting diagnosed and during the time that she was treated, she could work her eight-hour temple shift and then she would go straight to bed after getting home. After a couple of hours nap, she would get up for a short time to get small tasks done before retiring to bed for the night." This student summarized the interview experience as "I dislike the idea of physical suffering so hearing about someone getting a disease made the idea of getting a disease if I don't get vaccinated seem more real." These students both became PV with VAS increases of 7 and 6 points, respectively.

In keeping with this idea, many VH students with smaller VAS gains generally reported less serious physical suffering and physical limitations from the people they interviewed. A

student who gained 4 points and interviewed a shingles patient wrote: “She considered her case very minor and she did not suffer physically much. She had some difficulty sleeping for a couple of weeks. She was a stay-at-home wife at that time, so she wasn’t missing work [or] school.” Similarly, a student who gained 3 points and interviewed a German measles case remarked, “Mother developed typical rash for about 3 days with high fever and remained bed bound. She is a school teacher and didn’t work for a few days.” While some VH students who gained low to middle VAS points had extreme examples, overall the tone was more moderate than the students who gained the greatest VAS points.

4.4 Discussion

In this study, we succeeded in improving student vaccine attitudes through either (1) having students interview individuals who had experienced a VPD or (2) providing intensive vaccine- and VPD-related course material. Combining intervention styles allowed us to assess the strength of each intervention. VPD interviews (intervention group) were most successful at swaying student vaccine attitudes when the coursework did not discuss vaccines or if the interviews had strong themes of physical suffering and limitations. The majority of students in the intervention group who became pro-vaccine and the resulting increase in vaccine attitude scores mirrored those achieved through intensive education (MMBio 261). Thus, encouraging students to conduct VPD interviews may be an easy and effective intervention when the course has little to do with VPDs or lacks vaccine-related content.

In courses that do address vaccines, it may be advantageous to first rigorously introduce students to VPD consequences before addressing, lightly, vaccine safety and societal implications. While Bio 100 introduces vaccines through a homework assignment that seeks to

correct misconceptions about vaccine safety and societal implications, MMBio 261 begins with rigorous weeks-long sections on immunity and VPDs but only briefly discusses vaccine safety and herd immunity. This may explain why Bio 100 VH students did not have significantly increased post-intervention VASs, only a suggestive upward trend, whereas MMBio 261 VH students had significantly improved post-intervention VASs. The comparison between Bio 100 and MMBio 261 students mirrors earlier research that discussing VPD ramifications has a greater impact on combating vaccine hesitancy than correcting flawed assumptions or asserting an absence of risk about vaccines [344-347, 468].

This study does have limitations. We did not examine whether an interview-based intervention would be successful in a non-science course. Any biological instruction discussing vaccines might provide some boost to vaccine attitudes. Additionally, for logistical reasons, we did not assess whether the increase in VAS is meaningful by following whether students vaccinate their current and future children. Furthermore, this study focuses on college students and may not be expandable to the general population. Nonetheless, despite these limitations, interview-based interventions and intensive VPD-dependent vaccine education does significantly increase vaccine attitudes, in a population susceptible to anti-vaccine attitudes. Vaccine hesitancy is a complex, situation-dependent problem, and requires unique and tailored interventions. Interview-based interventions are easy to implement and can supplement courses or even community outreach programs seeking to address vaccine hesitancy. Predisposing students to think more favorably about vaccinations by openly discussing the consequences of vaccine-preventable diseases may improve their prospective individual and familial vaccine uptake. Future research should tease apart the contributions of science education and personal familiarity with VPDs towards improving vaccine attitudes in diverse populations.

4.5 Conclusions

There are two major conclusions of this work. First, an interview-based intervention, where students discuss vaccine-preventable diseases with people who have actually experienced these diseases, can significantly improve attitudes towards vaccination. Second, the subject matter used while teaching about vaccine-preventable diseases matters. In the class with extensive discussion of the diseases themselves, there was a strong increase in vaccine attitudes among vaccine-hesitant students, while this effect was not seen in the class that discussed mostly vaccine safety. Taken together, these findings indicate that increasing familiarity with vaccine-preventable diseases leads to improved attitudes towards vaccination. This should help to create solutions to the worldwide problem of vaccine hesitancy or denial, by indicating aspects of education that are important for affecting those attitudes.

CHAPTER 5: Conclusions and Future Directions

The work presented in this dissertation summarizes research focused on understanding 1) T cell activation 2) the contribution of autoimmune disorders to viral incidence and cancer, and 3) opinions about vaccine hesitancy. Individual sections for these projects address concluding remarks and future directions for each of these three areas of research.

5.1 Helper T cell receptor affinity influencing T cell activation

Clinical research studies have focused mainly on T cell receptors (TCRs) expressed in killer T cells to directly eliminate cancer cells [142, 389]. Helper T cells can promote both indirect and direct immune responses to cancer, playing a key role in preventing tumor antigen escape [388] and may be less toxic against overexpressed tumor associated antigens [146]. Helper T cell activation is influenced by a confluence of factors including TCR-peptide:major histocompatibility complex II (TCR-pMHCII) affinity, coreceptor expression, and immunological conditions such as the proximal cytokine milieu [11, 36, 49, 50]. Here we described work testing the hypothesis that increased helper TCR-pMHCII affinity promotes T cell activation and that CD4 expression would modulate IL-2 expression in both full length TCR (flTCR) and TCR-single chain signaling chimeric antigen receptors (TCR-SCS CARs). We determined that while increased TCR-pMHCII affinity does promote increased T cell activation, CD4 expression moderated or abrogated IL-2 production for intermediate and high affinity constructs. While the main inhibitory mechanism was Lck sequestration by CD4, CD4 was independently inhibitory at intermediate affinity for full length TCRs and at high affinity for TCR-SCS CARs. This work revealed several important T cell activation phenotypes and more work is needed to understand the mechanisms involved. Below are a series of questions that could be addressed in the future:

1. *How does increased TCR affinity in helper T cells alter novel downstream phosphorylation cascades?* Much is known about how increased TCR affinity affects killer T cell activation, yet increased TCR affinity in helper T cells may alter novel downstream phosphorylation cascades, affect helper T cell persistence and apoptosis rates, alter T cell subset fate and the production of other cytokines such as IFN-γ, IL-4, and IL-10. These traits could be measured in murine T cells ($CD4^+$ and $CD4^-$) transduced with high affinity TCRs through: A) measuring *in vitro* common signaling phosphorylation amounts on CD3ζ and ERK (a measure of T cell activation readiness) by phospho-flow or western blot; B) using flow cytometry to measure *in vivo* rates of T cell proliferation and apoptosis; and C) stimulating T cells with various LLO peptide concentrations and measuring cytokine output with a BD bead cytometric array to determine the T_h subtype.
2. *How does our high affinity TCRs affect T cell efficiency clearing Listeria monocytogenes infection in established in vivo mouse models?* This could be measured by injecting $CD4^+$ or $CD4^-$ murine T cells transduced with high affinity TCRs into mice, followed by *L. monocytogenes* injection, and then plating dilutions of the infected spleens at Day 3 post-infection to count bacterial colonies. For controls, mice injected with our transgenic WT LLO56, LLO118, or no T cells would be assessed for their bacterial clearance. Mice would be closely monitored for physical signs of adverse reaction. During a pre-experiment check, all TCR-transduced T cells would screened for reactivity to self-peptide by incubating them with splenocytes from C57/Bl.6 mice and measuring IL-2 production and T cell proliferation by CFSE flow cytometry. Alternatively, T cells transduced high affinity TCRs *in vivo* effectiveness could be measured in reaction to tumor cells engineered to express LLO peptide.

3. *What is the mechanistic root of the Lck-independent and CD4-dependent IL-2 production inhibition? Does CD4 have an activation regulatory role?* A CD4-specific inhibition phenotype has been noted in literature [64], but the mechanism itself has not been described. Furthermore, constructs expressed in the Lck-free T cell hybridoma line, $58^{-/-}$ $CD4^{-}$, had massive amounts of non-peptide-specific constitutive IL-2 production, indicating that a regulatory mechanism was disabled by the absence of CD4. It is possible that CD4 is more of a negative regulator in peptide-specific activation than previously understood. An easy experiment would be to sort our T cell hybridoma clones for high and low expression of TCR-SCS to see if constitutive activation is TCR-SCS concentration dependent. We could also sort T cells with stringent TCR expression into $CD4_{low}$ and $CD4_{high}$ populations for each of our $CD4^{+}$ clones to see if CD4 expression dictated constitutive IL-2 production and the observed activation phenotypes.
4. *How does CD4T binding pMHCII enhance activation when it cannot interact with Lck directly? Can we replicate these findings with MHCII (I-A^b) that have mutated CD4 binding domains?* We noted CD4T, an intracellular truncated form of CD4, enhanced the activation of low affinity flTCRs and intermediate affinity TCR-SCS. This is surprising, because CD4T cannot interact directly with Lck to phosphorylate CD3 ITAMs. It is therefore likely that CD4T provides a structural help by perhaps prolonging the contact between MHCII and the TCR. Additionally, it is possible that the CD4T truncated form adversely affected CD4 function. Thus repeating the experiments with splenocytes that have I-A^b molecules that do not bind CD4 would clarify whether CD4T provides structural support to improve activation *and* whether CD4 truncation altered our initial observations about CD4 extracellular domain inhibition.

5. Would expressing high affinity MHCII-specific TCRs in CD4$^-$ cell lines improve immune responses? As CD4 expression in helper T cells abrogates IL-2 production for intermediate flTCR and high affinity TCR-SCS CARs, expressing the constructs in alternative cells lines such as CD8$^+$ killer T cells or natural killer cells (NK) may produce novel immunological responses. CD8 does not bind Lck as well as CD4 [471], thus more free-Lck may be available to promote TCR-MHCII activation where CD8 would be excluded. CD8 may also act as a dominant-negative inhibitor when it is not recruited to the TCR-MHC complex [62]. Additionally, helper TCRs transduced into CD8 T cells may promote more cytotoxic activity against MHCII$^+$ tumors [389]. CAR T cells can have substantial toxic effects to patients. CAR-NK cells have been shown to have reduced cytokine release syndrome, neurotoxicity, graft-versus host disease, and maintain baseline levels of inflammatory cytokines such as IL-6 [472-474]. Natural killer cells express both CD4 and Lck [475, 476], thus it is unknown whether NK cells transduced with helper TCRs would maintain the gains seen in CAR NK cells or would have abrogated or altered immunological responses.

5.2 Future directions SLE-virus promoting cancer risk

Systemic lupus erythematosus (SLE) and viruses have a reciprocal relationship. Not only do viruses promote and exacerbate SLE onset [251], viral infection can also promote the development of certain cancers such as hematological malignancies [289-291], cervical cancer and liver cancer [273, 274]. In our study we demonstrated that SLE patients have an increased risk for viral infections and these infections interacted with SLE to promote hematological and overall cancer development. We also demonstrated that SLE and Multiple sclerosis (MS) uniquely affect cancer development. While SLE by itself did not promote cancer development, MS may increase overall cancer risk. There were some limitations to this study, including that

our cohort was too small to detect enough HPV and hepatitis ICD-9/10 codes and lab codes to assess the contribution of those viruses to cervical cancer and liver cancer, respectively. We also did not have enough viral tests in our MS cohort to detect viral patterns for this autoimmune disorder. Therefore, follow up studies could include the following:

1. *Can we improve the robustness of our results with a larger cohort?* Because we had so few viruses reported in the MS cohort, and too few incidences of rare cancers, repeating the study with a larger cohort of SLE and MS patients could improve the power of these rare incidences and expand our predictive capabilities.
2. *Do multiple risk factors for hematological cancers contribute to cancer risk independently or concomitantly?* The improved cohort could also be used to investigate whether there are interactions between three SLE patient hematological cancer development risk factors: cyclophosphamide, glucocorticoid use, or EBV activity [274]. It is possible that the immunosuppressive drugs increase EBV activity thereby increasing cancer risk or that they are separate risk factors for hematological malignancy development. This kind of work could inform clinicians about which patients are at risk for adverse cancer events, and might uncover ways to create preventative screening tools to identify at-risk patients prior to prescribing cyclophosphamides and glucocorticoids.
3. *Do vaccination recommendations improve patient cancer rates and survival?* At the end of our paper, we recommended preventative vaccines such as the HPV vaccine (Gardasil) for SLE patients to prevent oncological viral infections. Again, a longitudinal electronic medical records study may be an excellent way to follow up on whether this recommendation decreases cervical cancer incidence in SLE patients.

4. *Could a mouse model help uncover the links between SLE, EBV and hematological cancer incidence?* This is a whole grant in and of itself. Developing this model requires choosing the correct lupus murine model and murine virus as mice cannot be infected with EBV. However, this model could reveal important triggers for cancer development that could lead to therapeutics or models of care for at-risk SLE patients.

5.3 Future directions vaccine hesitancy

Vaccine hesitancy is a complex and variable problem driven by factors that change over time, place and vaccine [311]. There is no "one size fits all" solution. Confronting inaccurate vaccine information directly often causes further entrenchment in erroneous beliefs [346, 347]. Children and adolescents may be more likely to influence their family's current and future behaviors [373-375]. In this paper, we sought to educate a specific young adult population with shared cultural values (BYU undergraduates) about the importance of vaccines by teaching them about vaccine preventable diseases (VPDs) through classroom instruction or interviews with community members. We found that students who either interviewed a community member about a VPD and received no education about vaccines, or students who received education about VPDs and the immune system significantly improved their vaccine attitude scores (VAS). Students that interviewed a community member about an autoimmune disorder or received vaccine-safety education did not improve their before and after VAS. This work has led to the following questions that would be important to address in the future.

1. *Did the intervention have longevity? Will it improve vaccine rates for the students and their families over time?* To answer these questions, the study would need to be extended with follow-ups over 5-10 years tracing students and their children from birth-kindergarten. We could also observe the whether the students themselves choose adult vaccinations such as

yearly influenza vaccines or the HPV vaccine. If there is no persistent effect, it is possible that an intervention like this could be used in the short-term to encourage students to get influenza vaccines or HPV vaccines.

2. *Would an interview-based intervention work for a non-science course like a history or English course? Are there course topics such as discussing the Black Plague in a European history course or consumption (tuberculosis) in Gothic literature that would complement the intervention and improve its efficacy?* Designing a second study to investigate this would include controls such as running the original study in science courses as positive controls, in addition to deploying different interventions in non-sciences course sections taught by the same instructor. These interventions would include the interviews compared to sections that receive a few lectures discussing either vaccine safety or VPD/immunology in a way that complements the usual course content. The interview sections could be designed to support class goals as either a writing project for an English or journalism course or as a tool to teach interviewing skills to history students.
3. *Would stories based on VPD-interviews make an effective intervention in a doctor's office and increase vaccine uptake by patients?* Previous research using stories from the CDC about a child sick with measles had mixed results [347]. Could this be because the CDC story is about a child told in the third person? Are stories told retroactively in the first person more effective? Is it more effective to have video clips vs written stories or does the interaction need to be live with someone the listener knows well? A study could be designed with a set story told in multiple perspectives from third person to first person using active to retroactive language. These stories could be deployed in doctors' offices and administered randomly to patients with children due for shots. As clinician use of presumptive language

and conversational language has a decided effect on patient vaccine uptake [370], clinicians would need to use a set script. The vaccine uptake could be tracked and compared across treatments and between doctors' offices.

4. *What content creates stories that change VAS?* In our study, we noted that severe physical suffering and limitations to normal activity were effective at changing minds. We could generate a panel of stories with increasing pain or limitations and measure the effect on VAS for a variety of participants including students and parents. This could potentially be deployed in clinics in a similar manner as discussed above.

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APPENDIX I: Journal of Immunology Abstracts

The following appendix contains two published abstracts from the Journal of Immunology: **Abstract 1:** Johnson, D. J., Myers, S., Magoffin, W., Orton, T., Tellez-Frietas, C.M., Christensen, K., and Weber, K. S. Effects of high affinity engineered class II-restricted full length and single chain CAR-format T cell receptors specific for a naturally occurring Listeria monocytogenes epitope on T cell activation and specificity. *The Journal of Immunology* 202, 196.16 (2019). The data for this abstract was presented at the American Association of Immunologists (AAI) Annual Meeting in San Diego, CA, 2019.

Abstract 2: 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 1: *Effects of high affinity engineered class II-restricted full length and single chain CAR-format T cell receptors specific for a naturally occurring* Listeria monocytogenes *epitope on T cell activation and specificity*

High affinity T cell receptors (TCRs) are absent from the native T cell repertoire. While current hypotheses propose that high affinity TCRs are more sensitive to antigen and have a competitive advantage in immune responses, emerging evidence suggests that higher affinity T cells may reach a threshold where they experience decreased functionality, loss of binding specificity, and become prone to anergy. High affinity TCRs are especially attractive to facilitate immunotherapies as native TCR binding affinity may be too low to effectively initiate activation. However, the effects of increased TCR affinity for $CD4^+$ T cells specific for naturally occurring epitopes is poorly understood. To address this question, we engineered via yeast display several

class II-restricted high affinity TCRs specific for a naturally occurring peptide from *Listeria monocytogenes* protein listeriolysin O. Our high affinity clones have K_D values as low as 8.7 nM and half-lives as long as 174 minutes when measured by tetramer dissociation assays. This panel of class II restricted high affinity TCRs specific for a naturally occurring *Listeria monocytogenes* epitope provides a novel means of testing the role of affinity and $CD4^+$ T cell activation responses in the context of an infection.

Abstract 2: *CD5 Expression Influences T cell Metabolism and Mice Behavior*

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 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.

APPENDIX II: Presentations

Presentations

1. "Contradictory CD4: an inhibitory activating T cell co-receptor." *BYU Department of Microbiology and Molecular Biology Annual Retreat*, Provo, UT; Aug 2019

2. "Combating vaccine hesitancy with vaccine preventable disease familiarization: An interview and curriculum intervention for college students." *American Society for Microbiology Intermountain branch meeting*, Provo, UT; 13 Apr 2019.

3. "Role of affinity for antigen in T cell activation and memory generation." *American Society for Microbiology Tri-branch meeting*, Durango, CO; 7 Apr 2018

4. "The role of CD5 in helper T cells in activation and metabolism." *Autumn Immunology Conference*, Chicago, IL; 19 Nov 2017.

5. "The sky is the limit: Detecting activation thresholds for high affinity T cell receptors." *Simmons Center for Cancer Research Summer Final Seminar Series*, Provo, UT; 11 Aug 2017

6. "T cell receptors specific for a naturally occurring *Listeria monocytogenes* epitope engineered in vitro for higher affinity." *American Society for Microbiology Intermountain branch meeting,* Ogden, UT; 15 Apr 2017.

7. "Turbocharged: The daring and intense pursuit to harness the immune system's serial killers to fight cancer (a snapshot)." *BYU Department of Microbiology and Molecular Biology Annual Retreat*, Provo, UT; 25 Aug 2016

8. "'Turbocharging' the immune system to fight cancer." *Simmons Center for Cancer Research Summer Final Seminar Series*, Provo, UT; 13 Aug 2016

9. "Time Management for Undergrads." *BYU Phage Phield Day (Phage Hunters Conference),* Provo, UT; Feb 2016.

10. "A Tale of Two T cells: The effects of affinity, cautionary co-receptors, and injurious insertions." *BYU Department of Microbiology and Molecular Biology Annual Retreat*, Provo, UT; Aug 2015

11. "Engineering chimeric antigen receptors to target infectious disease." *Simmons Center for Cancer Research Summer Final Seminar Series,* Provo, UT; 12 Aug 2015

Posters

1. **Deborah Johnson,** Emily Mello, Trent Walker, Spencer Hood, Jamie Jensen, and Brian Poole, "Improving attitudes towards vaccination by emphasizing vaccine-preventable diseases," *Precisions Vaccines, Boston Children's Hospital,* Boston, MA, Oct 2019.
2. **Deborah Johnson**, Wyatt Magoffin, Jordan Finnell, Sheldon Myers, Becca Nemrow, Ken Christensen, and K. Scott Weber, "Effects of high affinity engineered class II-restricted full length and single chain CAR-format T cell receptors specific for a naturally occurring *Listeria monocytogenes* epitope on T cell activation and specificity," *American Association of Immunologists*, San Diego, CA, May 2019.

3. Emily Mello, **Deborah Johnson,** Trent Walker, Spencer Hood, Jamie Jensen, and Brian Poole, "Effectiveness of Interviewing Persons Affected by Vaccine-preventable Diseases as an Intervention for Vaccine Aversion," *Utah Conference on Undergraduate Research*, Ogden, UT, Feb 2019.

4. **Deborah Johnson,** Claudia Tellez, John Hancock, Josie Tueller, Sheldon Myers, Carlee Raymond, Garrett Hamblin, and Scott Weber, "CD5 expression influence helper T cell metabolic state," *Autumn Immunology Conference*, Chicago, IL, Nov 2017.

5. **Deborah Johnson,** Kiara Vaden, Claudia Tellez, Sheldon Meyers, Becca Nemrow, and Scott Weber, "Role of affinity for antigen in T cell activation and memory generation," *LDS Life Sciences Symposium*, Lehi, UT, July 2016. **First Prize Winner*

6. **Deborah Johnson,** Bryce E. Anderson, Kemais Ehlers, Stephen Persuad, and Scott Weber, "Engineering high affinity T-cell receptors specific for *Listeria monocytogenes*," *Midwinter Immunologist Conference*, Monterey, CA; 24-27 Jan 2015

APPENDIX III: Compiled Publications

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

vaccines

MDPI

Article

Combating Vaccine Hesitancy with Vaccine-Preventable Disease Familiarization: An Interview and Curriculum Intervention for College Students

Deborah K. Johnson [1], Emily J. Mello [1], Trent D. Walker [1], Spencer J. Hood [1], Jamie L. Jensen [2] and Brian D. Poole [1,*]

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Abstract: In 2019, the World Health Organization (WHO) listed vaccine hesitancy in its top ten threats to global health. Vaccine hesitancy is a "delay in acceptance or refusal to vaccinate despite availability of vaccination services". Urban areas with large amounts of vaccine hesitancy are at risk for the resurgence of vaccine-preventable diseases (VPDs). Many vaccine-hesitant (VH) parents may be unfamiliar with the consequences of VPDs, and thus might be swayed when confronted with the symptoms and dangers of VPDs. As such, we sought to educate college students (future parents) in an urban vaccine-hesitant hotspot by assigning them to interview family or community members who had experienced a VPD. Student vaccine attitudes were assessed by surveys before and after the interviews. Vaccine-hesitant students who conducted a VPD interview but received no additional vaccine educational materials were

significantly more likely (interaction term $p < 0.001$) to become pro-vaccine (PV) (68%) than students who conducted an autoimmune interview and received no additional educational materials. Additionally, students whose interviewees experienced intense physical suffering or physical limitations or students who were enrolled in a course with intensive VPD and vaccine curriculum had significantly increased vaccine attitudes. This suggests that introducing students to VPDs can decrease vaccine hesitancy.

Keywords: vaccine hesitancy; college student; vaccine; vaccine curriculum; interview intervention for college students (future parents) in Provo, Utah might help improve vaccine attitudes and future vaccine uptake for themselves and their families.

Introduction

Vaccines are victims of their own success. Due to the effectiveness of vaccination programs, many people have limited or no experience with vaccine-preventable diseases (VPDs) [1]. Parents increasingly assume that the risks associated with VPDs are minimal compared to potential health and safety risks of vaccinations themselves [2–5]. This has led to a rise in vaccine hesitancy by parents that results in a "delay in acceptance or refusal to vaccinate despite availability of vaccination services" [6]. Urban centers with large clusters of vaccine-hesitant individuals are particularly vulnerable to VPD outbreaks among exposed, unimmunized children. In the 2016–2017 school year, Utah County (Provo) in Utah, USA ranked sixth nationally for the total number of entering kindergartners that were under-vaccinated as measured by non-medical exemption (NME) waivers (n = 662 NME) [7]. As many of these parents may have never experienced VPDs, we hypothesized that designing an intervention Influencing students before they become parents will likely encourage pro-vaccination behaviors for their future and current families, as children and adolescents who participate in health

education activities in school can positively influence family health management [8–10]. However, there is evidence that correcting erroneous assumptions about potential health and safety risks may be ineffective, causing a "backfire effect" and further entrenching vaccine-hesitant individuals' beliefs [11,12]. Vaccine hesitancy often arises from deep-rooted ideological beliefs and conspiracist ideational tendencies. As this kind of thinking has been shown to become further entrenched when those holding it are presented with contradictory information, correcting misinformation is often counterproductive [13–15]. Further, it is difficult to provide convincing data for the absence of risk; consequently, vaccine-hesitant parents may be recalcitrant to messages aimed at alleviating concerns about vaccine safety and side effects [16,17]. Rather, pro-vaccine interventions may be more effective if they warn of health dangers to individuals from VPDs [17]. Additionally, the vaccine-hesitant movement uses emotionally charged stories with dire long-term consequences to effectively convey anti-vaccine ideology. Combating this rhetoric with a similarly emotional appeal may be an effective preventative strategy [18]. Therefore, we predicted that hearing about the effects of VPDs from family and community members who suffered from VPDs would improve the students' attitudes towards vaccination. We further predicted that classroom education could improve attitudes towards vaccines.

In this study, we analyzed three courses with different vaccine instructional approaches varying from none to intensive. Enrolled students were also assigned to interview a family or community member about their personal experience with either an autoimmune disease (control) or a VPD. Students exposed to either intensive VPD-focused vaccine instruction or who interviewed individuals who had had a VPD had statistically significant and meaningful gains in vaccine attitude.

2. Methods

2.1. Study Population

Students at a large private institution in the Western United States were enrolled in a quasi-experimental survey-based intervention study. Students were eligible if they attended one of three courses in the Winter 2018 semester: General education non-science major Bio 100 (Principles of Biology, one section), and microbiology and molecular biology (MMBio) major-specific courses MMBio 240 (Molecular Biology, two sections) and MMBio 261 (Infection and Immunity, one section). Vaccination principles were taught in Bio 100 and MMBio 261, but not in MMBio 240. All students enrolled in the courses were encouraged to participate and offered extra credit for their time and efforts. Our study sample consisted of 425 students who completed the study requirements (574 began the study). The study was conducted in accordance with the Declaration of Helsinki, and study procedures were approved by the Institutional Review Board at the institution (study #E17263). All participants received and signed a consent form that included a description of the study and were assigned a random number to protect their identities. Students were informed that their instructors would never see their names associated with any of the survey results, and steps were taken to avoid such instructor access.

2.2. Assigning Vaccine Attitude Groups and Randomization Process

To determine initial vaccine attitudes, students took a pre-interview survey (see Box 1) and were asked to rate each question from 1–5 where 1 is “strongly disagree” and 5 is “strongly agree”. Each question concerning vaccine attitude was chosen to cover a specific aspect of vaccine hesitancy. Pre-intervention vaccine attitude scores (VASs) were tallied from questions 1, 4, 9, 11, and 13 (Box 1). Question 1 is a test of general attitude towards vaccines. Question 4

addresses side effects, relevant since many vaccine-hesitant individuals are afraid of these side effects. Question 9 is about the common belief that vaccines cause autism, a major concern for many vaccine-hesitant people. Question 11 gives an opportunity for the participants to opine on the positive aspects of vaccination in terms of how well they work. Question 13 is a question about how the vaccine attitude would affect action and give it a more real-world, rather than theoretical, effect. To avoid answer bias, students were not informed that the study was about vaccination opinions and additional questions about autoimmune diseases and depression were included in the survey. Scores from questions 4 and 9 were reverse coded to account for the negative nature of the question. These questions were written in a negative way to avoid biasing the study by presenting vaccines in only a positive light in the questions. Students with VASs between 20 and 25 points were categorized as "Pro-Vaccine" (PV) and students that had a VAS less than 20 points were categorized as "Vaccine-Hesitant" (VH). A cutoff value of 20 was chosen because it meant, on average, that the student at least "agreed" (score or converted score of 4) with all of the vaccine attitude questions. A score of less than 20 would mean that the student on average either did not at least "agree" with the pro-vaccine statements, or that they had a serious disagreement with at least one statement about vaccines. Students were assigned to interview groups by alternating autoimmune (negative control for survey and interview effects) and vaccine-preventable disease (VPD) interview assignments alphabetically within PV and VH categories so that equal numbers of students were assigned to each interview intervention.

Box 1. Pre-interview survey.

Rate each question from 1–5 where 1 is "strongly disagree" and 5 is "strongly agree"

1. Vaccines are more helpful than harmful
2. Treatment for autoimmune diseases is more helpful than harmful
3. Medications for depression are more helpful than harmful
4. Vaccines often have severe side effects
5. People with autoimmune diseases suffer considerably
6. Medication for depression is effective at treating depression
7. There are effective treatments for autoimmune diseases
8. Depression can be overcome using willpower
9. Vaccines cause autism
10. Exercise is the best treatment for autoimmune diseases
11. Vaccines are effective at preventing disease
12. Medications for depression have severe side effects
13. I am likely to fully vaccinate my children/I have fully vaccinated my children

Students were emailed their survey assignments and related paperwork, and based on their group assignment, were asked to interview members of the community who had experienced either a VPD or an autoimmune disease before the end of the semester using the interview questions shown in Box 2. To encourage study completion, students received full points for extra credit with completed survey submission (Bio 100 10 points, 1% of grade; MMBio 240 20 points, 2.3% of grade; MMBio 261 20 points, 2.3% of grade). At the end of the semester, students were administered a post-interview survey (see Box 3) that reiterated the pre-interview survey questions and included follow-up questions about the survey itself. These questions (14–18 in the post-interview survey) were written to identify the aspects of the interview that had the most significant impact on vaccine attitudes. They provided both an opportunity to rank several factors and the ability to explain in their own words how the various aspects of the interview affected them. We then assigned students a post-intervention VAS and assessed for changes in overall vaccine scores between the pre-and post-intervention surveys. Included in this analysis was determining whether students moved from the vaccine-hesitant to pro-vaccine group, or vice-versa.

Box 2. Interview questions.

1. What is your relationship to the person who had the disease?
2. When did they develop the disease?
3. Which disease was involved?
4. What type of physical suffering did the disease cause? How bad was it?
5. How did the disease limit the person's ability to do normal activities?
6. How did the disease affect the person's interaction with other people?
7. How did the disease affect the person's friends, family, or loved ones?
8. How did the disease affect the person financially?
9. Were there any other effects of the disease?

Box 3. Post-interview survey.

1. Vaccines are more helpful than harmful
2. Treatment for autoimmune diseases is more helpful than harmful
3. Medications for depression are more helpful than harmful
4. Vaccines often have severe side effects
5. People with autoimmune diseases suffer considerably
6. Medication for depression is effective at treating depression
7. There are effective treatments for autoimmune diseases
8. Depression can be overcome using willpower
9. Vaccines cause autism
10. Exercise is the best treatment for autoimmune diseases
11. Vaccines are effective at preventing disease
12. Medications for depression have severe side effects
13. I am likely to fully vaccinate my children/I have fully vaccinated my children

    Complete next section only if you interviewed a VPD-subject

    Circle an answer: much more opposed, slightly more opposed, no effect, slightly more in favor, much more in favor

14. How did hearing about the subject's physical suffering affect your opinion of vaccines?
15. How did hearing about how the disease limited normal activity affect your opinion of vaccines?
16. How did hearing about how the disease affected the subjects' interactions with other people affect your opinion of vaccines?
17. How did hearing about how the disease affected the subject's family, friends, or loved ones affect your opinion of vaccines?
18. How did hearing about the disease's financial impact on the subject affect your opinion of vaccines?

    Rank the following:

    (a) Physical suffering
    (b) Limitation of activities
    (c) Interactions with other people
    (d) Effect on family, friends, or loved ones
    (e) Financial impact

19. Please explain briefly, what effect, if any, the project had on your attitude towards vaccination and why it has that effect.
20. If this interview did NOT affect your attitude towards vaccination, why not?

2.3. Analyses

Changes between groups' pre- and post-intervention VASs were assessed with factorial ANOVAs. Individual group changes over time were assessed by paired sample *t*-tests, and differences between two groups at specific time points were assessed by independent sample *t*-tests. Bonferroni corrections were applied to any multiple comparisons to account for alpha inflation. Standard deviations were reported for statistics less than 5 points. All other statistics reported 95% confidence intervals (CI).
All analyses were performed using SPSS Statistics 25 (IBM). Figures were generated in Prism 8 (GraphPad) and tables were generated in Excel 2016 (Microsoft).

3. Results

3.1. Overview and Pre-Interview Intervention Vaccine Attitudes

A total of 574 students volunteered to take the pre-interview survey during the Winter 2018 semester. Based on their pre-intervention vaccination attitude scores (VASs), students were designated either pro-vaccine (PV) (87%) or vaccine-hesitant (VH) (13%) and assigned to the control group (autoimmune survey, $n = 286$) or the intervention group (vaccine-preventable disease (VPD) survey, $n = 288$). Of the students, 74% ($n = 425$) completed all requisite parts of the study (pre-interview survey, community/family interview, post-interview survey) and were included in the final analysis (Figure 1). The VH group was defined as VAS < 20 and the PV group was defined as VAS ≥ 20 based on the pre-interview survey responses. There were no statistically significant differences in the sociodemographic characteristics among the classes nor did the course they were enrolled in significantly affect the assignment to VH and PV groups (Table 1). Course year explains the age difference between the courses: Bio 100 is a general

education course for first year students, MMBio 240 is a second-year major-specific course, and MMBio 261 is a second to third year major-specific course. Furthermore, sex, race, and age were not significantly correlated with pre-intervention vaccine attitudes (Table 2). Student willingness to vaccinate current/future children was significantly different between VH and PV groups (scale of 1–5 from strongly disagree to strongly agree) with means of 3.84 and 4.92, respectively (independent *t*-test CI 95% 0.814–1.355; $p < 0.001$).

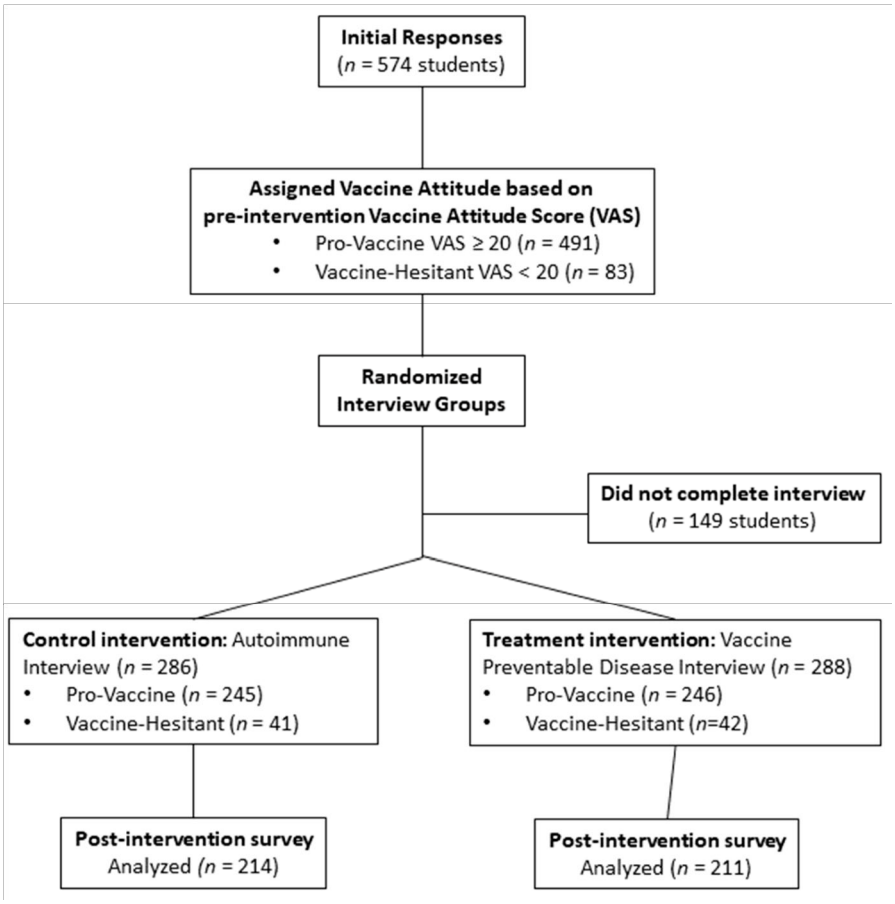


Figure 1. Participant flow through the randomized treatment.

Table 1. Baseline characteristics of the participating classes ($n = 425$).

Class Demographics	Total % (*n*)	Bio 100 % (*n*)	MMBio 240 % (*n*)	MMBio 261 % (*n*)
Total	100% (425)	13% (56)	70% (298)	17% (71)
Gender Male	62% (263)	70% (39)	61% (182)	59% (42)
Female	38% (162)	30% (17)	39% (116)	41% (29)

Age	21.2 ± 0.21	19.7 ± 0.50	21.3 ± 0.24	22.1 ± 0.40
Pre-Intervention Vaccine Attitude Score				
Vaccine-Hesitant	13% (56)	18% (10)	14% (41)	7% (5)
Pro-Vaccine	87% (369)	82% (46)	86% (257)	93% (66)

There are no statistically significant differences among the classes for gender distribution, age, or Pro-Vaccine or Vaccine-Hesitant group assignment.

Table 2. Baseline characteristics of vaccine-hesitant and pro-vaccine groups (n = 425).

Sociodemographic Characteristic	Total % (n)	Vaccine-Hesitant % (n)	Pro-Vaccine % (n)
Total	100% (425)	13% (56)	87% (369)
Gender Male	62% (263)	14% (36)	86% (227)
Female	38% (162)	12% (20)	88% (142)
Age	21.2 ± 0.21	21.0 ± 0.74	21.3 ± 0.21
Race/Ethnicity African American	1% (3)	-	100% (3)
Asian	3% (11)	27% (3)	73% (8)
Caucasian	87% (370)	13% (48)	87% (322)
Hispanic	3% (12)	17% (2)	83% (10)
Native American	0.2% (1)	100% (1)	-
Other	6% (26)	8% (2)	92% (24)

There were no statistically significant differences in ethnicity, gender, or age between vaccine opinion groups.

3.2. Interview Intervention Improves Student Vaccine Attitude Scores

Vaccine attitudes improved when the participants gained a personal understanding of how vaccine-preventable diseases affect individuals and communities. Vaccine-hesitant students enrolled in MMBio 240 (no vaccine curriculum) who were part of the intervention group (n = 19) showed a significant increase in VAS; average VAS shifted from 17.58 ± 0.84 to 20.53 ± 0.94 (paired t-test CI difference (diff) 95% 4.077–0.817; $p < 0.001$), an average increase of 2.95 ± 2.34 points (Figure 2). Of these students, 68% (n = 13) had sufficient increases in their VASs to move from the vaccine-hesitant group to the pro-vaccine group. Conversely, vaccine-hesitant students who were part of the control group (n = 22) had no significant increase in VAS (paired t-test CI diff 95% 1.856–0.038; p = 0.059) which shifted only 17.27 ± 0.87 to 18.18 ± 1.31, an

average increase of 1 ± 2.05 point (p = 0.059). Only 27% (n = 6) of students in the control group increased their scores sufficiently to move from the vaccine-hesitant group to the pro-vaccine group. Post-intervention VASs are significantly different between control and intervention VH groups (independent t-test CI diff 95% 4.066–0.623; p = 0.009), whereas post-intervention VASs are still significantly different between VH and PV students in the intervention group (independent t-test CI diff 95% 3.702–1.733; p < 0.001). α = 0.0125.

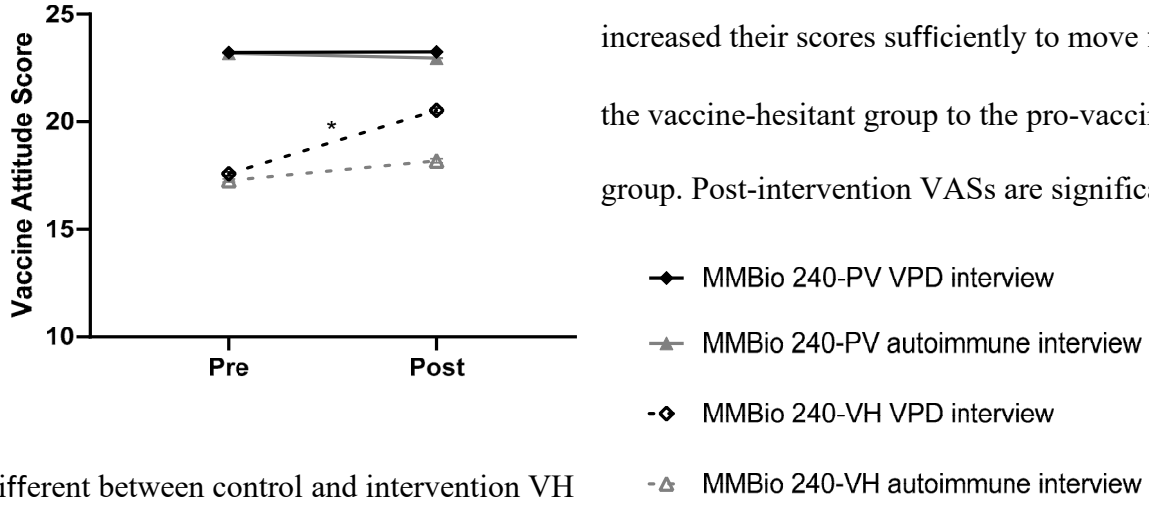


Figure 2. Vaccine-preventable disease interview significantly improves attitudes towards vaccines. Treatment makes a significant difference (interaction term p < 0.001) for vaccine-hesitant (VH) students in MMBio 240.

3.3. Vaccine Education Likely Improves Student Vaccine Attitudes

Intensive vaccine education may be even more effective at improving vaccine attitudes than interviewing individuals who have had a VPD. All vaccine-hesitant students (n = 5) enrolled in MMBio 261 (intensive immune, VPD, and vaccine education) significantly increased their VASs by

7.00 ± 1.41 points on average regardless of survey intervention (p < 0.001), (pre-control group MMBio 261 VH mean 16.50, CI 95% 14.41–18.59; post-control group VH mean 23.500, CI 95% 12.616–25.384; pre-intervention group VH mean 14.000, CI 95% 12.29–15.71; post-intervention group VH mean 21.00, CI 95% 19.46–22.54) (Figure 3a). For all VH students, including intervention and control groups, the pre-intervention VAS mean was 15.00 ± 2.06 and

the post-intervention VAS mean was 22.00 ± 2.23, decidedly in the pro-vaccine range. Four out of five VH students increased their VASs sufficiently to move from the vaccine-hesitant category to the pro-vaccine category with an average increase of 7.50 ± 1.00 points. The final student, who participated in the intervention group, increased their VAS from 13 to 18 points, an increase of 5 points. There is a significant difference between pre- and post-intervention group MMBio 261 VH students ($p = 0.026$, $n = 3$ paired t-test CI diff 95% 11.97–2.03). Statistics cannot be run across time between pre- and post-control group VH students since there are a low number of respondents ($n = 2$). Although these results are promising, large in magnitude, and statistically significant, they are based on a small number of vaccine-hesitant students in the class ($n = 5$). Furthermore, students in MMBio 261 are majoring in a life sciences degree and may be more prone towards persuasion by scientific reasoning than the non-major students in the general education Bio 100 course.

To highlight the need for tailored and intensive vaccine education, vaccine-hesitant students in Bio 100 had a non-significant yet distinct upward trend over time regardless of survey intervention (Figure 3b). Overall, VASs do significantly change across time ($p = 0.036$) and vaccine attitude/survey groups ($p < 0.001$). All students in Bio 100 received brief instruction on how vaccines work, the rarity of vaccine side effects, the benefits of herd immunity to society, and no specific conversation about VPDs. The average VH student increased 1.9 ± 2.37 points between pre- and post-intervention VASs. There is no significant difference after the survey intervention between post-control group and post-intervention group VH students (independent t-test CI diff 95% 3.47–7.47; $p = 0.42$), or between post-control group PV and post-control group VH students (independent t-test CI diff 95% 8.32–1.57; $p = 0.136$). There is a significant

difference after survey treatment between post-intervention group PV and post-intervention group VH students (independent *t*-test CI diff 95% 8.24–3.38; $p < 0.001$).

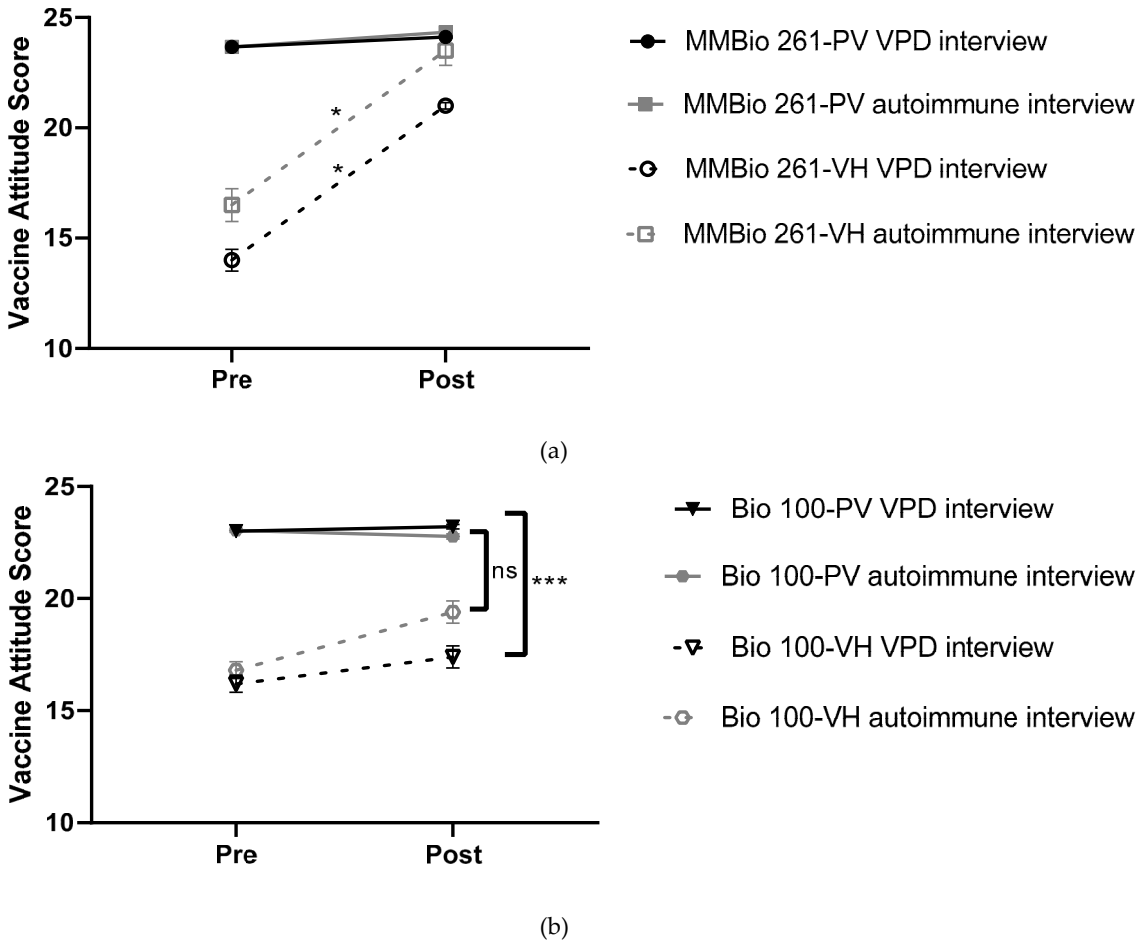


Figure 3. Education can significantly increase vaccine attitude. (**a**) Vaccine attitude scores (VASs) of MMBio 261 vaccine-hesitant students significantly increased regardless of survey intervention ($p < 0.001$), Difference between pre-control group VH and pre-intervention group MMBio 261 VH students is not significant (CI diff 95% 4.72–9.71; $p = 0.35$). (**b**) While there is an upward VAS trend for all Bio 100 VH students, it is not significant, suggesting that education has more influence than intervention.

3.4. Vaccine-Hesitant Students' VAS Change Dependent on Pre-Intervention VASs and Class

This intervention focuses on the vaccine attitudes and responses of vaccine-hesitant students to an interview intervention. To better understand what aspects of the interview intervention positively influenced VH students, we focused on analyzing the scores of VH students by comparing pre- and post-intervention VASs. Overall, most VH students (75%) have increased

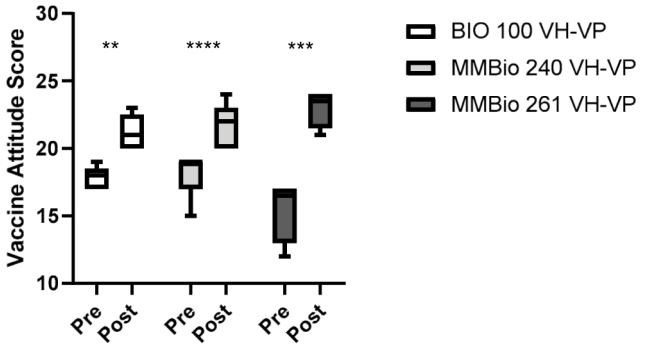
VASs, while 50% of all VH students advance to PV scores by the end of the study (Table 3). This gain, however, depends on class or interview group as previously described. For example, interview group determines the fate of MMBio 240 VH students but not Bio 100 VH students (Table 3). Class enrollment predicts pre- to post-intervention VAS changes (Figure 4a). MMBio 261 students have the greatest increase (7.50 ± 1.00), while students in Bio 100 and MMBio 240 had similar gains (3.40 ± 1.50 and 3.50 ± 2.00 points, respectively).

Yet, once students are broken into groups based on pre-intervention VASs, it becomes clear that not all VH students are alike (Figure 4b, Table 4). Students with the lowest pre-intervention VASs (11–15 points) are unlikely to become PV (n = 2.18%) and only gain an average of 2.91 ± 2.74 points ($p < 0.001$). This average is clearly defined by survey groups: intervention group students gain an average of 4.67 ± 2.65 points, whereas control group students gain an average of 0.8 ± 1.92 points. Students in this low score category who gained 5+ points (n = 4) were all part of the intervention group. This suggests that the most vaccine-hesitant students are swayed by VPD interviews. Students with middle VH pre-intervention VASs (16 or 17 points) gain an average of 4.00 ± 3.07 (p = 0.0095) and are more likely to become PV (n = 9, 60%). Overwhelmingly, students in this middle category who gained 5+ points were either in MMBio 261 (n = 3) or had conducted a VPD survey in Bio 100 or MMBio 240 (n = 4). Two students in this category were not in MMBio 261 and conducted autoimmune surveys, thus their reasons for change are not predictable. The final group of VH students with the highest pre-intervention VAS (18 or 19 points) gained the least, an average of 1.27 ± 2.02 points (p = 0.0018, n = 17.57%). As these students are near the highest range already, it is not surprising that no students gained more than 5 points as a 6 point gain places them at the top of the VAS range.

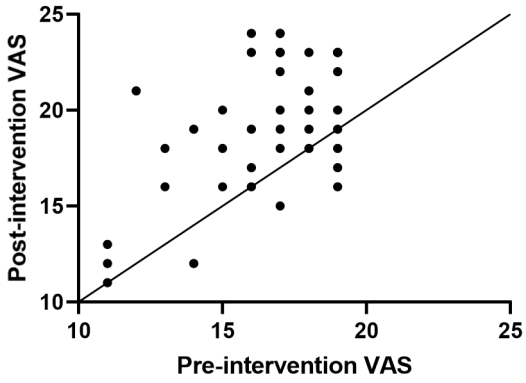
Table 3. Survey intervention and education significantly improves vaccine-hesitant student VASs.

Change Post-Treatment	Overall VH % (*n*)	BIO 100 % (*n*)	MMBio 240 % (*n*)	MMBio 261 % (*n*)
Total	56	10	41	5
VAS Increased	75% (42)	80% (8)	71% (29)	100% (5)
VAS No Change	11% (6)	0% (0)	17% (7)	0% (0)
VAS Decreased	14% (8)	20% (2)	12% (5)	0% (0)
Pro-Vaccine VAS (20+ points)	50% (28)	50% (5)	46% (19)	80% (4)

Breakdown of all vaccine-hesitant (VH) post-intervention VASs regardless of survey treatment. All students who reached a VAS of 20+ were reassigned as pro-vaccine (PV). "VAS Increased" includes students who became PV.



(a)



(b)

Figure 4. Vaccine-hesitant students make varying gains based on starting score and class attended. (**a**) VAS changes for VH students with PV post-intervention VASs. VH to PV students in MMBio 261 had an average VAS increase of 7.5 ± 1.0 points, whereas students in Bio 100 and MMBio 240 gained an average of 3.4 ± 1.5 and 3.5 ± 2.0 points, respectively. (**b**) VH students' gains are determined by pre-intervention VASs. Plotting pre-intervention VASs against post-intervention VASs for VH students shows student responsiveness is dependent on pre-intervention VASs. The line indicates no change between pre-and post-intervention scores, so the farther away from the line the larger the change.

Table 4. Numerical breakdown of all VH students by pre-intervention VAS.

Pre-Intervention VAS	11–15 Points	16 or 17 Points	18 or 19 Points
Total	11	15	30

Post-intervention VAS			
VH	82% (9)	40% (6)	43% (13)
VP	18% (2)	60% (9)	57% (17)
Change (average)	2.91 ± 2.74	4.00 ± 3.07	1.27 ± 2.02
VAS Decreased	9% (1)	7% (1)	20% (6)
Age (average)	20.9 ± 0.9	21.0 ± 0.2	21.1 ± 1.3

All VH students broken down by pre-intervention VAS. Age is not significantly different between the groups.

3.5. Vaccine-Hesitant Student Post-Intervention VAS Increase Correlated with Perceived Physical Suffering and Physical Limitations

In the post-survey interview, students in the intervention group were asked to assess how much each of the following characteristics affected their opinion of vaccines: physical suffering, limited normal activity, limited interaction with others, impact on family and friends, and financial costs (Methods, Box 3). These attributes were assessed from "strongly more opposed to vaccination" to "strongly more in favor of vaccination" and assigned the values of 1–5 points. VH students' post-intervention VASs are significantly and moderately correlated with physical suffering (4.08 ± 0.845, $r^2 = 0.405$, $p = 0.04$) (Figure 5a) and limitation on normal activities (3.88 ± 0.653, $r^2 = 0.518$, $p = 0.007$) (Figure 5b). VH students with a positive pre- to post-intervention VAS change agree or strongly agree that physical suffering is of major importance; although the amount change in VAS compared to strength of agreement is not significant, there is a visible upward trend (one-way ANOVA, $p = 0.3089$) (Figure 5c). Even more strikingly, VH students with the greatest VAS change (6–9 points, $n = 5$) strongly agree that normal activity limitations affect their vaccine opinions (one-way ANOVA, $p = 0.0206$) (Figure 5d). This suggests that VH students are more influenced by stories from VPD victims that include physical suffering and activity limitation.

3.5.1. Interview Examples Correspond to Student Perceptions of Physical Suffering and Physical Limitations

Examples of interview responses for physical suffering and physical limitations from students with the greatest VAS change (6–9 points) suggest that extreme cases enhance student response. One student interviewed a member of their church congregation who had shingles: “The pain was so bad that she ended up at a pain management clinic where they did steroid shots into her spine. The pain meds didn’t even touch [reduce] her pain, even the heavy ones. For months she couldn’t leave the house.” This interview led the student to explain (Methods, Box 3, question 25) that “The project showed how the lack of vaccination is essentially accepting the pain and suffering that comes with disease.” Another student interviewed his or her grandmother about tuberculosis: “Before getting diagnosed and during the time that she was treated, she could work her eight-hour temple shift and then she would go straight to bed after getting home. After a couple of hours nap, she would get up for a short time to get small tasks done before retiring to bed for the night.” This student summarized the interview experience as “I dislike the idea of physical suffering so hearing about someone getting a disease made the idea of getting a disease if I don’t get vaccinated seem more real.” These students both became PV
with VAS increases of 7 and 6 points, respectively.

In keeping with this idea, many VH students with smaller VAS gains generally reported less serious physical suffering and physical limitations from the people they interviewed. A student who gained 4 points and interviewed a shingles patient wrote: “She considered her case very minor and she did not suffer physically much. She had some difficulty sleeping for a couple of weeks. She was a stay-at-home wife at that time, so she wasn’t missing work [or] school.”

Similarly, a student who gained 3 points and interviewed a German measles case remarked, "Mother developed typical rash for about 3 days with high fever and remained bed bound. She is a school teacher and didn't work for a few days." While some VH students who gained low to middle VAS points had extreme examples, overall the tone was more moderate than the students who gained the greatest VAS points.

3.5.2. Student Ranking of Influential Factors Does Not Match Actual Impact

We sought to confirm this finding by examining the ranking data (Methods, Box 3). We asked students to rank which factor (physical suffering, limitation of activities, interactions with other people, effects on family/friends, and financial impact) they perceived to have the greatest impact on the interviewee. Interestingly, 62% of all students ranked physical suffering as having the most impact, and 54% of students ranked financial costs as having the least impact. However, neither of these factors was significant for the whole population. There were no clear ranking distinctions for limitation of activities, and interactions with other people and family/friends. These findings are replicated for VH students. Therefore, in many cases, physical suffering of the interviewee is represented as the greatest symptom reported by the interviewer, but does not surpass other factors in influencing post-intervention VASs in general, at least as perceived by the student's ranked understanding. This may be because what was most important to the interviewee does not necessarily directly correlate with the interviewers "take away". For example, while students perceived that the interviewee might have emphasized characteristics other than physical suffering in their interview, the students themselves perceived pain as an important concept. This may be important to consider while designing interventions.

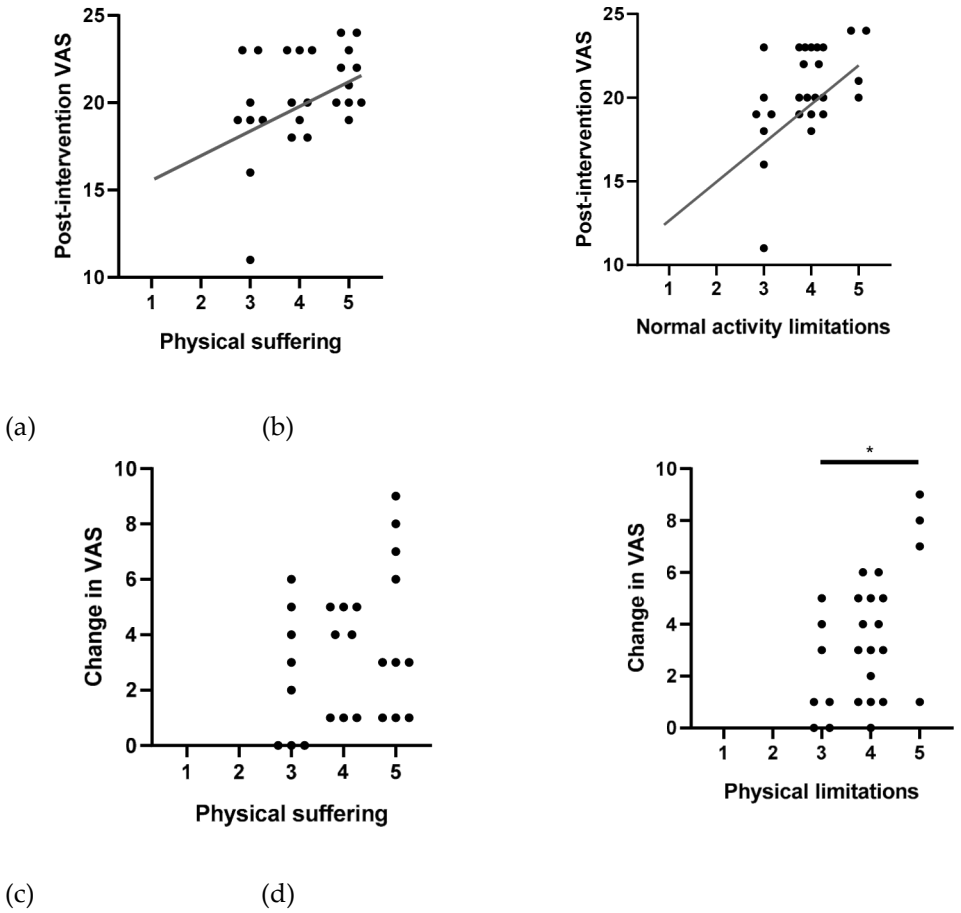


Figure 5. Post-intervention VAS and positive pre- to post-intervention VAS changes are influenced by (**a**,**c**) physical suffering and (**b**,**d**) physical activity limitations. (**a**) Post-intervention VAS is predicted by physical suffering ($r^2 = 0.405$, $p = 0.04$) and (**b**) physical limitations ($r^2 = 0.518$, $p = 0.007$). (**c**) While the student's perception of physical suffering did not predict the amount of VAS change ($p = 0.3089$), (**d**) the student's perception of normal activity limitations is significantly predicted ($p = 0.0206$). * $p < 0.05$.

4. Discussion

In this study, we succeeded in improving student vaccine attitudes through either (1) having students interview individuals who had experienced a VPD or (2) providing intensive vaccine- and VPD-related course material. Combining intervention styles allowed us to assess the strength of each intervention. VPD interviews (intervention group) were most successful at swaying student vaccine attitudes when the coursework did not discuss vaccines or if the interviews had strong themes of physical suffering and limitations. The majority of students in the intervention group who became pro-vaccine and the resulting increase in vaccine attitude

scores mirrored those achieved through intensive education (MMBio 261). Thus, encouraging students to conduct VPD interviews may be an easy and effective intervention when the course has little to do with VPDs or lacks vaccine-related content.

In courses that do address vaccines, it may be advantageous to first rigorously introduce students to VPD consequences before addressing, lightly, vaccine safety and societal implications. While Bio 100 introduces vaccines through a homework assignment that seeks to correct misconceptions about vaccine safety and societal implications, MMBio 261 begins with rigorous weeks-long sections on immunity and VPDs but only briefly discusses vaccine safety and herd immunity. This may explain why Bio 100 VH students did not have significantly increased post-intervention VASs, only a suggestive upward trend, whereas MMBio 261 VH students had significantly improved post-intervention VASs. The comparison between Bio 100 and MMBio 261 students mirrors earlier research that discussing VPD ramifications has a greater impact on combating vaccine hesitancy than correcting flawed assumptions or asserting an absence of risk about vaccines [11–15].

This study does have limitations. We did not examine whether an interview-based intervention would be successful in a non-science course. Any biological instruction discussing vaccines might provide some boost to vaccine attitudes. Additionally, for logistical reasons, we did not assess whether the increase in VAS is meaningful by following whether students vaccinate their current and future children. Furthermore, this study focuses on college students and may not be expandable to the general population. Nonetheless, despite these limitations, interview-based interventions and intensive VPD-dependent vaccine education does significantly increase vaccine attitudes, in a population susceptible to anti-vaccine attitudes. Vaccine hesitancy is a complex, situation-dependent problem, and requires unique and tailored interventions.

Interview-based interventions are easy to implement and can supplement courses or even community outreach programs seeking to address vaccine hesitancy. Predisposing students to think more favorably about vaccinations by openly discussing the consequences of vaccine-preventable diseases may improve their prospective individual and familial vaccine uptake. Future research should tease apart the contributions of science education and personal familiarity with VPDs towards improving vaccine attitudes in diverse populations.

5. Conclusions

There are two major conclusions of this work. First, an interview-based intervention, where students discuss vaccine-preventable diseases with people who have actually experienced these diseases, can significantly improve attitudes towards vaccination. Second, the subject matter used while teaching about vaccine-preventable diseases matters. In the class with extensive discussion of the diseases themselves, there was a strong increase in vaccine attitudes among vaccine-hesitant students, while this effect was not seen in the class that discussed mostly vaccine safety. Taken together, these findings indicate that increasing familiarity with vaccine-preventable diseases leads to improved attitudes towards vaccination. This should help to create solutions to the worldwide problem of vaccine hesitancy or denial, by indicating aspects of education that are important for affecting those attitudes.

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SCIENTIFIC REPORTS

OPEN 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 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).

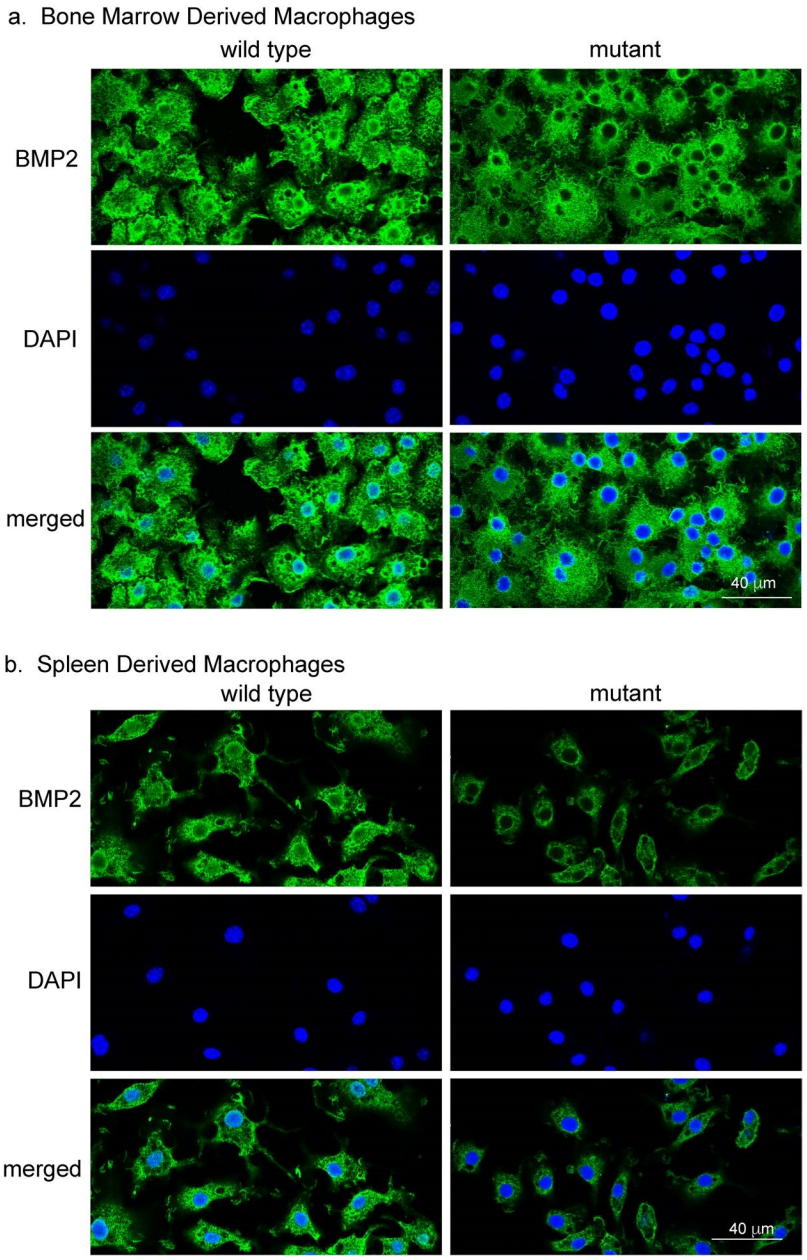
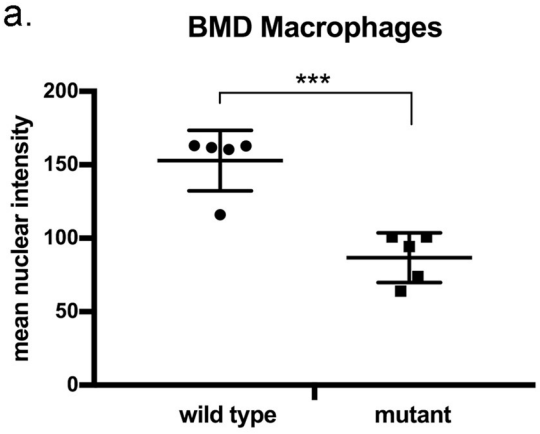


Figure 1. BMD macrophages and splenic macrophages express nBMP2, which is decreased in the nuclei of nBmp2NLS^{tm} mutant macrophages. (**a**) BMD macrophages and (**b**) splenic macrophages were stained with anti-BMP2 antibody (green) and counterstained with DAPI (blue), demonstrating that nBMP2 is 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.

a.



b.

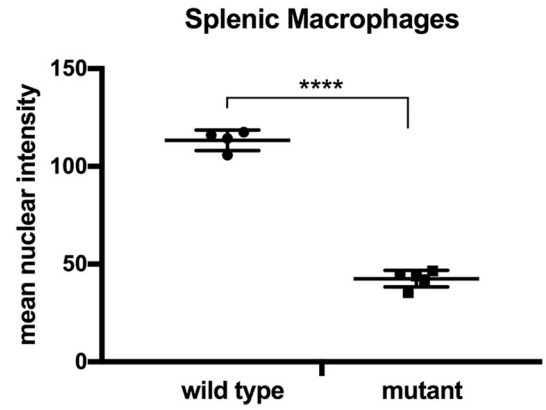


Figure 2. Quantification of nBMP2 nuclear staining intensity. Five images each were analyzed for wild type and mutant BMD macrophages and for mutant splenic macrophages. Four images were analyzed for wild type splenic macrophages. Each image contained between 10 and 93 cells, and the number of cells 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, $p < 0.0001$.

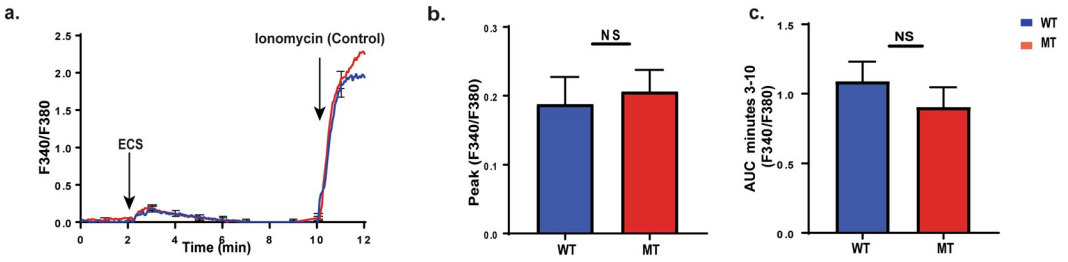


Figure 3. Naïve bone marrow derived (BMD) macrophages from nBmp2NLS^{tm} mutant mice and wild type mice have a similar Ca^{2+} response. Naïve BMD macrophages from wild type (WT) and nBmp2NLS^{tm} mutant

(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.

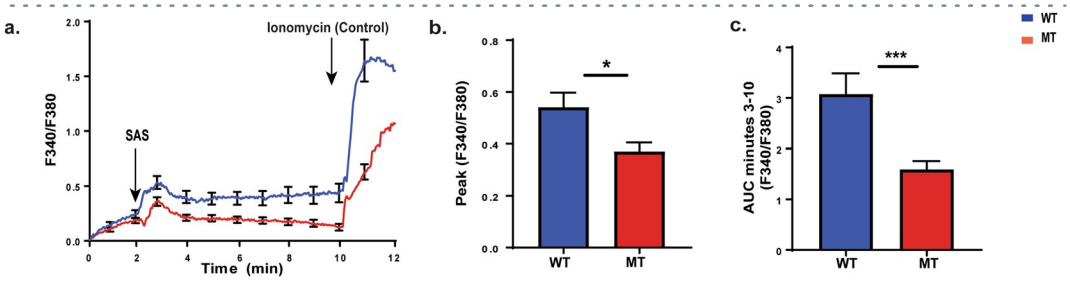


Figure 4. Splenic macrophages collected from nBmp2NLS^{tm} mutant mice after secondary infection have an impaired Ca^{2+} response. Splenic macrophages from wild type (WT) and

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

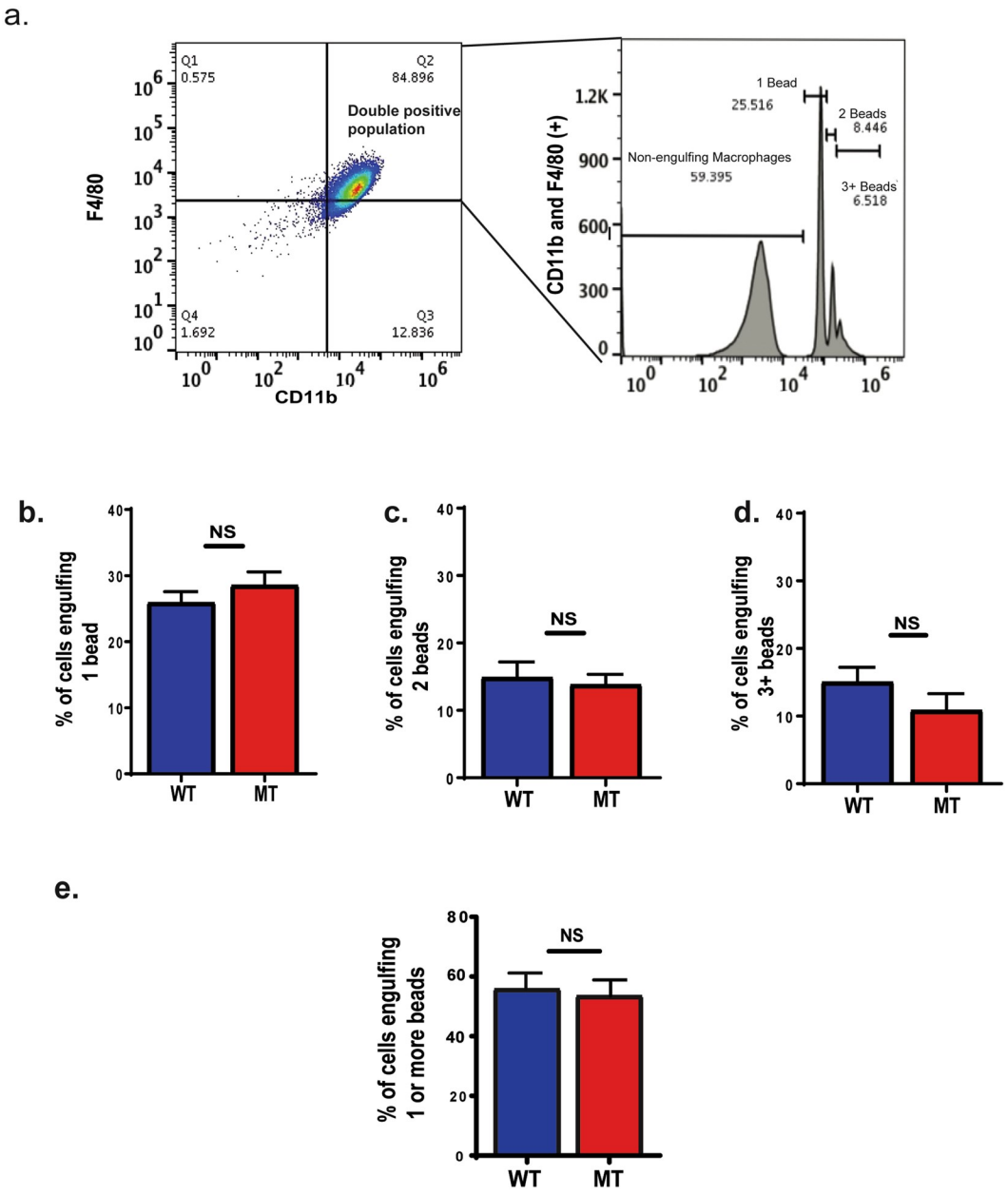


Figure 5. Naïve bone marrow derived (BMD) macrophages from nBmp2NLS^{tm} mutant mice and wild type mice show similar phagocytic activity. After incubation with fluorescent microspheres, macrophages were analyzed by flow cytometry. (**a**) A representative analysis is shown. The F4/80 and CD11b double 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.

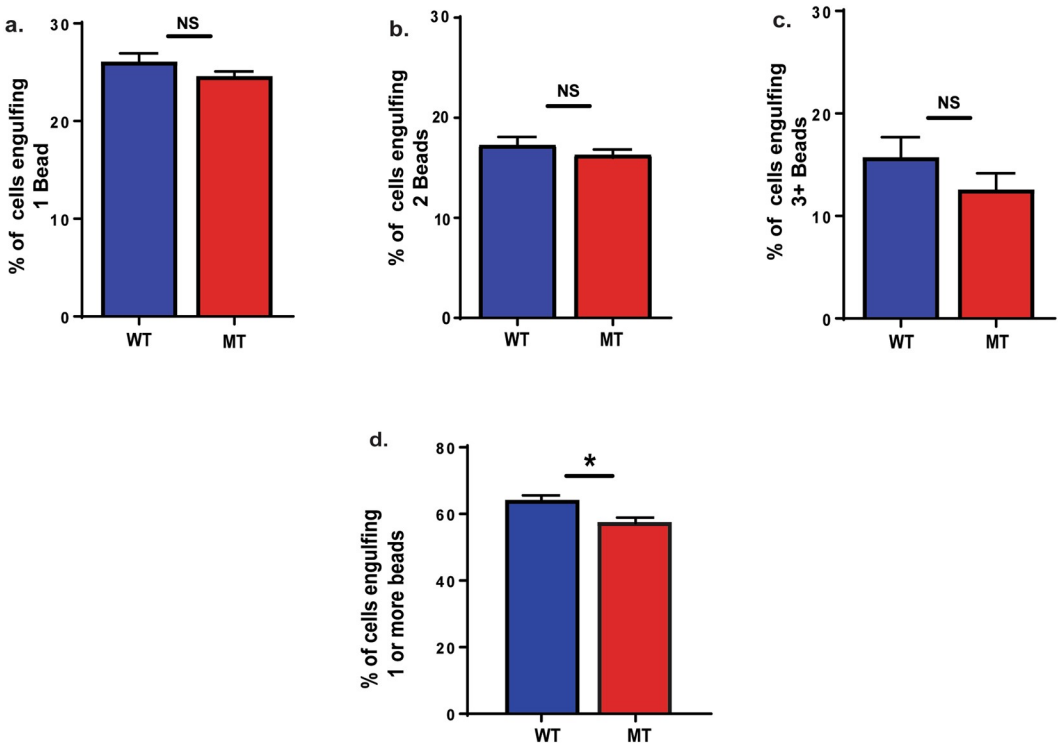


Figure 6. Splenic macrophages collected from nBmp2NLS^{tm} mutant mice after secondary infection show impaired engulfment activity. After incubation with fluorescent microspheres, macrophages were analyzed by flow cytometry as described in Fig. 3. (**a**) Percent of cells engulfing 1 bead, (**b**) percent 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™ 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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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.

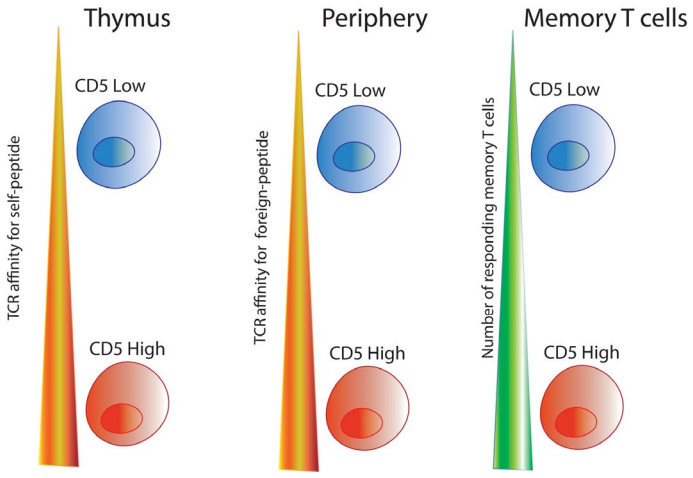

Figure 1. Effects of CD5 on different stages of T cell development. CD5 expression on thymocytes is directly proportional to the signaling intensity of the TCR:self-pMHC interaction. In the periphery, T cells with higher CD5 levels ($CD5^{hi}$) are better responders to foreign-peptide. Long-lived memory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

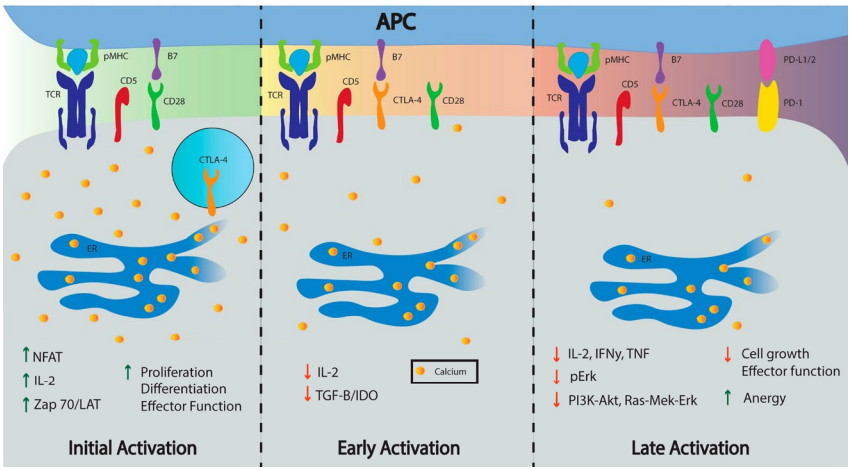

Figure 2. Inhibiting co-receptors modulate T cell activation by increasing (green arrows) or decreasing activity (red arrows). CD5 is present in naïve T cells and localizes to the TCR:pMHC complex during activation. Initial activation cascades signal for the release of CTLA-4 from vesicles to the cell 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.

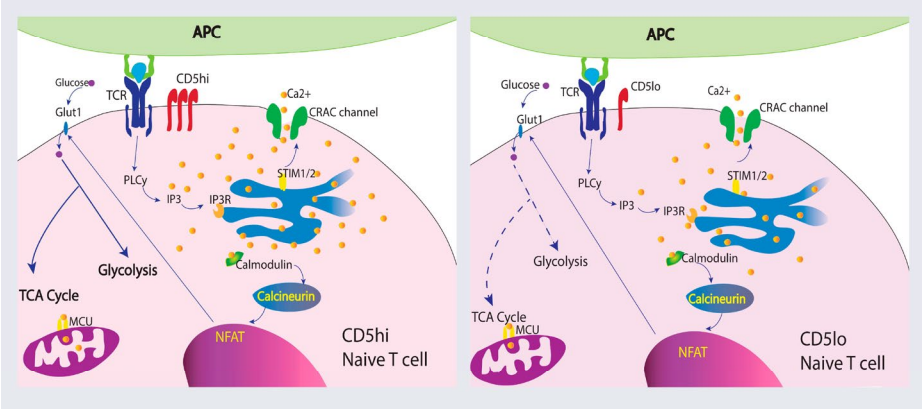

Figure 3. CD5 expression levels in naïve T cells may influence T cell metabolism and function. Differential levels of CD5 result in differences in Ca^{2+} mobilization in naïve T cells. $CD5^{hi}$ naïve T cells have higher Ca^{2+} influx than $CD5^{lo}$ naïve T cells upon TCR:pMHC interaction [35]. Ca^{2+} 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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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

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1. INTRODUCTION

Incidence rates of type-2 diabetes and obesity are rising worldwide. In adition 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, 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 (**Figure 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 βcell, 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 agreggation 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 βcells 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 **Figure 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). 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 hours 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). 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 hours at 37ºC, 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 1M NaOH over the course of 1 hour. The NaOH was then placed in a liquid scintillation counter and counted. Data were expressed as means ± SEM 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) 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, California, 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 DGlucose, 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 37ºC in 5% CO_2 to allow for adherence. Following adherence, cells were pretreated for 4 hours with 10% FBS/1% PSA DMEM containing the test compounds (5 and 10 μM) or vehicle only (≤0.1 % DMSO). After the 4-hour pretreatment, 500 μM H_2O_2 was added to injure the cells, and the microplate was subsequently incubated for an additional 4 hours. 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 $1.5 \times$

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-1 832/13 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 hours, treated with test compounds at 24 hours, and harvested at 48 hours. Stock solutions of test compounds were made at 100mM, and diluted in media for assays at final concentrations of 0100 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-1 832/13 β-cells were plated and grown to confluency in standard 24-well plates. Upon reaching confluency, cell were cultured with test compounds at 0 −100 μM in complete media for 24 hours. Following the 24 hour treatment, cells were washed with PBS and preincubated in secretion assay buffer (SAB) for 1.5 hours (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 hour (basal), followed by 1 hour 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 hour 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-1 832/13 β-Cell Oxygen Consumption Rate—Oxygen consumption rate (OCR) was measured using an XFp Extracellular Flux Analyzer (Agilent Technologies). INS-1 832/13 β-cells were seeded at 2.0×10^4 cells/well in complete 832/13 RPMI 1640 medium (L-glutamine, 11.2 mM glucose supplemented, 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 hours of culture with the compounds, cells were incubated in 2.5mM glucose SAB for 3 hours. Following incubation, buffer was exchanged for 180 µL fresh pre-warmed 2.5mM glucose SAB per well. A XF Cell Mitochondrial Stress Test was completed to assess the bioenergetic status

of the cells by injecting glucose (16.7mM, 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 ± SEM. For activity assays, data were analyzed by 1- or 2way 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 highglucose treatments to compare each dose to the vehicle (0 μM) control. For 1-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 $P < 0.05$. Statistical anslyses 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 3 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 **Figure 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 (**Figure 2D**) and increase the ratio of complete: incomplete oxidation at 10 μM (**Figure 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 (**Figure 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 (**Figure 3A**). While HVA had no apparent activity, both 5PVA and HA were able to simulate glucose metabolism at 25 μM (**Figures 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 **Figure 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 **Figure 4A-B.** None of the compounds tested significantly enhanced basal respiration (**Figure 4C**), ATP-dependent respiration (**Figure 4E**), maximal respiration (**Figure 4F**), or respiratory reserve (the difference between basal and maximal respiration, which reflects reserve bioenergetic capacity available to the cell, **Figure 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 (**Figure 4H**). HA and 5PVA both modestly enhanced 'leak' respiration at 10 μM (**Figure 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 repiration 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 **Figure 5**. Peroxide treatment induced mitochondrial injury as assessed by increased 'leak' respiration (respiration after oligomycin roughly doubled) (**Figure 5D**) and lower rates of maximal respiration (FCCP), ATP-dependent respiration (**Figure 5E**), respiratory reserve capacity (**Figure 5G**), and coupling efficiency (**Figure 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 (**Figures 5D-H**). As

observed for uninjured cells, one metabolite actually worsed 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 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 (**Figure 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 (**Figure 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-1 832/13 β-cells but only at 100 μM (**Figure 6A)**, which is not physiologically relevant, suggesting minimal relevance for activity in vivo. Interestingly, all three

microbial metabolites demonstrated significant induction of GSIS at concentrations from 5–100 μM (**Figure 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-1 832/13 β-cells, we examined the cellular insulin content under stimulatory conditions (16.7 mM glucose) to determine if treatment impacted insulin expression (**Figure 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 (**Figure 7A**), but the effect was inconsistent across doses. Interestingly, increases in insulin content were vastly different across the metabolites (**Figure 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 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 (**Figure 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 (**Figure 8C**). The same results were also observed under glucose stimulation and maximal respiration (although the level of glucose induced respiration is surprisingly less that what has been observed in other studies) (**Figure**

8D-E). None of the compounds tested significantly affected respiratory reserve (**Figure 8F**). It is important to note that the low means and comparatively high SEMs 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 (**Figure 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 (**Figure 6**), these respiration data suggest several novel findings. First, EC does not enhance GSIS except at extremely high doses despite enhacing 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 **Figures 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 enhance 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.

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 (**Figure 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-cellinsulin 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 determine 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 significantlyprotect 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 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 interindividual variability in microbial metabolism affects subsequent metabolite profiles and bioactivities [49]. This knowledge will be 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- Microbial metabolites of flavonoids possess potent activities
- Metabolites stimulated glucose-stimulated insulin secretion
- Metabolites stimulated beta-cell respiration
- Metabolites protected skeletal muscle from oxidative injury
- Metabolites did not generally uncouple mitochondrial respiration

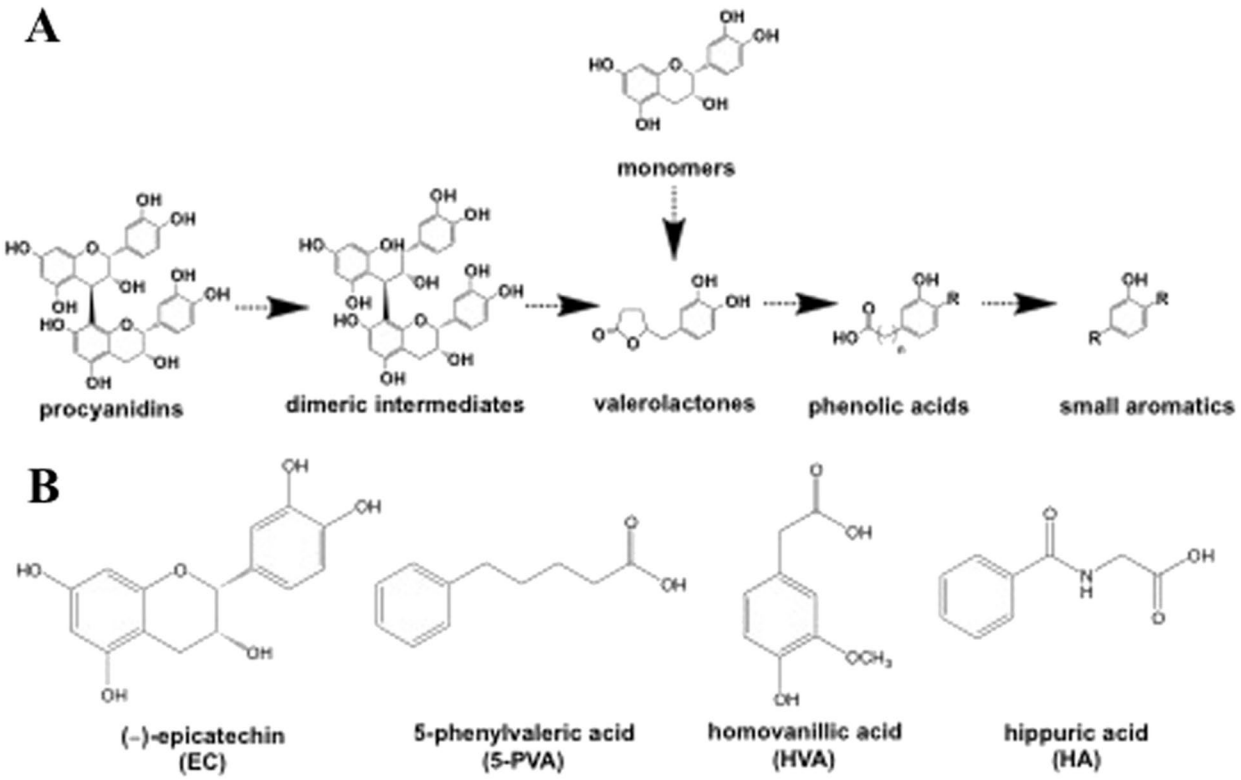

Figure 1.

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.

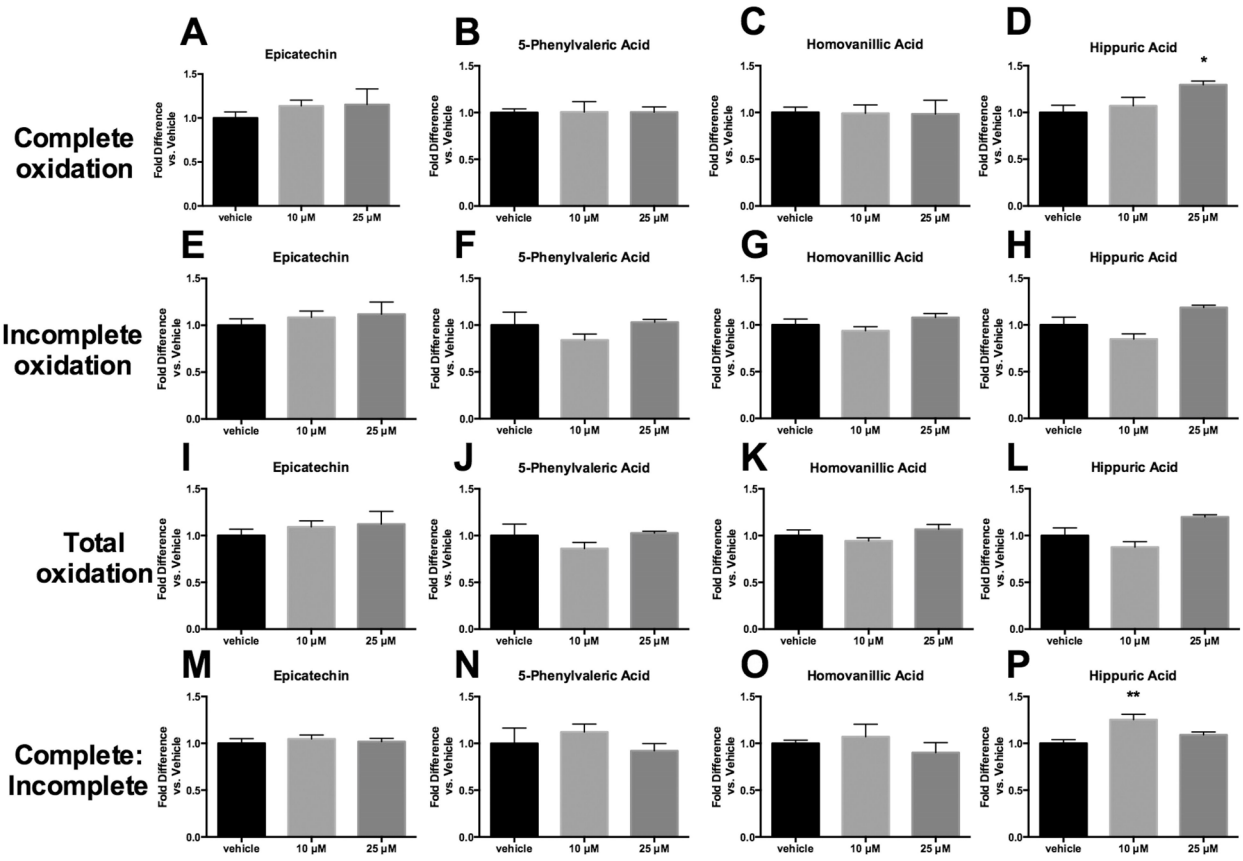

Figure 2.

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 (ASM) from ^{14}C-labeled palmitate. Total oxidation represents the sum of complete and incomplete oxidation. Values represent mean ± SEM from n=4 replicates, normalized to vehicle (vehicle expressed as 1). Data were analyzed by 1-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: *$P \leq 0.05$, **$P \leq 0.01$.

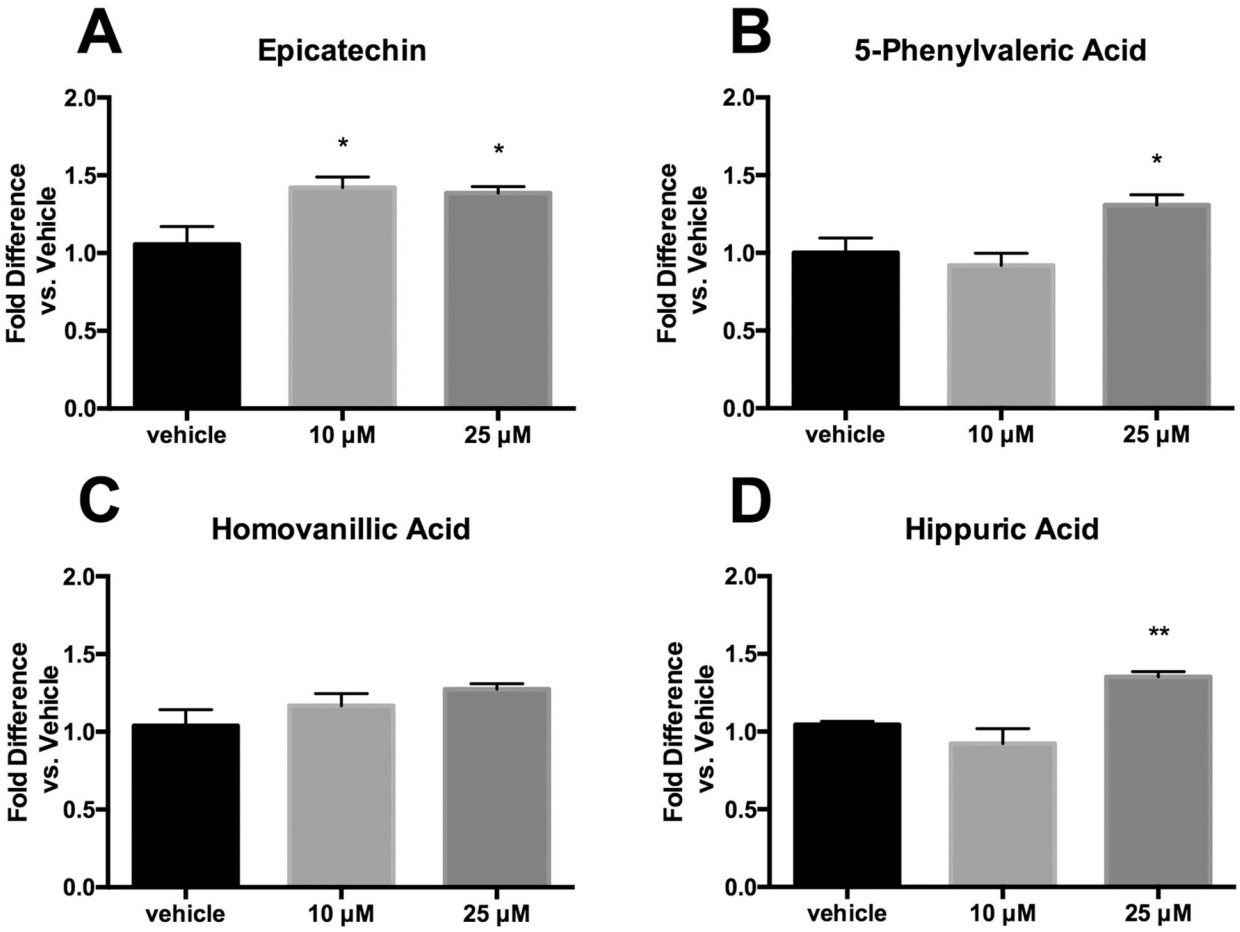

Figure 3.

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 ± SEM from n=4 replicates, normalized to vehicle (vehicle expressed as 1). Data were analyzed by 1-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 ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001.

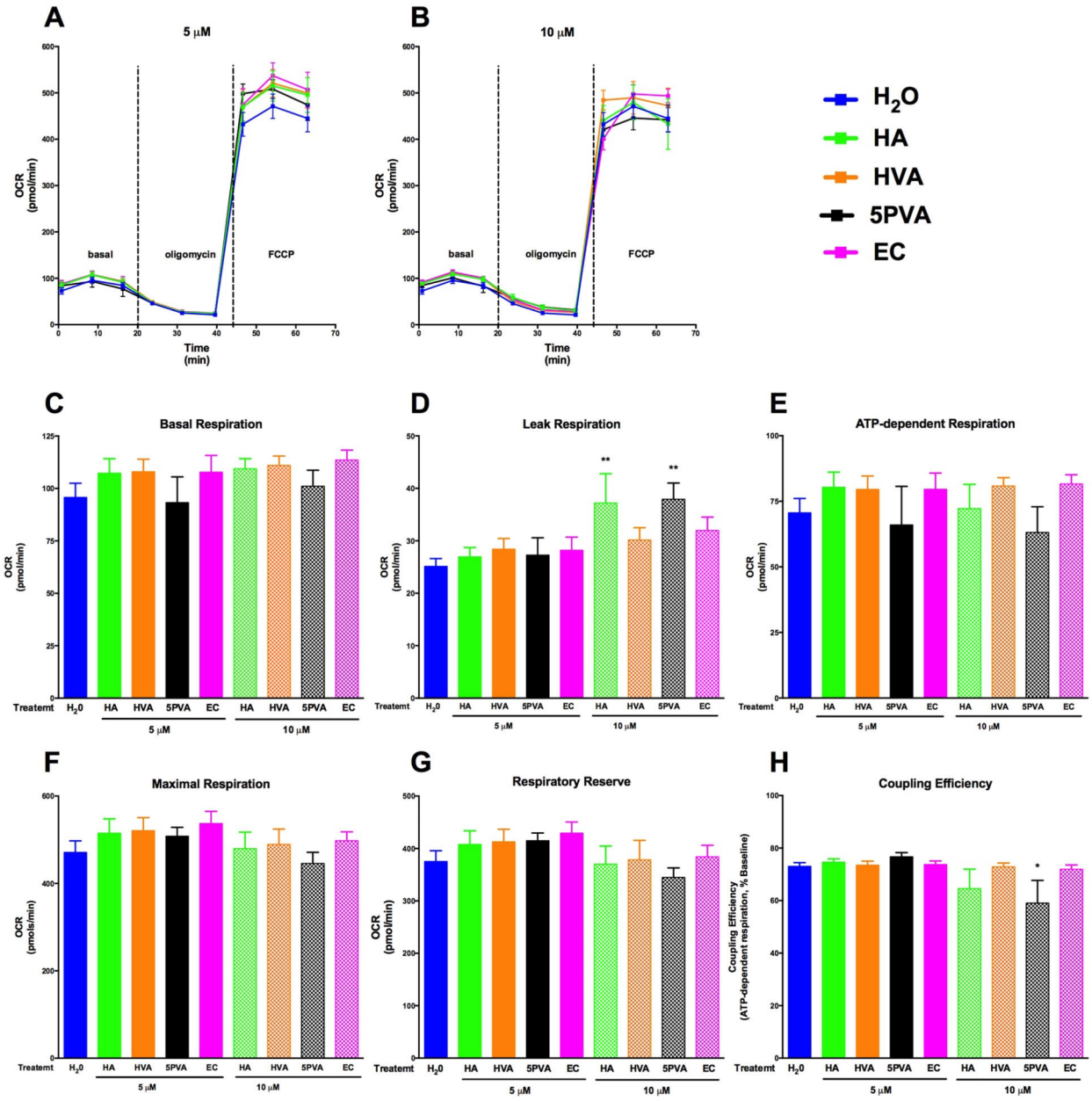

Figure 4.

Corrected mitochondrial respiration data for C2C12 cells cells cultured acutely (4h) 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 ± SEM from n=8 replicates. Data were analyzed by 1-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 ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001.

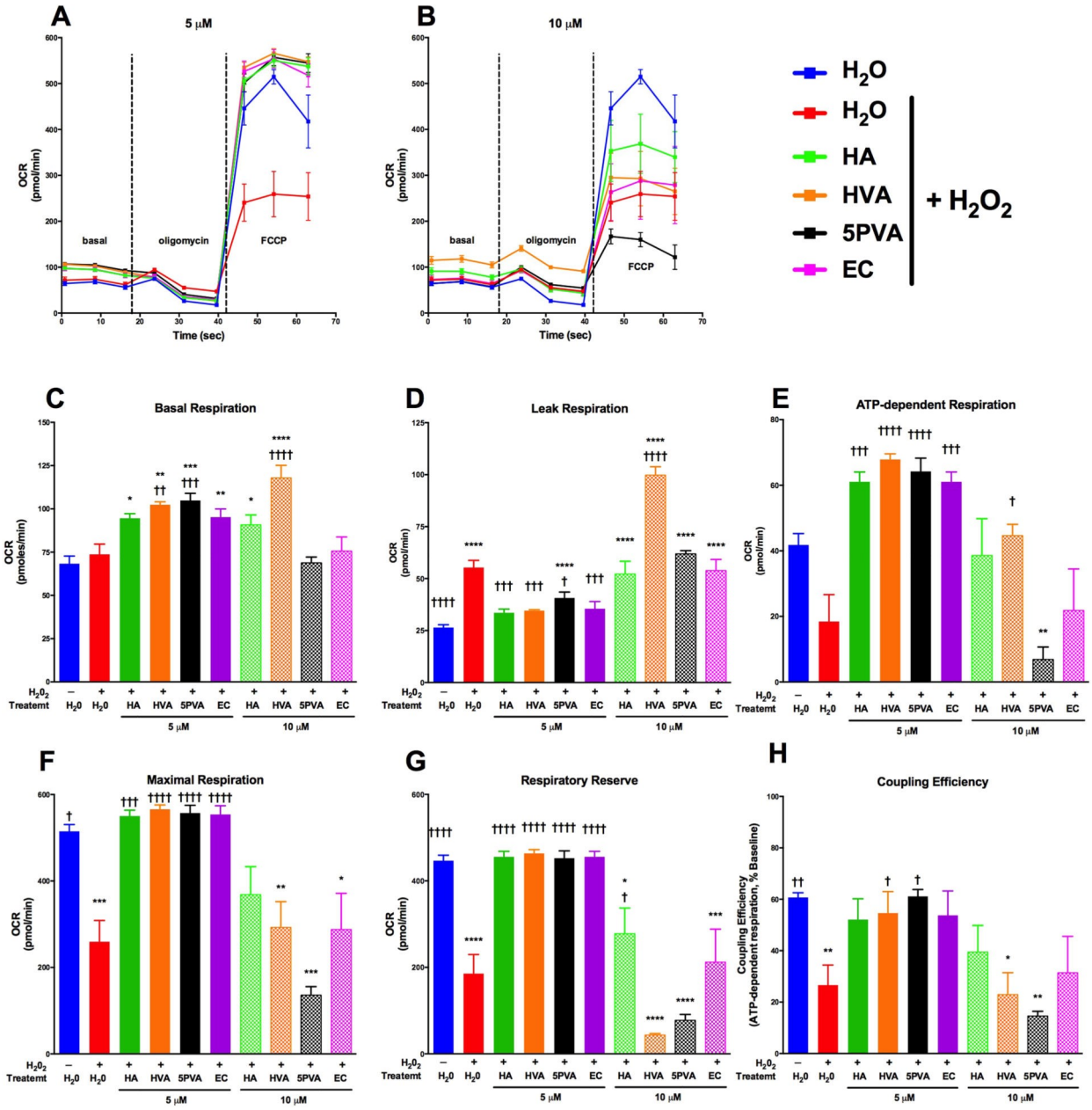

Figure 5.

Corrected mitochondrial respiration data for H_2O_2-injured C2C12 cells cultured acutely (4h) 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), ATPdependent 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 ± SEM from n=8 replicates. Data were analyzed by 1way 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_2). Significance vs. vehicle control is indicated by: *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001; significance vs. injury control is indicated by: †P ≤ 0.05, ††P ≤ 0.01, †††P ≤ 0.001, ††††††P ≤ 0.0001.

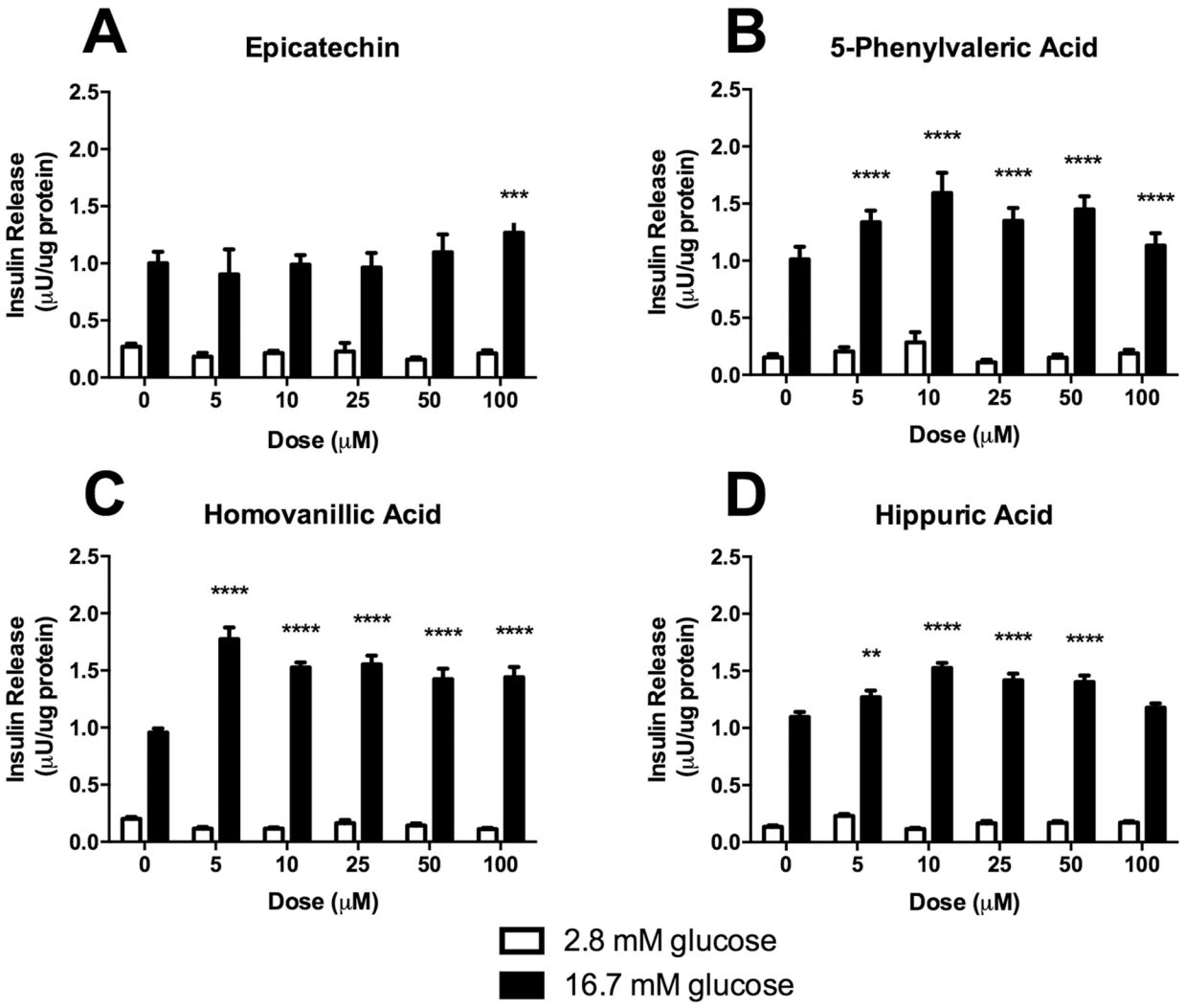

Figure 6.

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 ± SEM 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 ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001.

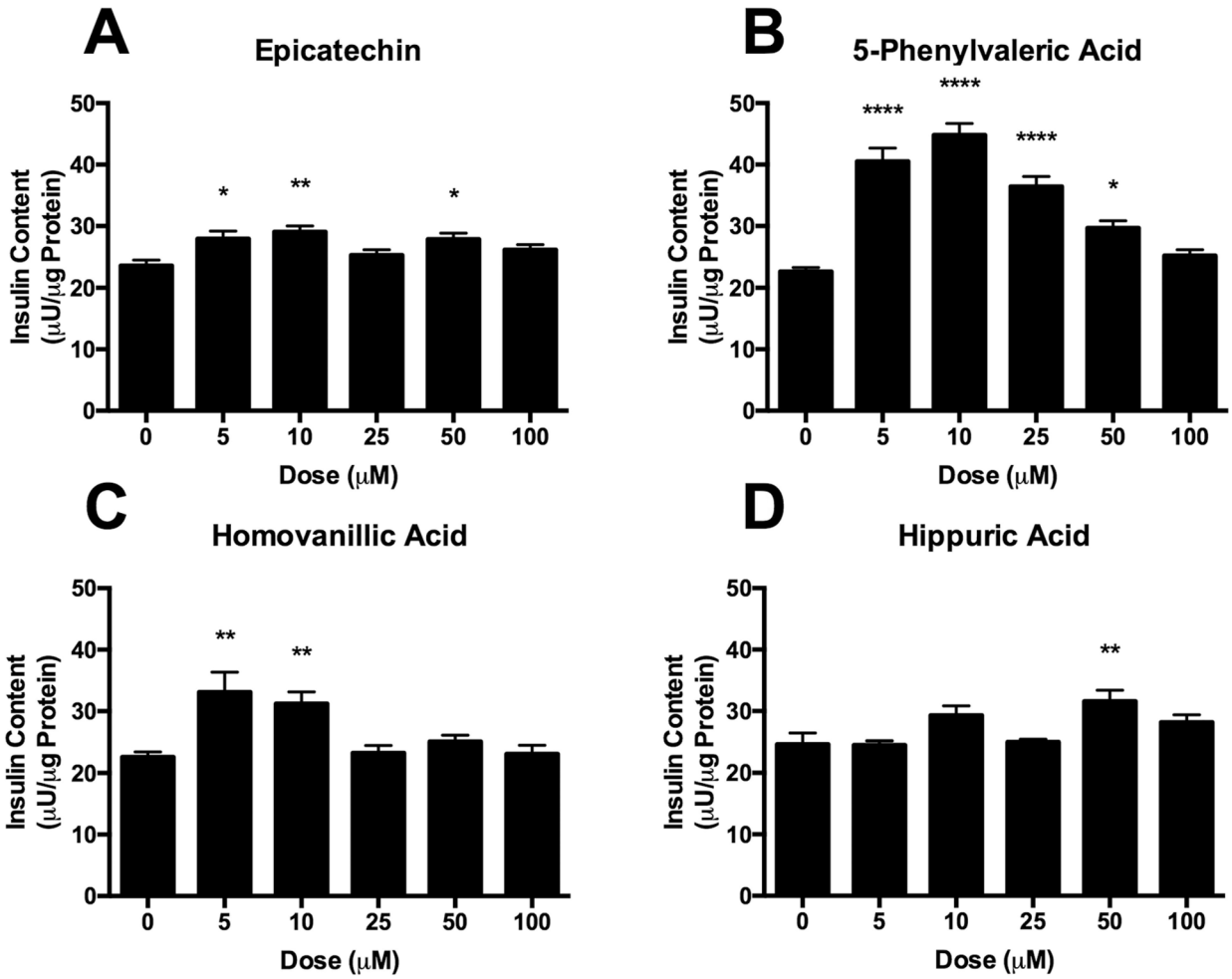

Figure 7.

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 ± SEM from n=6 replicates. Data were analyzed by 1-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: *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$.

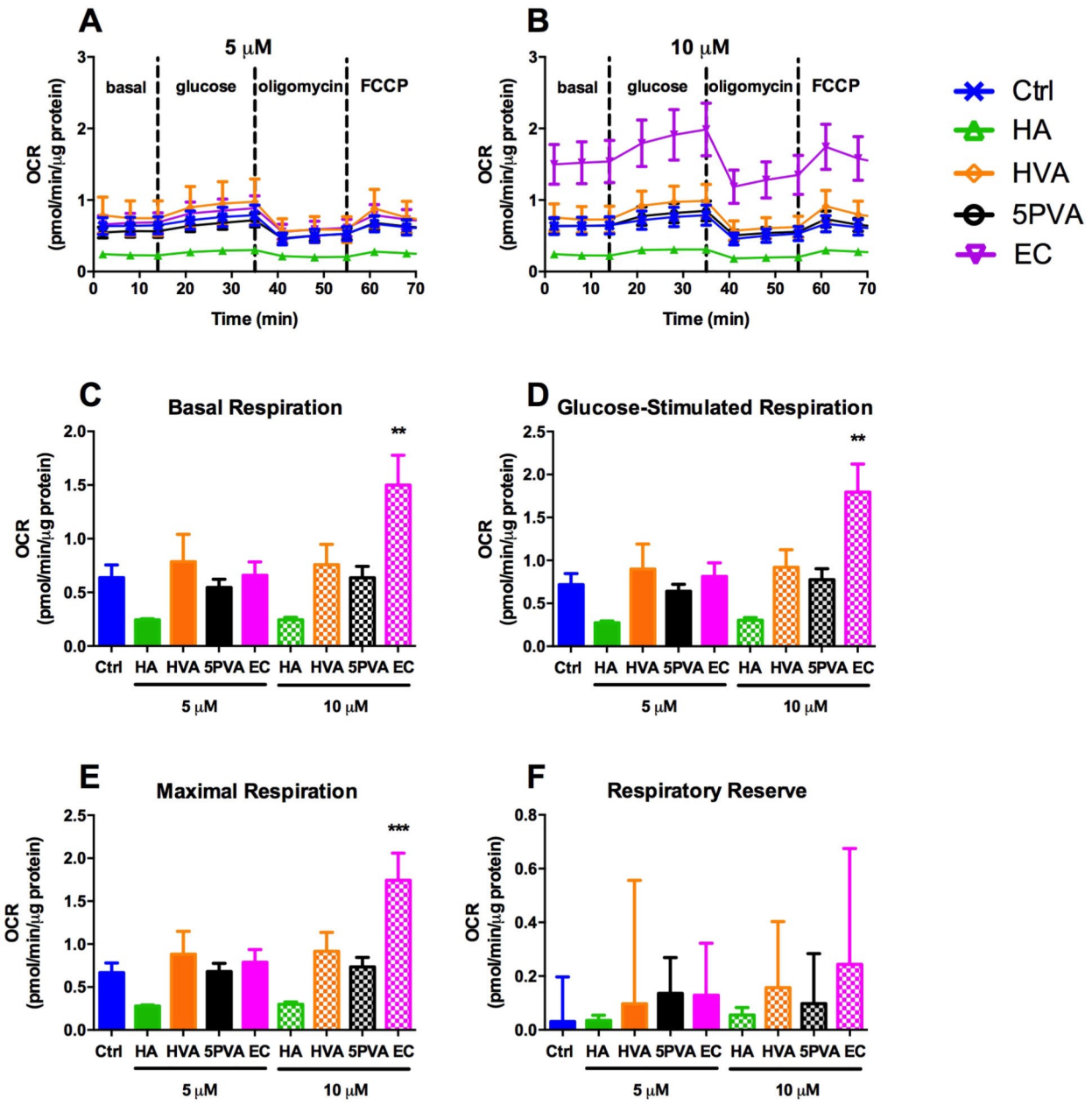

Figure 8.

Corrected mitochondrial respiration measured after culturing INS-1 832/13 β-cells for 24 h in the presence of 0, 5 or 10 μM hippuric acid (HA), homovanillic acid (HVA), 5phenylvaleric 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 (maxmal −

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 ± SEM from n=5 replicates. Significance vs. untreated control is indicated by: *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001.

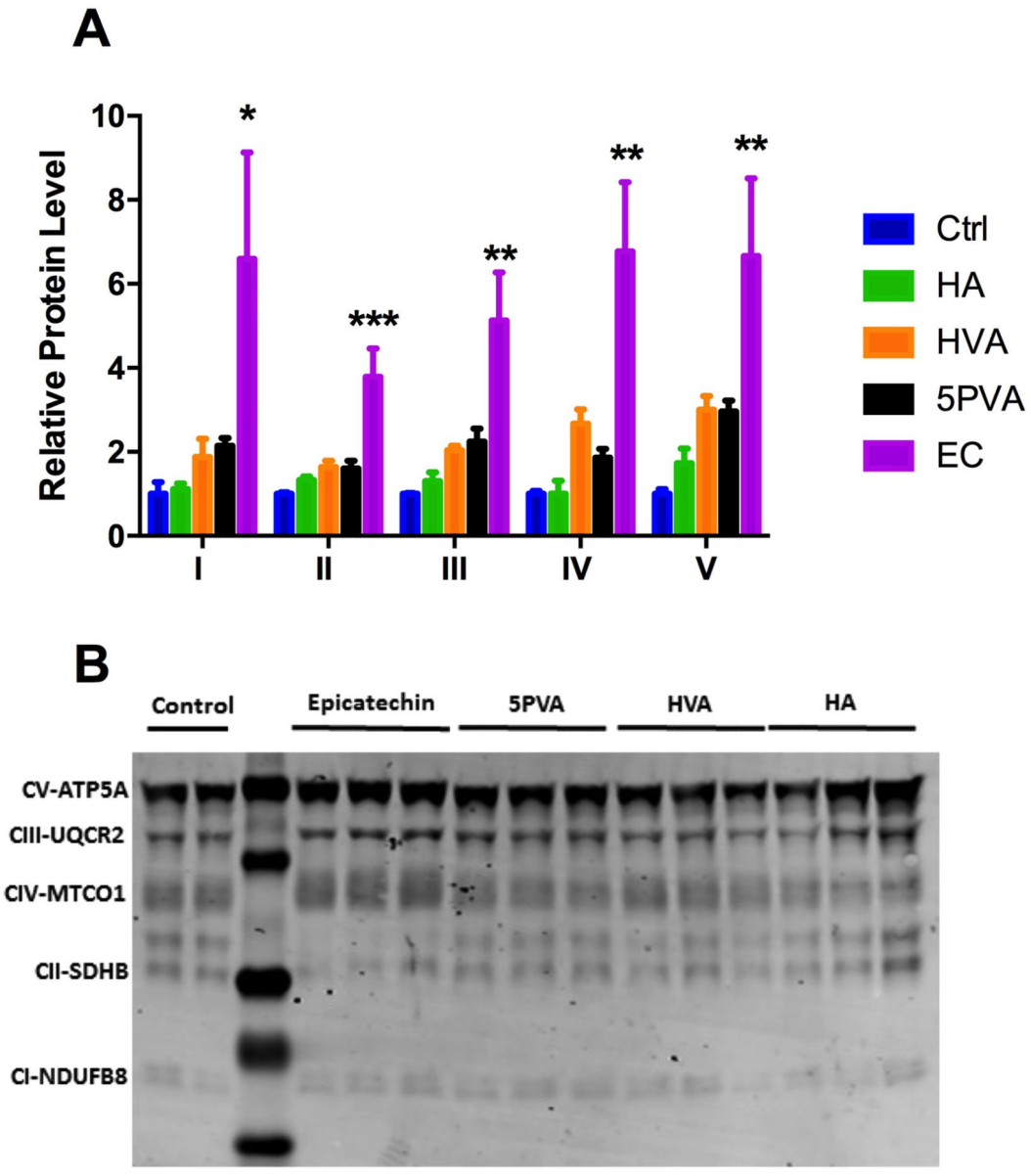

Figure 9.

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 ± SEM from n=3 replicates per condition. Data were analyzed by 1-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: *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$. **B**) Representative Western blot.