Determining the Role of Innate Immune Elements on RNA Viral Replication and Understanding Factors that Impact Adaptive T cell Anti-tumoral Activity Against Metastatic Osteosarcoma (mOS)

by

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ABSTRACT

The innate immune system serves as an immediate response to pathogenic infection and an informant to the adaptive immune system. The 2′,5′-oligoadenylate (2-5A) synthetase (OAS)–RNase-L system is a component of the innate immune system induced by interferons (IFNs) and serves to eliminate viral infections. In humans, three enzymatically active OAS proteins exist, OAS1, OAS2, and OAS3. Recent evidence suggests variations in cellular localization of OAS proteins may influence the impact and influence of those proteins on viral replication. However, viral suppression mechanisms involving specific OAS proteins are still unclear for most viruses. Here, I overexpress different isoforms of OAS and determined that though viruses within the same family have similar replication strategies, the extent to which each OAS protein impacts viral replication for *Flaviviruses*, and *Alphaviruses* varies.

In contrast to the innate immune system, the adaptive immune system provides specific and long-lived immune responses. In the context of cancer, T cells have been shown to play a prominent role in tumor regression. It has previously been demonstrated that administration α-CTLA-4/α-PD-L1 immune checkpoint blockade (ICB) to mice inoculated with a K7M2 metastatic osteosarcoma (mOS) cell line resulted in ~50% survival. Here, I sought to determine biological differences among murine responders and non-responders to ICB for mOS to understand better what factors could increase ICB efficacy. A prospective culprit is a variance in circulating antibodies (Abs). I have shown that sera from mice, before inoculation with mOS or ICB, display distinct differences in Ab repertoire between responders and non-responders, suggesting the presence or absence of particular Abs may influence the outcome of ICB. Recent studies have also shown that

malleable environmental factors, such as differences in microbiome composition, can yield subsequent changes in circulating Abs.

Strong associations have been made between host-microbiome interactions and their effects on health. Here, I study potential associations of microbiome-mediated impacts on ICB efficacy for mOS. Additionally, I sought to determine potential changes in T-cellular response to mOS due to modulations in microbiome composition and showed that ICB efficacy can change in conjunction with microbiome composition changes in a murine model.

DEDICATION

I want to dedicate this dissertation to my family, especially my dad Joseph E DiPalma MD, who encouraged, supported, and loved me for years. He inspired me to dream bigger and always believed I was capable of whatever I set my mind to. He always knew the right advice to give me when I was in a difficult situation and encouraged me to be my best through his hard work. Dad, I'll always treasure every memory I have with you and will continue to try to make you proud. I also want to dedicate this dissertation to my mom, Maria A DiPalma. She has taught me a tremendous amount of empathy and resilience and has loved me even when I am at my worst. Mom, you are one of the strongest women I know, and I am so thankful to have you as my mother. I want to thank my siblings, Nick, Maria, and Vittoria, for your support, love, and friendship throughout my life. I love you all with all of my heart. To my Aunt Diane Faraone, my Grandma and my Nonni, for the many years of love, and to Stefania Pifferi, Sean Pifferi, and Carlo Marchi for being my family and for all the Italian food and laughs over the years.

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LIST OF ABBREVIATIONS

Ab	Antibody
Ag	Antigen
ADP	Adenosine Diphosphate
AID	Activation-Induced Deaminase
AMP	Adenosine Monophosphate
APC	Antigen Presenting Cell
APE-1	Apurinic/Apyrimidinic Endonuclease 1
ATP	Adenosine Triphosphate
AVN	Ampicillin, Vancomycin, and Neomycin
AVNM	Ampicillin, Vancomycin, Neomycin, and Metronidazole
BALS	Breathing, Ambulation, Lethargy, and Scruffiness
BCR	B Cell Receptors
BiKEs	Bi-Specific NK Cells Engagers
BLAST	Basic Local Alignment Search Tool
BM	Bone Marrow
CAD	Caspase-3– Activated DNase
CARD	Caspase Activation and Recruitment Domain
CCL2	C-C Motif Chemokine Ligand 2
CCL20	C-C Motif Chemokine Ligand 20
CHKV	Chikungunya Virus
CLP	Common Lymphoid Progenitor

CLS	Cell Lysis Buffer
CMP	Common Myeloid Progenitor
CR2	Complement Receptor 2
cGAS	Cyclic GMP-AMP Synthase
cGAMP	Cyclic Guanosine Monophosphate -Adenosine Monophosphate
DAMPs	Damage-Associated Molecular Patterns
DC	Dendritic Cell
DENV-2	Dengue Virus (stereotype 2)
DNA	Deoxyribonucleic Acid
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DPI	Days Post Infection
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-linked Immunosorbent Assay
eIF2α	Eukaryotic Translation Initiation Factor 2 Subunit 1
EMPs	Epitope Prediction Models
ER	Endoplasmic Reticulum
FBS	Fetal Bovine Serum
FCS	Fetal Calf Serum
FDCs	Follicular Dendritic Cells
FMO	Fluorescence Minus One
GFP	Green Fluorescent Protein
GLAM2	Gapped Local Alignment of Motifs

GMP	Guanosine Monophosphate
GTP	Guanosine Triphosphate
HEPES	4- (2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid
HEV	High Endothelial Venules
HiLo	High-Efficiency Low Background
HSC	Hematopoietic Stem Cells
IAV	Influenza A Virus
iCAD	Inhibitor of Caspase-3– Activated DNase
ICB	Immune Checkpoint Blockade
Ig	Immunoglobulin
IFN	Interferon
IFNR	Interferon Receptor
IκB	Inhibitor of Nuclear Factor Kappa B Alpha
IRF	Interferon Regulatory Factor
IKK	Inhibitor of Nuclear Factor Kappa B
ISG	Interferon Stimulated Gene
ISW	Isotype Switching
JAK	Janus Kinase
KUJV	West Nile Virus, Kunjin Strain
LCMV-Arm	Lymphocytic Choriomeningitis Virus- Armstrong Strain
LCMV-13	Lymphocytic Choriomeningitis Virus- Clone 13
LDH	Lactate Dehydrogenase
LN	Lymph Node

LPS	Lipopolysaccharide
OAS	Oligoadenylate Synthetase
OASL	Oligoadenylate Synthase-Like
PAP	Polyadenylate Polymerase
PAM	Plaque Assay Media
PAMP	Pathogen-Associated Molecular Pattern
PBS	Phosphate-Buffered Saline
PMA	Phorbol 12-Myristate 13-Acetate
PMN	Polymorphonuclear
PFU	Plaque Forming Unit
PSG	Penicillin, Streptavidin, and Glutamine
PKR	Protein Kinase RNA-Activated
P/S	Penicillin and Streptavidin
PRR	Pathogen Recognition Receptor
Pur	Puromycin
pHSC	Pluripotent Hematopoietic Stem Cells
mOS	Metastatic Osteosarcoma
MAC	Membrane Attack Complex
MBL	Mannose-binding Lectin
MHC	Major Histocompatibility Complex
NEMO	Nuclear Factor-kappa B Essential Modulator
NETs	Neutrophilic Extracellular Traps
NF-κB	Nuclear Factor Kappa-light-chain-enhancer of Activated B Cells

NOD	Nucleotide-binding Oligomerization Domain
NLRs	Nucleotide-binding Oligomerization Domain-like Receptors
NK	Natural Killer
NKT	Natural Killer T
OS	Osteosarcoma
RIG	Retinoic Acid-inducible Gene
RIP2	Receptor Interacting Protein 2
RLR	Retinoic Acid-inducible Gene I-like Receptors
R-loop	Recombination Loop
RNA	Ribonucleic Acid
SAS	Saturated Ammonium Sulfate
SCC	Single Color Controls
SCFA	Short Chain Fatty Acid
SCFAA	Short Chain Fatty Acid Analysis
scFv	Single Chain Variable Fragment
SDS-PAGE	Sodium Dodecyl Sulfate-polyacrylamide Gel Electrophoresis
SMH	Somatic Hypermutation
SINV	Sinbis Virus
SINV-GFP	Sinbis Virus Expressing GFP
SNVs	Single-nucleotide Variants
STAT	Signal Transducer and Activator of Transcription
STING	Stimulator of Interfeuron Genes
TAA	Tumor-associated Antigen

TBK1	TANK-binding Kinase 1
TSA	Tumor-specific Antigen
TBST	Tris·HCl Buffer Saline with 0.5% Tween-20
TCVs	Therapeutic Cancer Vaccines
TCR	T Cell Receptor
T_{CM}	Central Memory T cell
T_{EM}	Effector Memory T cell
TET-ON	Tetracycline On
TME	Tumor Microenvironment
TILs	Tumor Infiltrating Lymphocytes
TNF	Tumor Necrosis Factor
TRAIL	Tumor Necrosis Factor-related Apoptosis-inducing Ligand
TRAF	Tumor Necrosis Factor Receptor-associated Factor
TRiKEs	Tri-specific NK Cell Engagers
T_{RM}	Tissue Resident Memory T Cell
UNG	Uracil-DNA Glycosylase
WBC	White Blood Cell
WS	Working Solution
WNV	West Nile Virus
ZIKV	Zika Virus
2-5A	2’-5' Oligoadenylate Synthetase

CHAPTER 1

INTRODUCTION

The Establishment of Immunology: A Historical Perspective

The study of Immunology, the understanding of the immune system and the body's response to infection, was not formally characterized until the late 18th century; however, the notion that the body could protect itself against disease was documented even as early as 429 BC in ancient Greece during the Plague of Athens, when Thucydides detailed that persons who previously recovered from disease, did not develop disease upon exposure to those who were ill (Page 1953). Centuries later, in the 1400s, the Middle East and China further began to demonstrate an understanding of the body's ability to protect itself from disease. They practiced a preventative measure against Smallpox, a devastating viral illness at the time. This preventative measure was achieved by inhaling dried, presumably inactivated, pustules transferred from a patient previously exhibiting Smallpox symptoms to someone unexposed to the virus that caused Smallpox; in modern day, this virus is known as *Variola*. This measure intended to instigate protective immunity to Smallpox in the person inhaling the inactivated pustules, should they ever be exposed to the virus that causes the disease again, a practice later termed "variolation" that proved to be largely successful at preventing disease in those who were inoculated (A. K. C. Leung 2011).

As Smallpox spread across the globe in subsequent decades, it maintained a malevolent reputation by exhibiting a 30-50% death rate for those infected, claiming the lives of millions of people and leaving severe bodily scarring on those fortunate enough to survive (Stewart and Devlin 2006). In response, the practice of variolation became increasingly popular for both individuals and national leaders who aimed to improve the

chances that countries would be able to survive an outbreak, despite the risks associated with variolation, which included a variable 3-10% death rate for those who were inoculated (Stewart and Devlin 2006). Prominent national leaders such as Emperor Kangxi, who became emperor of China after surviving Smallpox himself and after his father Emperor Fu-lin died of Smallpox, mandated variolation across China in 1661 A.D., increasing support and popularity for the practice in Asia (Jiafeng Zhang 2002). In England, Lady Mary Wortley Montagu, who previously observed variolation in Turkey, had survived Smallpox and later chose to have her children variolated. This action increased interest in the practice in England, as she was a prominent member of the British Royal Society (Grundy 2000). The practice of variolation circulated to other continents, including North America, and remained the primary method of immunization of Smallpox for close to a century.

Many decades after the establishment of variolation and its increase in popularity among nations, in 1796, a medical doctor named Edward Jenner noted the disease that resulted from infection with Cowpox, a disease related to Smallpox but native to bovine, was significantly milder in humans than that of Smallpox. Jenner noted that infection with the virus that causes Cowpox in humans could additionally confer protection against Smallpox (Jenner 1798). In 1796, Jenner demonstrated the ability for Cowpox to protect against Smallpox disease by inoculating human subjects with Cowpox and further challenging the same subject with Smallpox, from which the human subjects were immune (Jenner 1798). Jenner referred to this practice as “vaccination,” a term still used today to describe the inoculation of a healthy individual with a weakened or attenuated strain of a disease-causing agent, also known as a pathogen, to prevent disease upon future exposure.

The work of Jenner was further analyzed and developed by other scientists who aimed to understand better the cause of disease and protection from those diseases in humans. In the late 19th century, Robert Koch, a German physician, and microbiologist, contributed to our understanding of disease and immunology by demonstrating that microorganisms are the causative agents of disease; he developed Koch's postulates to establish a causal relationship between disease and microorganisms (Koch 1876). This work and his various contributions to tuberculosis, cholera, and anthrax research revolutionized our understanding of diseases caused by microorganisms and paved the way for completing causative research for other scientists. Louis Pasteur utilized the knowledge of immunology available to him and added to developments in vaccine research by developing effective vaccines against diseases such as cholera and rabies. His rabies vaccine proved efficient upon its first use in a boy bitten by a rabid dog (Pasteur 1885). At this time, the mechanisms that resulted in immunity caused by vaccinations were still poorly understood, but successes by scientists led to an interest in understanding mechanisms behind effective vaccination, and vaccine efforts continued to develop further into the late 19th and 20th centuries.

Components and principles of the immune system were further characterized by Emil von Behring, and Shibasaburo Kaitaso, who detailed the activity of what we now know are antibodies, showing that not only could immunization with diphtheria toxin prevent disease caused by diphtheria but also that immunity could be transferred from one subject to another via serum transfer (Behring 1890). In 1895, Jules Bordet showed that the complement cascade acts in tandem with antibodies, first demonstrating its existence as a heat-sensitive component of the immune system and then demonstrating that when

combined with the heat-insensitive component of the immune system (antibodies), enhancement in immune activity ensued (J. Bordet 1895).

Not long after these initial characterizations of the immune system, several immune principles were applied to treatment for diseases other than those of communicable transmission. In 1891, William Coley, a surgical resident in a New York hospital, injected a patient who had an inoperable soft tissue sarcoma tumor with heat-inactivated *Streptococcus pyogenes* and *Bacillus prodigiosus,* intending to shrink the patient's tumor. Coley’s attempt at treating the patient's tumor was successful and served as the first known example of immunotherapy (Loughlin 2020; McCarthy 2006). Over his career, Coley injected over 1000 patients with his heat-inactivated bacterial mixture, termed Coley’s toxin, with physicians who chose to do the same reporting favorable prognoses for their patients. Though the immunology surrounding the success of Coley’s work was not yet fully understood, our modern-day understanding of immunology supports Coley’s principles in that his treatment regimen likely instigated innate immune responses via pathogen recognition receptors (PRRs), increasing pro-inflammatory cytokine release as well as immune cell recruitment to the site of cancers, which then also increased antigen presentation to adaptive immune cells and successive adaptive responses against tumor antigens.

The establishment of the innate and adaptive immune systems was later described in greater depth as scientists observed initial non-specific immune reactions. These observations include those by Ilya Mechnikov, who 1908 described the non-specific functions of macrophages as an initial defense against invading pathogens, arguing that cellular components were the primary source of immune protection in a host (Metchnikoff

1882). Though, it would later be understood that both cellular and humoral components of the immune system were vital for protection against infection with a pathogen. Paul Elrich later contributed to advances in antiserum development based on the understanding that antibodies can confer specific protection against a considerable range of substances (Ehrlich 1891). Following this work and the work of others, principles of both non-specific innate and specific adaptive immunology were established and expanded on, principles that are still being unraveled and further understood today.

The Innate Immune System

The innate immune system is the body's first line of defense against pathogens; it non-specifically eliminates these potentially harmful invaders (Janeway and Medzhitov 2003). The immune system's non-cellular anatomic and chemical barriers primarily offer initial protection against pathogens. Many of these components reside on the body's epithelial surfaces. For example, β defensins, which are produced by the epithelia of the respiratory and urogenital tracts, skin, and tongue, are members of a family of cationic antimicrobial peptides that insert themselves into the outer layers of invading pathogens and collectively form a pore in these pathogens, disrupting their osmotic balance, acting as a primary non-specific defense against foreign invaders (Schneider et al. 2005). Other host factors, like sebum secretions on epithelial surfaces and unfavorable pH, prevent pathogens from colonizing on the surface of a host and from entering a host. Further, all cellular components of the blood, including all white blood cells (WBCs) that make up all cellular components of the immune system, originate from hematopoietic stem cells (HSCs), which reside in the bone marrow (BM) (Till 1961). HSCs undergo a process known as

hematopoiesis which is the formation of all blood cells, including those of adaptive and innate origin as well as platelets. The innate immune system contains some of these cellular components activated when non-cellular anatomic and chemical barriers have been breached. These cells are primarily activated by recognizing pathogens via innate PRRs, whose functions will be discussed further in depth in later sections.

The Common Myeloid Progenitor (CMP) is the cellular precursor of innate immune cells, including macrophages and other granulocytes such as neutrophils, eosinophils, basophils, mast cells, and dendritic cells (DCs) (Murphy and Weaver 2016). Macrophages reside in most tissues and constitute a significant source of phagocytosis of pathogens, resulting in the potential killing of pathogens and serving as a source of antigen presentation to adaptive immune cells; these cells play a crucial role in activating B cells in the lymph node (Ginhoux and Jung 2014). Monocytes perform the same function as macrophages but circulate in the blood and migrate to tissues, eventually differentiating into macrophages (van Furth et al. 1972). Further, the complement system is a collection of proteins produced by the liver that can disrupt the ionic potential of an invading pathogen and coat invading pathogens for recognition by some immune cells, including macrophages, by PRRs (Sarma and Ward 2011; Jules Bordet 1895).

Granulocytes contain cytotoxic granules and multilobed nuclei, giving them the designation of "polymorphonuclear" (PMN) cells (Murphy and Weaver 2016). Neutrophils are the most numerous circulating WBCs, often the primary innate immune cell to respond to an invading pathogen via the release of neutrophilic extracellular traps (NETs) (Lacy 2006). NETs are typically only released in response to invading pathogens and are composed of neutrophilic DNA adorned with cytotoxic granules within the cell and

degrative enzymes that allow neutrophils to kill invading pathogens directly (Lacy 2006). Eosinophils and basophils are granulocytes that function via degranulation after recognition of IgE or IgG Abs. They serve to clear parasites and are also major driving factors in the allergic response via the release of histamines, cathepsins, peroxidases, and other inflammatory factors that result in increased capillary permeability and immune cell recruitment. Mast cells also contain granules that function as inflammatory mediators, such as histamine or proteases, that aid in the defense and response to parasites but migrate from the BM to peripheral tissues before maturing (Stone, Prussin, and Metcalfe 2010). One of the most critical cells in the immune system is the dendritic cell (DC). DCs engulf particulates by micropinocytosis and, through this process, degrade pathogens to prepare them for presentation to T immune cells. DCs contain PRRs, and through PRR recognition, they release a range of protein mediators called cytokines, which direct other immune cells to make individual responses suited for the type of pathogen to which a response must be made (Ferrantini, Capone, and Belardelli 2008).

The Adaptive Immune System

The Common Lymphoid Progenitor (CLP) is the cellular precursor of adaptive immune cells (Murphy and Weaver 2016). The adaptive immune system cells are called lymphocytes; they contain specific and highly variable regions on their surface that they use to recognize foreign proteins. Through interactions between these antigen receptors on the surface of lymphocytes and foreign antigens, adaptive immune cells acquire both effector functions and memory capabilities, which allow these cells to respond to specific infections upon repeated pathogen exposure (Murphy and Weaver 2016). Naïve

lymphocytes are lymphocytes that have not yet confronted antigen; after encountering antigen, lymphocytes become activated and differentiate into lymphocytes that encompass effector functions (Mitchison 1971). Two central lymphocytes are in the adaptive immune system: B lymphocytes (B cells) and T lymphocytes (T cells). B cells and T cells contain differences primarily in the expression of their surface antigen receptors which subsequently serve various functions.

B cells express B cell receptors (BCRs) produced from the same genes that encode antibodies (Abs); the secreted form of BCRs are known as immunoglobulins (Igs). When BCRs bind to a foreign antigen, B cells proliferate and differentiate into plasma cells that secrete antigen-specific antibodies that increase specificity to a particular protein upon repeated exposures to an antigen (Cooper, Peterson, and Good 1965). B cells require assistance from T cells in the form of costimulatory ligands and cytokines, allowing them to differentiate and mature fully (Miller and Mitchell 1968). Initially, naïve B cells are activated in the lymph node. Antigen (Ag) enters the lymph node via passive diffusion or active transport carried by macrophages. Ag that is brought to the lymph node (LN) is coated in iC3b, which can bind to complement receptor 2 (CR2) present on Subscapsular space (SCS) macrophages that line the subcapsular space of the LN (Fearon and Carter 1999). iC3b is an inactivated component of the complement protein C3; it coats antigens through attachment via a labial thioester bond. (Law, Lichtenberg, and Levine 1980) SCS macrophages further transfer Ag to non-Ag specific B cells, which further transfer Ag to Follicular Dendritic Cells (FDCs). FDCs hold Ag, and when B cells that express a cognate receptor come into contact with Ag and iC3b, which is bound to the Ag and binds further to CR2 on B cells, B cells are fully activated and begin to express transcription factors AP-

1, NFκB, and cNFAT (Murphy and Weaver 2016). In naïve B cells, expression of these transcription factors leads to the release of cytokines and trafficking of the now-activated B cell to the cortical: paracortical region of the LN where B cells can receive necessary co-stimulatory signals from T cells.

Additionally, the B cell internalizes the Ag and expresses its peptides on MHC II. The B cell is then present at the cortical: paracortical junction, where it comes into contact with a cognate T cell. Here, CD4 T helper cells contact B cells that express peptides on MHC II via contact with the T cell receptor (TCR). Further, the CD40 ligand (CD40L) on T cells binds to CD40 on B cells, and T cells release cytokines to B cells that provide it with instructions on what type of response to make (Armitage et al. 1992). B cells begin to express CXCR5, which allows the B cell to form what is known as a germinal center. Here the B cell can undergo somatic hypermutation (SMH) processes, enabling antibodies to become higher affinity and Isotype Switching (ISW). This process will allow B cells to produce an isotype required for a particular immune response against a pathogen. ISW is made possible by the cytokine secretions provided by T cells; specific cytokines cause low-level transcription of short complementary mRNA called S-transcripts that bind to its complementary sequence in the portion of the locus that codes for the constant region of an antibody-forming a recombination loop (R-loop). Activation-induced deaminase (AID) changes cysteine (C) to uracil (U), and Uracil-DNA glycosylase (UNG) cleaves U sites, leaving behind an abasic site. This abasic site is recognized by Apurinic/apyrimidinic endonuclease 1 (APE-1), which creates dsDNA nicks, splicing out the entire contained DNA segment in between the beginning and end of the R-loop. DNA repair enzymes such as Ku70/80 and DNA repair the DNA fragment, resulting in a non-reversible process that

yields a new fragment of DNA, coding for a particular isotype (Murphy and Weaver 2016). The affinity maturation process then occurs by testing B cell Antibody affinity to its cognate antigen and ensuring the most efficient antibody responses are maintained (Wabl and Steinberg 1996).

T cells serve multiple functions in the adaptive immune response and can subsequently differentiate into diverse types of effector T lymphocytes, each with different roles in the immune response. Primarily, αβ T cells, T cells that are comprised of an α and β subunit, recognize linear peptides (Hayday et al. 1999). CD4 T cells conventionally recognize MHC II and are called "helper" T cells. These cells have a variety of functions and are associated with helping B cells to produce complete Ab responses via secretion of cytokines that direct B cells to isotype switch to the necessary Ab type. CD4 T cells also secrete cytokines to recruit other immune cells and expand cell populations. The type of CD4 T cell that is differentiated is dependent on initial activation signals and cytokines that DCs provided during the activation of the cell in the LN, based on the specific PRRs that were activated within the DC. Several CD4 T cells exist, including Th_1, Th_2, Th_{17}, T_{reg}, and T follicular helper cell populations, though many new diverse populations of CD4 T cells are being discovered and characterized though they will not be covered here (O'Garra 1998).

Each CD4 T cell population functions to create a detailed response to a pathogen. Th_1 CD4 T cells are often produced in response to systemic infections and IL-12 secretion by DCs. They are associated with the release of IFNγ and further the production of IgG made after signaling through IFNγR and further signaling through STAT1, which dimers to induce Igγ S-transcripts and production of IgG, typically used to clear systemic

infections of viral and bacterial origin. Th_1 cells also recruit and activate macrophages, NK cells, and CD8 T cells (Trinchieri 1993; Murphy and Weaver 2016). Th_2 CD4 T cells are often produced in response to IL-4 cytokine signals provided by DCs. These T cells produce IL-4, IL-5, and IL-13, among other cytokines that allow for tailored responses typically associated with extracellular pathogens. IL-4 signals through IL-4R, which further signals through STAT 3/6 to induce Igε S-transcripts which cause isotype switching to IgE, which binds to mast cells and enhances immunity at epithelial surfaces. Cytokines produced by Th_2 CD4 T cells typically activate and recruit Eosinophils, basophils, mast cells, and macrophages to help combat an extracellular infection (Walker and McKenzie 2017). Th_{17} CD4 T cells are often produced in response to IL-17 and IL-22 provided by DCs. TGFβ signals through TGFβR, which further signals through Smad2/3 to induce Igα S-transcripts which cause isotype switching to IgA; this allows for an effective response against mucosal pathogens (Korn et al. 2009). T regulatory cells (Tregs) are often made in response to TGFβ and IL-2, they secrete TGFβ and IL-10 to regulate and suppress T cells. These responses are often made in response to the presentation of self-peptides to a T cell, and they increase during metastasis (Roncarolo, Levings, and Traversari 2001). T follicular helper (T_{FH}) cells play a significant role in the affinity maturation of B cell responses, allowing B cells to re-enter what is known as the cyclic reentry model of affinity maturation. These cells are differentiated in response to IL-21, they secrete IL-10 (Crotty 2019). CD4 T cells are necessary for a complete adaptive immune response, they in turn can also impact the degree of CD8 T cells present.

CD8 T cells are known as cytotoxic T cells (Zinkernagel and Doherty 1974). They typically can kill virus-infected or cancerous cells after a foreign antigen has been

presented on MHC I. These cells kill target cells via perforin granzyme B-dependent killing or death receptor pathways, including Fas/FasL and the TNF/TNFR family death receptors (Wong and Choi 1997). The perforin granzyme B pathway is typically activated under conditions of high antigen stimulus, like those associated with a viral infection. Perforin is a molecule that polymerizes and forms pore-like structures in the membrane of a target cell upon activation, acting as a transporter for granzyme B. Granzyme B then causes activation of caspase 3, through cleavage of pro-caspase 3, which further degrades the inhibitory subunit of the Inhibitor of caspase-3– activated DNase (iCAD), yielding a functional Caspase-3– activated DNase (CAD), which degrades cellular DNA and results in apoptosis.

Death receptor pathways are more likely to be activated under conditions of low antigen stimulus, such as those associated with antigen presentation during malignancy. In one commonly utilized death receptor pathway, the Fas cytolysis pathway, FasL on a cytotoxic cell binds to Fas on a target cell; this binding results in a conformational change in Fas, resulting in binding to the death domain-containing adaptor proteins. These adaptor proteins can activate caspase 8, which eventually activates Caspase 3, ultimately resulting in the activation of iCAD and apoptosis of the cell. Other death receptor pathways, such as TNF/TNFR, function similarly to the Fas/FasL pathway described here, resulting in cellular apoptosis. Both the perforin granzyme B-dependent pathway and the death receptor pathways described here function through the activation of caspase three and the subsequent activation of iCAD, resulting in the degradation of cellular DNA and apoptosis of the target cell (Trapani and Smyth 2002; Aggarwal 2003). These pathways serve a valuable role in the immune response to viral infections and cancer.

The Bridge Between the Innate and Adaptive Immune System

Though both the innate and adaptive immune systems serve different functions in their response to pathogens, both impact one another and effectively modulate the magnitude of the immune response. The innate immune system works to prime, direct, and establish the magnitude of adaptive immune responses in many ways. At the same time, the adaptive immune system provides a specific and overwhelming immune response to eliminate a pathogen. Still, it requires initial input from innate immune cells to create and deliver this response. The PRRs are a major driving factor in alerting adaptive immune cells to respond to a particular threat; they recognize common or conserved microbial components known as pathogen-associated molecular patterns (PAMPs). When a pathogen enters a cell and PAMPs associated with the pathogen make contact with a PRR, a range of downstream signaling events occur, often releasing cytokines that can act on neighboring cells to respond to infection (Iwasaki and Medzhitov 2010). These cytokines can instigate an inflammatory response within tissues. Releasing pro-inflammatory cytokines will ultimately recruit other immune cells, such as macrophages and DCs; these cells further alert and activate adaptive immune cells (Murphy and Weaver 2016). Activation of PRRs also can result in specific cellular processes, such as those associated with the degradation of cellular DNA or RNA (Akira, Uematsu, and Takeuchi 2006), processes which will be described further in later sections.

Though the complement system is considered innate, it plays a significant role in activating substantial adaptive immune responses. Components of the complement system are also needed to engage initial immune cell responses fully. In fact, deficiencies in

elements of the complement system often are supplementary to deficits not only in complement activation and subsequent innate responses to pathogens but also in the generation of significant adaptive responses, such as T cell activation and proper SMH and ISW regarding the generation of Abs by B cells (West, Kolev, and Kemper 2018; M B Fischer et al. 1996). Complement activation occurs through several pathways, the classical pathway, the mannose-binding lectin (MBL) or Lectin pathway, and the spontaneous or alternative pathway. Each pathway results in the cleavage of C2, C3, C4, and C5 proteins, which produce potent anaphylatoxins. Anaphylatoxins can act on blood vessels and increase vascular permeability. This permeability further allows immunoglobulin and complement components to enter the area through leakage from the blood (José, Forrest, and Williams 1981). Cleavage of C5 results in C5a and C5b; C5b complexes with C6, C7, and C8. C8 inserts into the membrane and binds C9 molecules, recruiting many additional C9 molecules. These additional C9 molecules form a pore known as the membrane attack complex (MAC) that causes loss of membrane potential in the target cell or viral membrane in which the MAC was formed.

Additionally, the vascular permeability caused by anaphylatoxin release during the complement cascade increases the migration of macrophages, polymorphonuclear cells (PMNs), and other lymphocytes. Macrophages are essential to cells in alerting and activating B cells in the lymph node. Macrophages require binding to the anaphylatoxin C5a to phagocytose C3b coated bacteria or other pathogens via binding of CR1 on macrophages to C3b on bacteria in conjunction with binding the C5a receptor on macrophages to C5a in serum. Further, C3a serves a similar function in recruiting and activating DCs (Gutzmer et al. 2004). Both macrophages and DCs travel to the LN to

activate adaptive immune cells, such as B and T cells, to allow for adaptive responses to infection. Factor I in the complement system cleaves C3b to iC3b, which is required to present Ag to B cells in the LN properly and fully activate B cells via recognition of iC3b by CR2 on B cells. In these ways, the innate immune system and the adaptive immune system encompass separate functions but still work in conjunction with one another to provide adequate responses against pathogens.

The Innate Immune System's Response to Viral Infection

The innate immune system is the body's first response to infection within a host before a tailored reaction by the adaptive immune system can occur. Primarily, innate immune cells respond to conserved pathogenic sequences and signals recognized by PRRs. Several PRRs have been identified in recent decades, including Toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (Creagh and O'Neill 2006). Recognition by PRRs can result in the release of inflammatory cytokines that can either directly act on pathogen-infected cells by initiating intracellular mechanisms that limit pathogen spread or by alerting other innate immune cells to the site of infection.

TLRs are present both inside of a cell and on the surface of a cell. Intracellular TLRs often recognize ssRNA or dsRNA, whereas extracellular TLRs often recognize pathogen components such as Lipopolysaccharide (LPS) (TLR4) that might be present on the surface of extracellular pathogens. Signaling through TLRs generates a downstream signaling cascade tailored to the detected pathogen, often, this response will generate the release of pro-inflammatory cytokines or type I interferons (IFNs) (Uematsu and Akira

2007). Type I IFNs, which can be produced by all nucleated cells, play a significant role in the immune system's antiviral response by causing apoptosis in virus-infected cells and poising neighboring cells to prepare for viral infection through the production of hundreds of interferon-stimulated genes (ISGs) which are expressed after IFN binds to the IFN receptor (IFNR) and allows for downstream JAK/STAT signaling to occur. STAT proteins subsequently dimerize and translocate to the nucleus, resulting in the transcription of ISGs (Raftery and Stevenson 2017). ISGs, when translated, can cause many different responses within a cell, including the activation of mechanisms that alter cellular function to combat infection. One family of proteins known as the oligoadenylate synthetase (OAS) proteins, when activated, will cause other components constitutively expressed in a cell to activate and cut RNA within a cell to protect against viral infection (Li et al. 2016); these mechanisms regarding OAS proteins will be discussed further in future chapters.

Recently, it has been established that the retinoic acid-inducible gene-I (RIG I) /melanoma differentiation-associated gene 5 (MDA5)–mitochondrial antiviral-signaling protein (MAVS) axis represents the primary mechanisms associated with the innate immune response to the cytosolic presence of RNA, often indicative of viral infection (Ni, Ma, and Damania 2018). Both RIG-I and MDA5 are members of the RLR family of PRRs. RIG-I and MDA5 play non-redundant roles in the recognition of RNA, as each recognizes a different group of viral RNA. RIG-I 5' triphosphate double-stranded RNA, while MDA5 typically recognizes long dsRNA originating from viral genomes (Wu and Chen 2014). Activation of both RIG-I and MDA5 induces polymerization of MAVS, which recruits and activates E3 ligases, tumor necrosis factor receptor (TNFR)-associated factor proteins, TRAF2, TRAF3, TRAF5, and TRAF6. Activation of TRAFs causes the synthesis of

polyubiquitin chains which bind to the Nuclear factor-kappa B Essential Modulator (NEMO). NEMO recruits inhibitor of nuclear factor kappa B kinase (IKK) and TANK-binding kinase 1 (TBK1), which complexes to MAVS, where the kinases are able to phosphorylate and activate the inhibitor of nuclear factor kappa B alpha (IκBα) and interferon regulatory factor 3 (IRF3), which lead to the induction of type I IFNs as well as other anti-viral cytokines (Wu and Chen 2014).

The IFN-induced protein kinase RNA-activated (PKR) pathway is another central anti-viral pathway cells utilized to combat viral infections. As previously mentioned, IFN is typically released from neighboring cells and binds to the IFNR when a viral infection is present, triggering the production of ISGs, including PKR. The promoter region of PKR contains elements to which Type I IFNs bind, inducing transcription of PKR genes. PKR is generally further activated in response to dsRNA within the cytoplasm. PKR can recognize dsRNA regardless of whether the dsRNA is of cellular, viral, or synthetic origin as long as it is larger than 30 nucleotides. This recognition results in the dimerization of PKR's kinase domains, resulting in an auto-phosphorylation reaction and activation of the protein. Once PKR is activated, it phosphorylates the protein synthesis initiation factor, eukaryotic translation initiation factor 2 subunit 1 (eIF2α) (Gal-Ben-Ari et al. 2019). The now activated and phosphorylated eIF2α forms a complex with another protein called eIF2B. eIF2B is responsible for the exchange of GTP for GDP, which is required for protein translation initiation. Phosphorylation of eIF2α prevents this exchange reaction, resulting in the sequestration of eIF2 bound to GDP and in its inactive state. As a result, the availability of active eIF2-GTP complexes decreases, and protein synthesis is stifled (J. J. Chen and London 1995). This protein synthesis ablation results in the cell's eventual

apoptosis and death, preventing further viral replication. Additionally, PKR activates the kinase complex IKKα/IKKβ, which activates nuclear factor-κB (NF-κB) transcription factor by phosphorylating its IκBα inhibitor. This activation results in the transcription of many ISGs, including Type I IFNs which can act on neighboring cells to establish an anti-viral state (García, Meurs, and Esteban 2007; Li et al. 2016; Baglioni, Minks, and Maroney 1978; Roberts et al. 1976).

Supplementary, the cyclic GMP-AMP synthase (cGAS)–stimulator of interferon genes (STING) pathway is primarily associated with the innate immune response to cytosolic DNA, typically indicative of infection with DNA-based viruses that have entered a cell and are present in the cytoplasm. However, it also has recently been shown to be involved in restricting viral RNA infection (Ni, Ma, and Damania 2018). Canonically, cGAS binds to cytosolic dsDNA in a non-specific albeit length-dependent manner (Andreeva et al. 2017). cGAS catalyzes cyclic guanosine monophosphate (GMP)-adenosine monophosphate (AMP) (cGAMP) in the presence of GTP and ATP, which results in binding to STING in the endoplasmic reticulum (ER) membrane. STING dimerizes and traffics from the ER to the Golgi complex, where it recruits TBK1, activating IRF3 and NF-κB. These translocate to the nucleus, where they induce type I interferon (IFN) transcriptional activation and the production of other inflammatory cytokines (Barber 2015). In mice, deficiencies in the cGAS-STING pathway have been associated with increased susceptibility to DNA virus infection (Ma and Damania 2016). However, defects in the cGAS-STING pathway have also been described for multiple RNA viruses such as vesicular stomatitis virus (VSV), Sendai virus (SeV), dengue virus (DENV), and

West Nile virus (WNV) (Schoggins et al. 2013). However, the mechanisms underlying how cGAS is involved in suppressing RNA viral replication are still being elucidated.

One proposed mechanism of the cGAS-STING pathway's impact on the suppression of RNA viral replication is that certain viruses, like Dengue virus (DENV), cause host cell damage and release cellular DNA into the cytoplasm, such as through mitochondrial damage. In doing this, cGAS may bind host DNA and activate (B. Sun et al. n.d.). Further, STING has been found to interact with RIG-I and MAVS, both components of the RNA sensing pathway. Additional select studies showed that loss of STING might impact IFN production required to create a complete anti-viral response for RNA viruses (Ni, Ma, and Damania 2018). In fact, recently, STING, but not cGAS, was found to be needed for full interferon production caused by infection with enveloped RNA viruses such as influenza A virus (IAV) (Holm et al. 2016). STING is also required to produce antiviral cytokines such as C-C motif chemokine ligand 2 (CCL2) and C-C motif chemokine ligand 20 (CCL20), which impact the replication of some RNA viruses (H. Chen et al. 2011).

NOD-like receptors (NLRs) are similar to TLRs in structure and serve as an additional way for cells to combat viral infection and defend against other microbial pathogens. There are 22 known NLRs in humans; NLRs can recognize a large variety of microbial ligands and changes in intracellular ions that contradict a homeostatic environment (Hong, Yoon, and Wilson 2012). A more recent publication detailed that NLRs can bind ssRNA and dsRNA, indicating that NLRs may be able to bind RNA directly. The functions of NOD-like receptors are broken into four categories: inflammasome formation, signaling transduction, transcription activation, and autophagy (Y. K. Kim, Shin, and Nahm 2016). Inflammasome formation is able to be activated by

eight NLRs (NLRP1, NLRP2, NLRP3, NLRP6, NLRP7, NLRP12, NLRC4, and NAIP) and can occur in response to the binding of microbial products to NLRs causing activation and a conformational change leading to multimerization. Activation of the inflammasome mainly results in the activation of Caspase 1 and subsequent cleavage of pro-inflammatory cytokines such as IL-1β and IL-18. Inflammasome activation can also result in pyroptotic cell death (Jacobs and Damania 2012). NLRs can also play a role in signal transduction by signaling through receptor-interacting protein 2 (RIP2) after contact with microbial peptides, leading to NF-kB activation. NF-kB then translocates to the nucleus and enhances the transcription of proinflammatory cytokines (K. Kobayashi et al. 2002). NLRs take part in transcription activation in that several studies have shown that the expression of MHC I and MHC II depends not only on several transcription factors but also on the presence of NLRA and NLRC5 (K. S. Kobayashi and Van Den Elsen 2012; Motta et al. 2015). Autophagy is the normal cellular process where organelles are digested for cellular turnover. Multiple NLRs can induce autophagy to remove pathogens, allowing recognition of viral components in various portions of the cell (Y. K. Kim, Shin, and Nahm 2016; Jacobs and Damania 2012).

The antiviral proteins that recognize viral infections within a cell work together to maintain cellular integrity or to reduce the replication of a virus. For this reason, there exists a similarity between several innate proteins that allows for functional redundancy and additional supplementation of the anti-viral immune response. The RLR family of PRRs shows similarities with viral TLRS in that they signal the activation of NF-κB and IRF3 and ISGs such as type I IFNs (Creagh and O’Neill 2006). Additionally, the RLR family of PRRs is similar to the NLR family of PRRs NLRs because they contain CARD

domains which ultimately result in IRF3 and NF-κB activation (Creagh and O'Neill 2006). TLR pathways and NLR pathways also interact in that TLR pathways poise NLR signaling. For instance, TLR activation can lead to the production of pro-IL-1β and pro-IL-18, while NLR activation can lead to the production and activation of caspase 1. Caspase 1 can result in the cleavage of pro-IL-1β and pro-IL-18 into active IL-1β and IL-18, resulting in inflammation and can induce NLR pathways (R. S. T. Tan et al. 2014). Additionally, NLR pathways can poise TLR signaling, secreted active IL-1β binds to IL-1β receptor, which increases the expression of MyD88 and IRAK4, both of which are required for downstream signaling of TLRs (R. S. T. Tan et al. 2014). PRRs described here can detect viral RNA or DNA and intermediate viral products, with many of these receptors recently identified and still being understood (Koyama et al. 2008).

The Adaptive Immune System's Response to Viral Infection

As previously mentioned, the adaptive immune system is informed of pathogenic infection by the innate immune system, primarily through cytokine-mediated signals. In this sense, the innate immune system can regulate the adaptive immune system by informing it on what response is needed for a particular infection (Iwasaki and Medzhitov 2010). After recognition of a pathogen by PRRs, dendritic cells produce cytokines, such as IL-12, which direct naïve CD4 T cells to transition into T_H1 cells, capable of amplifying adaptive anti-viral responses (Macatonia et al. 1995). T_H1 cells are typically responsible for coordinating and strengthening the host response to viral infections, which can occur through various mechanisms, including classical activation of macrophages and continued release of pro-inflammatory cytokines, which reinforce innate anti-viral pathways and lead

to the recruitment of other immune cells (Janeway and Medzhitov 2003). Through cytokine release, T_H1 cells can further recruit other phagocytic cells to the site of infection. T_H1 cells secrete hematopoietic growth factors such as IL-3 and GM-CSF, which stimulate new monocytes in the bone marrow. Additionally, they secrete TNFα and lymphotoxin at the sites of infection, which alters the surface proteins on endothelial cells so that monocytes can better adhere to them (Murphy and Weaver 2016).

Under non-inflammatory conditions, CD4 T cells outnumber CD8 T cells in the spleen, lymph nodes, and serum. However, CD8 T cell populations expand during viral infections and disproportionately outnumber CD4 T cells (Butz and Bevan 1998). T_H1 cells typically activate CD8 cytotoxic T cells during viral infection. These cells are often also capable of recognizing a virus-infected cell and terminating it directly (Murphy and Weaver 2016). During the initial adaptive immune response to a virus, both CD4 and CD8 T cell populations proliferate and expand greatly and typically begin to secrete IFNγ in response to continued peptide stimulation, resulting in a massive expansion of antigen-specific cytotoxic T cells as well as reinforcement of innate and adaptive immune pathways (Butz and Bevan 1998). T-cell populations are the first adaptive immune cells to expand in response to viral infection. B cell activation in response to viral infections typically occurs after T cell activation. B cell responses begin with the secretion of low-affinity IgM antibodies; these can primarily bind to a pathogen and activate complement through the previously described mechanisms, releasing anaphylatoxins and reinforcing immune responses (Gonzalez et al. 2011).

Additionally, IgM Abs can neutralize viruses during early viral infection, preventing cell entry through antibody receptor binding interactions (Salvo et al. 2018).

Once the activation of a B cell has occurred in an LN, it travels to the cortical: paracortical region of the LN, where it attempts to make contact with a cognate CD4 T cell. Here the B cell receives cytokine messengers and the essential CD40L: CD40 interaction which allows a B cells to become fully activated and form germinal centers (GCs). In GCs, B cells undergo ISW and SMH, enabling them to have a higher affinity for the antigen and to become the correct isotype to generate a sufficient response to pathogen infection (den Haan, Arens, and van Zelm 2014). Adaptive immune responses will occur if the antigen is presented to B and T cells, regardless of whether or not it has been cleared from a host's system.

The Innate Immune Systems Response to Cancer

Innate immune cells also play a role in anti-cancer immune responses. DCs and macrophages can aid indirectly in eliminating cancerous cells by recognizing damage-associated molecular patterns (DAMPs), which allows for the recruitment and activation of other effector cells. Natural Killer (NK) cells, Natural Killer T cells (NKT), and γδ T cells have evolved mechanisms that allow for the direct killing and elimination of cancer cells (Woo, Corrales, and Gajewski 2015). Type I interferons released by macrophages characteristically activate NK cells. NK cells are then brought to the site of an immune response, where they begin to survey the cells in that area. NK cells have germline-encoded receptors that recognize molecules on the surface of cells, constantly monitoring for aberrant expression of MHC class I molecules (Waldhauer and Steinle 2008). When an NK cell comes into contact with MHC I, it is sent a signal instructing it not to attack the cell it is monitoring, as it is recognized as self. During the process of metastasis and subsequent

alterations in gene expression, cancer cells often downregulate the expression of MHC class I molecules (Cornel, Mimpen, and Nierkens 2020). For this reason, NK cells can directly kill and eliminate cancerous cells through two main mechanisms. First, upon activation, NK cells can release cytotoxic proteins, including granzymes and the pore-forming protein, perforin (Smyth et al. 2005). The second pathway that NK cells use to kill target cells is through a TNF family member known as tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). TRAIL is expressed on the surface of NK cells and interacts with two TNFR superfamily dearth receptors known as DR4 and DR5. TRAIL stimulated DR4 and DR5 to activate the proenzyme caspase 8, leading to downstream cleavage of caspase 3 and eventual degradation of host genetic material, leading to apoptosis of the cell (Smyth et al. 2005; Murphy and Weaver 2016). Due to their ability to eliminate cancerous cells upon receptor recognition, NK cells have been exploited to design some cancer immunotherapies, such as bi-specific NK cell engagers (BiKEs) and tri-specific NK cell engagers (TRiKEs) (Felices et al. 2016). BiKEs are comprised of an α-CD-16 single chain variable fragment (scFv), which activates NK cells when bound, and an scFv specific to a particular cancer epitope, while TRiKEs are comprised of an α-CD-16 scFv linked to An Activating cytokine which is further coupled to an scFv that is specific to a particular cancer epitope (Davis et al. 2017).

NKT cells are often considered part of the innate and adaptive immune system. They function differently from conventional NK cells because they are CD1d restricted, meaning they recognize antigens presented by a class Ib MHC on antigen-presenting cells (APCs) (Terabe and Berzofsky 2014). NKT cells recognize and kill CD1d-expressing tumor cells that present tumor-specific antigens to immune cells (Dhodapkar et al. 2003).

They further produce IFNγ that induces the maturation of DCs. Mature DCs further produce IL-12, which supplements IFNγ and IL-2 production by NKT cells and enhances anti-tumoral responses (Terabe and Berzofsky 2008).

γδ T cells function similarly to NKT cells in antigen recognition and killing of cells; they are typically tissue residents who recognize lipids and carbohydrates presented on a cell's surface. Recent studies have indicated that they impact the anti-cancer immune response peripherally by serving as an early source of IFN during the anti-cancer response. This IFN release by γδ cells activates and enables other immune cells to combat cancerous cells (Y. Gao et al. 2003).

The Adaptive Immune System's Response to Cancer

Much like the adaptive immune response to viral infections, the adaptive immune response to cancer is specific to a particular epitope. The adaptive immune response to cancer is typically generated in response to neoepitopes, which are unique protein fragments produced by genetic mutations in cancer cells. Neoepitopes are presented by cancerous cells on MHCI and MHC II and subsequent recognition by CD8 and CD4 T cells, respectively. Since the immune system does not recognize neoepitopes as self, adaptive immune responses, including CD4 and CD8 T cell responses to these epitopes, often follows (Hansen et al. 2020).

CD4 T cells play a pivotal role in the anti-tumor immune response as they are directly and peripherally involved, through effects on both innate and adaptive immune cells such as macrophages and CD8 T cells. Additionally, CD4 T cells can act directly on unhealthy cells that present neoepitopes to eliminate them through differentiation into Th1

cells and further elimination of these cells via perforin/granzyme B-dependent killing or target cell elimination via ligation of Fas/FasL (or other TNF/TNFR family) death receptors (K. U. Lundin et al. 2004). Activated CD4 T cells release cytokines such as IL-2 and IFN-γ, which promote the adaptive immune response. IL-2 drives the expansion of CD4 and CD8 T cells, increasing the magnitude of the response. IFN-γ can bind to its receptor on immune cells such as APCs, which further drives these cells to secrete IL-12, reactivation the IFN-γ production cycle (Jorgovanovic et al. 2020). Secretion of IL-2 and IFN-γ by CD4 T cells has also been found to elicit M1 macrophages to inhibit tumor propagation via secretion of nitric oxide synthase (iNOS), reactive oxygen species (ROS), and IL-12. Additionally, M1 macrophages secrete high levels of pro-inflammatory cytokines that act on neighboring cells to increase the anti-cancer immune response (Hao et al. 2012). These effector functions result in direct and indirect clearance of tumor cells.

The presence of CD8 T cells within the TME has been associated with improved tumor clearance and overall prognosis, often characterized by the simultaneous presence of pro-inflammatory cytokines such as Type I IFN (Trujillo et al. 2018). The mechanisms by which CD8 T cells within the TME are believed to eliminate cancerous cells are primarily via death receptor pathways such as through the ligation of the Fas ligand (Fas L) on T cells with the Fas receptor (FasR) on target cells, but they are also able to eliminate cancerous cells via the perforin/ granzyme B pathway, with a preference for FasL/FasR pathway for cancerous cells (Chávez-Galán et al. 2009). Though these mechanisms can aid in the clearance of tumors, tumor-infiltrating lymphocytes often display upregulation of inhibitory markers, such as PD-1 and CTLA-4, which bind PD-L1 and CD80/CD86, respectively, which can halt anti-tumoral effector functions and result in reduced effector

cytokine production (Ahmadzadeh et al. 2009). These inhibitory markers can be overexpressed on progressive malignancies for some cancers, making it less likely that T immune cells will generate responses to cancerous cells as metastasis progresses.

Several studies indicate that patients with progressive malignancies show substantial alterations in their circulating antibody repertoires compared to when no malignancy is present (Narang et al. 2019; Stafford et al. 2014a). These changes can be used to identify patients with progressive malignancy and patients that may have a particular form of cancer prior to a palpable tumor being detected (Stafford et al. 2014a). These changes in circulating Abs could be due to multiple factors, one being the adaptive response to cancerous neoepitopes causing an expansion in the pool of B cells specific to a given protein sequence. Generation of Abs against neoepitopes may be helpful if the neoepitopes that the Abs are against are present on the surface of the cancer cell, as this might be able to recruit immune cells such as neutrophils which can release cytotoxic elements and eliminate the cell. Abs against neoepitopes serve as a potential avenue for cancer immunotherapy. For instance, there are currently treatment methods in development that aim to utilize IgE that is engineered to be specific to a particular cancer neoepitope in the hopes that an immune response can be elicited against a cancer cell via degranulation of cells that function through the Igε receptor, such as basophils, eosinophils and mast cells (Daniels-Wells et al. 2013; Leoh, Daniels-Wells, and Penichet 2015). Overall, neoepitopes presented by cancerous cells are a major driver of the adaptive immune response to cancer.

Tumorigenesis

Tumorigenesis refers to the multi-step process that leads to the initial generation of tumor cells in the body. This process is characterized by increases in genomic instability and escape of cancer cells from immune recognition, resulting in tumor cell formation and immortal growth (Tabassum and Polyak 2015). The tumor surveillance hypothesis details a process known as "cancer immunoediting" in the context of the immune system's response to cancerous cells. It states that three distinct phases exist in which the immune system impacts the promotion or control of tumor cell formation, concluding in tumorigenesis. These three steps include elimination, equilibrium, and escape (Swann and Smyth 2007). It is usual for healthy cells to acquire damage that may disrupt the genetic stability of a cell. Factors that can cause this disruption include exposure to carcinogens, chronic inflammation, radiation, viral infection, and other factors that could alter the genetic makeup of healthy cells (Swann and Smyth 2007; Butel 2000). However, healthy cells have many repair mechanisms that prevent these cellular changes from evolving into cellular dysfunction, including DNA repair, senescence, and apoptosis of dysregulated cells (Figueroa-González and Pérez-Plasencia 2017). When these repair mechanisms fail, cells may begin to display tumor antigens or may secrete cellular products that are abnormal. The typical expression of proteins may also be altered at this point, such as the downregulation of MHC. The dysregulated cells may be more apparent to the immune system at this point.

This marks the first stage of the tumor immune surveillance hypothesis, the "elimination" stage. In this stage, the innate and adaptive immune system undergoes the process of cancer immune surveillance, in which cells that express tumor antigens that are

non-self may be eliminated by adaptive T cells specific to those antigens or cells that fail to express normal host proteins may be eliminated by innate immune cells such as NK cells (Waldhauer and Steinle 2008; Mehta, Gracias, and Croft 2018). In many cases, the immune system can protect the body from metastasis through extrinsic tumor suppression via the direct killing of tumorigenic cells or the release of anti-tumoral cytokines that can eliminate the cells during this phase (Swann and Smyth 2007). The cellular environment returns to homeostasis when the tumor cells are controlled or destroyed.

However, if the cancerous cells are not cleared, the next step of cancer progression under the tumor surveillance hypothesis, termed “equilibrium,” ensues. At this stage, cancerous cells persist and are not entirely eliminated though the immune system still reacts to and modulates their growth. For instance, cancerous cells may continue to multiply but do not reproduce in a large capacity because the immune system continues to eliminate and control dysregulated cells. During this phase, tumor cells may either remain stagnant or may begin to accumulate more mutations, resulting in additional neoepitopes. Tumor cells may undergo changes in protein expression within the cell, and on the cell surface, they may begin to express specific tumor-associated antigens (TAAs) resulting from accumulated mutations and subsequently translated proteins. As previously mentioned, the cancer cells may also start downregulating proteins typically represented by the cells, such as MHC, which can activate innate immune cells, such as NK cells (B. C. Taylor and Balko 2022; Waldhauer and Steinle 2008).

Cells may start to appear unrecognizable to the immune system or may instigate tolerance of immune cells. When control mechanisms by the immune system fail, tumor cells progress to the “escape phase”, in which cancer begins to progress. Several studies

have indicated that at this phase, even T cells with specificity to tumor antigens may be unable to perform typical effector functions when stimulated with tumor Ags, such as degranulation or release of IFNγ and release of pro-inflammatory cytokines (Anderson et al. 2007). Several mechanisms are believed to be responsible for this tolerization of immune cells, tumor cells themselves can secrete immunosuppressive cytokines such as TGF-β and IL-10 as well as enzymes such as indoleamine-2,3-dioxygenase (IDO), which catabolize amino acids that are required for proper T cell function (Belladonna et al. 2008; Katz, Muller, and Prendergast 2008). Tumor-associated DCs also release cytokine signals associated with immune suppression, such as PD-L1, arginase (ARG), and IDO (Hurwitz and Watkins 2012). Further, tumor cells across several cancers often begin upregulating immunosuppressive molecules on their surface, such as PD-L1, resulting in T cell dysfunction (Nakanishi et al. 2006; Abiko et al. 2013; Q. Gao et al. 2009). Collectively, these tolerization mechanisms reduce the ability of T cells to react and control tumor cells, leading to tumor progression and eventual metastasis.

Metastatic Osteosarcoma (mOS)

Osteosarcoma (OS) is the most common bone cancer in children and adolescents, with survival rates declining from ~70% to ~20% upon metastasis from the bone of origin (Lindsey, Markel, and Kleinerman 2017; Czarnecka et al. 2020). The incidence rate of osteosarcoma as of 2021 was at a projected ~1,000 cases diagnosed per year, with the majority of these cases in children and adolescents ("Home | American Cancer Society - Cancer Facts & Statistics" n.d.). It is predicted that cases of adolescent OS will only increase in the coming years, as past trends have shown that little progress in the

development of treatment for the disease has resulted in ongoing poor prognoses for patients, but increases in population and respective illness are expected to continue (Eyre et al. 2010).

Osteosarcoma is believed to originate from mesenchymal cells and is subclassified according to its predominant stroma, often comprised of osteoblastic, chondroblastic, fibroblastic, or giant cell-rich tumors, among others (Abarrategi et al. 2016). However, the predominant stroma is not believed to have prognostic importance or implications for the effectiveness of therapy but may be necessary to identify metastasis (Abarrategi et al. 2016). Most OS cases initiate in long bones, with common metastasis to the mucosa of the lungs (Lindsey, Markel, and Kleinerman 2017; Abarrategi et al. 2016). Pulmonary arteries are the most common site of metastases across many cancers, likely because the lungs are a rich source of vascularity and are the first reservoir for lymphatic drainage before entering the systemic venous system (Gerull, Puri, and Kozower 2021). For cancers like mOS, cells undergo many cellular component changes due to genetic alterations resulting in the metabolic reprogramming of pulmonary cells, changes that benefit from vascularization (Du et al. 2023). In this way, the lungs serve as an opportunity for mOS cells to flourish.

Traditional treatment methods for metastatic osteosarcoma (mOS) involve surgery, radiation therapy, chemotherapy, or a combination of all three. Limb-salvage surgery has also been pursued in which tumor cells and the bone of tumor origin are removed and subsequently replaced with a prosthetic. However, this process risks the return of metastasis if all cancerous cells are not removed during the initial procedure (Y. Yang et al. 2018). Still, current treatment methods have shown limited efficacy and high mortality rates even after the conclusion of treatment (Lindsey, Markel, and Kleinerman 2017). A

drive to elucidate and resolve these obstacles has led to discoveries of mOS resistance mechanisms to traditional treatment methods and has augmented the pioneering of alternative therapies (Chou and Gorlick 2006).

Progress In Immunotherapy for Metastatic osteosarcoma (mOS)

Immune checkpoint blockade (ICB) that employs α-CTLA-4 and α-PD-L1 has shown promise in improving survival rates amongst various malignancies (Y. J. Park, Kuen, and Chung 2018). We have previously demonstrated that human mOS cells express PD-L1, a ligand for PD-1 on cytotoxic T lymphocytes (CTL), implying that expression may limit CTL control of mOS (Lussier, O'Neill, et al. 2015). Similarly, murine mOS express PD-L1, and we have shown that combination α-PD-L1 treatment increases T cell killing of mOS cells, further reduces tumor burden, and extends lifespan in mice inoculated with mOS before treatment, resulting in prolonged survival but not complete eradication of mOS (Lussier, Johnson, et al. 2015). A combination of α-CTLA-4 and α-PD-L1 displayed improved progression-free survival rates among ICB-treated patients in preclinical and clinical trials for various cancers (Lindsey, Markel, and Kleinerman 2017; Chou and Gorlick 2006; Lussier, Nieves, et al. 2015; Topalian et al. 2014).

In addition, past studies have shown that the degree of T cells' tumor site infiltration is prognostic for tumor regression and respective patient outcomes (Al-Shibli et al. 2008; S. Liu et al. 2012). CTLA-4 is a regulatory protein expressed on T cells. It acts as a potent inhibitor of T cells by binding to B7-1 (CD80) and B7-2 (CD86) on the surface of antigen-presenting cells; this binding sequentially decreases CTL's ability to infiltrate and slow tumor progression (Lussier, Johnson, et al. 2015; Walunas et al. 1994). ICB comprised of

α-CTLA-4 and α-PD-L1 shows much promise as an effective immunotherapy candidate against several cancers, including OS. However, even though these therapies show much promise, it is not uncommon for human patients with the same malignancy to respond differently to the same cancer immunotherapies, posing an additional barrier in treatment advances (Sambi, Bagheri, and Szewczuk 2019). Response rates to therapies such as immune checkpoint blockade (ICB) in patients with various sarcomas show much inconsistency (Tawbi et al. 2017; D'Angelo et al. 2018; Petitprez et al. 2020). Unlike inbred laboratory mice, human patients often have numerous differences in genetic makeup, environment, and other circumstances that make it difficult to identify why patients with the same malignancy respond differently to the same treatment (Sambi, Bagheri, and Szewczuk 2019; Tawbi et al. 2017; D'Angelo et al. 2018; Petitprez et al. 2020). If the cause of these differences in response rate were better understood, treatment methods and overall patient prognosis would be improved.

The Impact of the Microbiome on Cancer Immunotherapy

Recent studies have demonstrated the impact of host-microbiome interactions on patient health, including disease presence and chronic inflammation (Czesnikiewicz-Guzik and Müller 2018; D. Kim, Zeng, and Núñez 2017). Further investigation in patients during cancer progression and treatment suggests the presence or absence of certain types of microbes in the gut could affect systemic pro- or anti-inflammatory processes, such as the upregulation of tumor necrosis factor (TNF), which could result in greater tumor regression (Iida et al. 2013). The presence of specific microbes in the gut can also impact ICB immunotherapy approaches. For instance, α-CTLA-4 treatment for melanoma has been

found to rely on the presence of *Bacteroides* species. At the same time, the therapy showed no effect on germ-free and antibiotic-treated mice (Vétizou et al. 2015). Depending on *Bifidobacterium's* presence, similar results were seen in the α-PD-L1 treatment of melanoma (Sivan et al. 2015). Metagenomic studies concluded that enrichment of anabolic pathways and differences in pro-inflammatory cytokines caused by some bacteria's presence affect some disparities seen in tumor response and serve as one possible reason differences in patients' microbiomes are associated with variance in ICB efficacy (Gopalakrishnan et al. 2018).

Strikingly, more recent investigations have shown fecal transplants from ICB responders to non-responders for the treatment of melanoma saw greater than one-third of human patients who previously were unresponsive to treatment became responsive after transplants, supporting a possible influence by the microbiome in ICB responsiveness and increasing curiosity to determine its role in the treatment across cancers. Discord in the microbiome has been recorded even in inbred lab mice (Laukens et al. 2016). Though the variation in microbiome composition is more significant among different strains than within the same strain, factors such as birthing conditions, cage-specific conditions, and the degree of stress experienced can cause a microbiome change composition (Laukens et al. 2016; Friswell et al. 2010; Moloney et al. 2014). Though many studies support that the microbiome and its variations profoundly impact treatment for some malignancies, investigations that characterize microbiome composition for most cancers have not been pursued (Mager et al. 2020; Vivarelli et al. 2019; Routy et al. 2018a; Almonte et al. 2021). For melanoma, studies have shown that ICB composed of α-PD-1 or α-CTLA-4 can even rely on the presence of particular microbes (Vétizou et al. 2015; Topalian et al. 2012).

Further studies showed that alterations of microbiome composition resulted in subsequent changes in the presence of metabolites thought to be secreted by these microbes, causing inhibition of ICB and chemotherapeutic drug efficacy, which can be primarily caused by antibiotic administration (J. H. Yang et al. 2017). This result is a significant finding because specific metabolites can impact cellular immune function via changes in inflammatory processes altering treatment regimen efficacy of treatments that rely on cellular immune function (C. H. Kim and Betz 2018).

Under less controlled conditions, attenuation of ICB efficacy for combination PD-1/PD-L1 has been seen in human patients to treat various cancers. For instance, antibiotics were associated with attenuated efficacy of α-PD-1/PD-L1 therapies in Chinese patients with advanced non-small-cell lung cancer (S. Zhao et al. 2019). Meta-analysis of 19 studies that involved antibiotic treatment during cancer treatments and possible effects on mainly PD-1 or CTLA-4 inhibitor therapies across human patients found that antibiotics use significantly reduced the progression-free survival in patients that were being treated with ICB among groups that were also given antibiotics when compared to those that were not (Huang et al. 2019).

On the cellular level, T cells' tumor site infiltration can significantly prognose tumor regression and the respective patient outcomes (S. Liu et al. 2012; Al-Shibli et al. 2008). Recent studies have demonstrated that antibiotic treatment can dramatically reduce CD3+ T cells in the lungs of saline-treated mice with viral infection compared to those not treated with antibiotics (Yaron et al. 2020). Since mOS metastasizes to the lungs, it is possible that a reduction in CD3+ T cells in the lungs can lead to poorer outcomes for mOS patients via a reduction in the ability for T cells to proliferate in the lungs (Abarrategi et al.

2016). Additional studies have shown that alterations of microbiome composition via antibiotic administration can lead to changes in metabolites present in patients (J. H. Yang et al. 2017). Metabolite changes can further impact cellular immune function, including the function of T regulatory cells (Arpaia et al. 2013; Rooks and Garrett 2016; C. H. Kim and Betz 2018). This could be significant for ICB for mOS because not only does mOS resonate in mucosal lung sites, but ICB to treat mOS is dependent on T cell function (Lussier, O'Neill, et al. 2015; Lussier, Johnson, et al. 2015). Microbiome composition has also been associated with the generation of initial T cell burst, which has implications regarding the overall response of T cells to tumors and can impact memory T cell formation (McCoy, Burkhard, and Geuking 2019).

T Cell Memory

T-cell memory was initially described in the context of acute or chronic viral disease. Memory T cells are essential in response to viral infections and tumor-specific antigens (TSAs), as they are the basis for cancer vaccine efficacy. In fact, immune memory to TSAs can prevent mice from developing cancer upon reinoculation with the same cancer cell line (Lussier, Johnson, et al. 2015). Memory immune cells can be divided into three different categories, which include Tissue Resident (T_{RM}), Central (T_{CM}), and Effector (T_{EM}) Memory T cells (Overacre-Delgoffe and Hand 2022). The different types of memory T cells are often characterized by their specific homing capacity and effector function; each can also be identified by differences in surface receptor expression that allows them to have differences in effector function.

Tissue Resident memory (T_{RM}) cells require inflammatory cytokines such as IFNα and IL-12 to develop in addition to transcription factors Blimp-1, Hobit, and Ahr (Behr et al. 2018). After they are activated, T cells begin to express CD69, which antagonizes S1PR1, resulting in a decrease in T cell egress and subsequent tissue residency (Overacre-Delgoffe and Hand 2022). T_{RM} cells that reside in sites outside of the LN will begin to upregulate adhesion molecules and chemokine receptors, allowing them to remain in their tissues while downregulating lymphoid-homing receptors (Dijkgraaf, Kok, and Schumacher 2021). In the tissue, T_{RMs} require additional cytokine signals from their local environment that can result in tissue retention and survival of T_{RM} cells (Nath et al. 2019).

Central memory T (T_{CM}) cells have little to no effector function and must return to an LN for full activation (Sallusto, Geginat, and Lanzavecchia 2004). Since human T_{CM} cells must return to the LN for full activation, they express surface receptors that allow them to enter the LN. T_{CM} cells are predominantly CD4+, CD450R0+, and constitutively express CCR7 and CD62L (Sallusto, Geginat, and Lanzavecchia 2004). These receptors are also present in naïve T cells, but T_{CM} cells are more sensitive to stimulation and have less of a requirement for co-stimulation. T_{CM} cells upregulate CD40L to a greater degree than naïve T cells, allowing them to provide more efficient activation of B cells and DCs. After they are activated T_{CM} cells typically produce IL-2 but further are able to produce large amounts of IFNγ or IL-4 (Sallusto, Geginat, and Lanzavecchia 2004).

Effector memory T (T_{EM}) cells typically migrate to inflamed tissues and are able to provide immediate effector function upon contact with a pathogen, this can provide a fast response to pathogens since these cells do not need to return to the LN to gain full T cell function like naive T cells must do (Sallusto, Geginat, and Lanzavecchia 2004). In

humans, T_{EM} cells no longer express CCR7, as they do not need to enter the high endothelial venules (HEV) of the LNs. Unlike T_{CM} cells, T_{EM} cells have primary effector functions upon contact with a pathogen. T_{EM} cells typically carry large amounts of perforin that can be expelled upon stimulation and are predominantly CD8+, though CD4+ T_{EM} cells do exist. Both CD4+ and CD8+ T_{EM} cells begin to produce inflammatory cytokines such as IFNγ, IL-4 and IL-5 shortly after antigenic stimulation (Sallusto, Geginat, and Lanzavecchia 2004).

Knowledge of immune memory can aid in the understanding and design of therapeutic models for cancer treatments. Typically, memory T cells can be detected even decades after exposure to antigen and a complete immune response. In fact, individuals primed with tetanus toxoid showed circulating antigen-specific T_{CM} and T_{EM} 10 years after initial priming and subsequent T cell population expansion upon immunization with boosters (da Silva Antunes et al. 2021; Warfel and Edwards 2015).

Tumor Antigens and Cancer vaccines

Antigens that are overexpressed or exclusively expressed on tumor cells are called Tumor-associated antigens (TAAs) or Tumor-specific antigens (TSAs), respectively. TAAs are proteins expressed on cells that may also be present in healthy cells but are frequently upregulated in tumor cells. TSAs are proteins expressed on cancer cells unique to tumor cells, often expressed because of non-synonymous single-nucleotide variants (SNVs) that developed during metastasis (Gubin et al. 2015). These tumor-specific proteins, called neoepitopes or neoantigens, help us identify and classify specific cancers.

Recent studies have demonstrated that therapeutic manipulation of both TAAs and TSAs has allowed for more specificity in the design of cancer immunotherapies. One avenue that aims to exploit the potential for neo-epitopes to be used as therapeutic cancer treatments is neoepitope vaccines, which seek to include mutation-associated neoantigens in the form of vaccination to create an immune response against proteins associated with tumors (Alcazer et al. 2019). In metastatic cancers, vaccination with neoepitope vaccines can alter a tumor's environment from one deficient in anti-tumoral T cells responses, a "cold" tumor, to one that increases the number of effector cells within cancer, a "hot" tumor (Alcazer et al. 2019).

Therapeutic cancer vaccines (TCVs) can target specific TAAs, target TSAs or be personalized based on a patient's tumor. Cancer vaccines targeting TSAs are typically considered safer, as they are less likely to cause off-target toxicity by reacting to proteins also present in noncancerous cells (Shemesh et al. 2021). Additionally, cancer vaccines may have the potential to be used as adjuvants for cancer therapy by administration of the vaccine in combination with other treatments such as chemotherapy or radiation, thereby increasing the effectiveness of both therapies and the overall response against tumors (Thomas and Prendergast 2016).

Methods to determine which TSAs can be used in cancer vaccines include algorithmic approaches identifying which tumor antigens might yield the most productive response. In human patients, this typically begins with a biopsy of the tumor and further characterization of the tumor-specific mutanome. Tumor biopsies are then sequenced using whole-exome and RNA sequencing, neoepitopes are then identified, and major

histocompatibility complex (MHC) class I epitope prediction algorithms are used to determine which proteins are the best targets for a particular patient (X. Zhang et al. 2017).

As of 2019, 800 TCVs are in development, with approximately half of these vaccines in clinical trials (Xin Yu, Hubbard-Lucey, and Tang 2019). Approximately 25 of those in clinical trials offer personalized vaccine approaches that employ methods mentioned previously. Additional research in both pre-clinical and clinical trials seeks to determine an effective way for future cancer vaccine development and could serve as a valuable complement to immunotherapy (Alcazer et al. 2019).

Chapter Organization

Chapter 2 of my dissertation, titled "Characterizing the Impact of Specific OAS Proteins on Viral Replication in a Human Lung, Adenocarcinoma Cell Line," discusses the impact that overexpression of the oligoadenylate synthetase (OAS) family of PRR proteins has on viral replication across several families of RNA viruses using high efficiency, low background (HILO), tetracycline induced (TET-ON) system, in a human lung adenocarcinoma (A549) model. Additionally, I examine the potential interdependence between specific OAS isoforms and RNase-L , which conically are believed to lead to viral suppression, supported by observations of impaired viral suppression or its absence during virus infections in an RNase-L-KO cell line that overexpresses the same OAS proteins.

Chapter 3 of my dissertation, titled "Distinguishing Circulating Antibody Repertoires of Responders vs. Non-Responders to Immune Checkpoint Blockade (ICB) for Metastatic Osteosarcoma (mOS) in Balb/c mice," discusses the replication of a previous

mouse model in which ICB composed of α-CTLA-4 and α-PD-L1 was used to treat mOS and the demonstration that responders to this immunotherapy regimen had distinct antibody repertoires before inoculation with tumor or ICB treatment compared to non-responders. I then analyze the sequences for which antibodies in responders show high reactivity, delving further into the implications of this reactivity and potential ways this knowledge can be applied to immunotherapy treatment for mOS.

Chapter 4 of my dissertation, titled "The Impact of Microbiome Dysbiosis on T Cell Function Within the Tumor Microenvironment (TME)," discusses first the conventional responses of T cells to cancer. Subsequently, it elucidates our current understanding of how alterations in the microbiome can influence T-cell immune function within the tumor microenvironment (TME), revealing additional dimensions that can be considered and potentially exploited for cancer immunotherapy.

Chapter 5 of my dissertation, titled "The Impact of Microbiome Modulations on T Cell-Mediated Immunotherapy Efficacy for Metastatic Osteosarcoma (mOS)," discusses initially whether pre-existing microbiome compositions influence treatment outcomes in response to cancer immunotherapy composed of α-CTLA-4 and α-PD-L1 in a Balb/c mouse model. I then discuss the impact of modulations in the microbiome on cancer progression, T cell functionality, and responsiveness to immune checkpoint blockade, analyzing how the microbiome can serve as a helpful modulatory tool to improve treatment outcomes for patients with mOS.

Chapter 6 of my dissertation, titled "Methods for Validation of a T cell Epitope Prediction Algorithm EnsembleMHC *in vivo,"* discusses potential avenues for testing the

efficacy of T cell prediction algorithms *in vivo*. Additionally, this chapter explores and considers the practical applications that can emerge from this research if pursued.

Chapter 7 of my dissertation, titled "Discussion," will discuss the broad implications of the preceding five chapters. I delve into potential insights that can be gained from this research regarding our understanding of treatments for viral illnesses and possible treatment strategies through leveraging innate immune mechanisms. Furthermore, I explore the potential application of the knowledge gained from this dissertation and how it can be used to improve immunotherapy treatments for mOS, enhancing patient prognosis and paving the way for tangible solutions to current issues with immune checkpoint blockade efficacy.

CHAPTER 2

CHARACTERIZING THE IMPACT OF SPECIFIC OAS PROTEINS ON VIRAL REPLICATION IN A HUMAN LUNG, ADENOCARCINOMA CELL LINE

Abstract

The 2',5'-oligoadenylate synthetase (OAS)-RNase-L pathway is an interferon (IFN)-induced cascade activated in response to viral infection. Upon IFN induction and subsequent double-stranded RNA (dsRNA) binding, OASs synthesize 2-5A, a potent activator of RNase-L, which results in nonspecific cleavage of cytoplasmic RNA, aiding in the clearance of viral infection. Though copious studies highlight the importance of specific OAS proteins in triggering robust antiviral responses against certain viruses, the significance of each OAS in suppressing the replication of particular viral families remains elusive. Here, we utilized a Tetracycline-on (TET-ON) system to engineer several human A549-derived cell lines that express OAS isoforms based on the three enzymatically active OAS proteins in humans--OAS1, OAS2, and OAS3. We show that although the activation of distinct OAS proteins has recently been attributed to the cellular localization of viral replication, the ability of unique OAS proteins to suppress viral replication when overexpressed exhibited considerable variation across RNA viruses even within the same viral family, indicating that additional undefined factors may impact the role of specific OAS proteins in suppressing viral replication for different viruses. We additionally show the antiviral capacity of OAS3 is abolished in an RNase-L KO cell line across several viruses, compared to a wildtype (WT) cell line that over-expresses the same OAS proteins. However, the ability of OAS1 and OAS2 isoforms to suppress viral replication is partially maintained when overexpressed in the same RNase-L KO cell line, albeit to a reduced

extent, indicating potential RNase-L independent mechanisms of viral suppression utilized by these proteins. Findings from this research aid in elucidating the impact of distinct OAS proteins on viral replication across different RNA viruses and contribute to a better understanding of what mechanisms govern the impact of the innate anti-viral responses to distinct viruses, which can be utilized in the development of anti-viral therapies.

Introduction

The innate immune system is comprised of a complex network of host proteins that work together to provide an initial defense against pathogens. Understanding how components of the innate immune system are responsible for inhibiting the replication of certain viruses is crucial to our understanding of innate viral immunity and can further frame the design of therapeutics that combat viral infection. The 2' 5' Oligoadenylate synthetase (OAS) proteins are a family of interferon (IFN)-induced enzymes that play a crucial role in the innate immune system's defense against viral infections. In humans, the main isoforms include OAS1, OAS2, OAS3, and OASL, though OASL is believed to lack enzymatic activity (Marques et al. 2008). Moreover, evidence supports that both RNase-L-dependent and RNase-L-independent pathways exhibit antiviral activity. The canonical OAS antiviral pathway, which is RNase-L dependent, has been extensively described several times (Roberts et al. 1976; Hovanessian, Brown, and Kerr 1977; Baglioni, Minks, and Maroney 1978). In the classical OAS-RNase-L pathway, OAS proteins aid in the defense against viruses by binding dsRNA that has entered the cell via a positively charged groove found at the interface of the OAS N- and C- terminal domains (Kristiansen et al. 2011). This interaction results in the allosteric activation of OAS and further synthesizing

of 2’-5' oligoadenylates (2-5A), utilizing adenosine triphosphate (ATP) as a substrate. 2-5A binds and activates RNase-L, which dimerizes and acquires RNase activity for both cellular and viral RNA, thereby halting the ability of a virus to exploit the machinery of an infected cell for viral replication. Additional research has demonstrated the presence of RNase-L-independent pathways. OASL in humans lacks enzymatic activity yet still displays antiviral activity in mammalian cells, likely due to its C-terminal domain, which displays homology to ubiquitin. Past studies have shown that OASL displays antiviral activity against Encephalomyocarditis Virus (EMCV) by requiring the ubiquitin-like domain but not the presence of RNase-L (Marques et al. 2008).

Several studies indicate that mutations in certain OAS proteins can impact the likelihood that a person will either contract a certain viral illness or experience severe outcomes after infection (Lim et al. 2009; Alagarasu et al. 2013; Barkhash et al. 2014), though our understanding of not only why these mutations lead to altered outcomes but also which proteins are most important for protection against certain viral illness is currently incomplete. OAS proteins exhibit distinct cellular localization patterns, with a proclivity towards different regions of a cell after translation. For instance, OAS1-46 can be prenylated in specific hosts, and these proteins tend to localize to endomembrane systems (Wickenhagen et al. 2021). For this reason, it is believed that patients who can produce prenylated forms of OAS1-46 are also able to inhibit SARS-CoV-2 replication in large part because the virus utilizes endomembrane systems to replicate within a cell, clinically evident by the improvement in patient outcome for those who produce prenylated OAS1-46 (Wickenhagen et al. 2021; Soveg et al. 2021). Conclusions from these studies have supported the notion that a particular innate immune protein’s impact on replication

for distinct viruses can be in large part attributed to cellular localization of viral replication since where the virus replicates can also impact what proteins viral RNA will interact with.

Previous studies have concluded that OAS3 substantially impacts RNase-L activation and further that OAS3 displayed a higher affinity for dsRNA than OAS1 or OAS2 in a length-dependent manner (Li et al. 2016; Wang et al. 2020). However, these studies were completed across few viruses and were completed *in vitro.* Additionally, various factors may cause disparate inhibitory effects among viral species compared to each other. Recent studies have shown that the length of dsRNA can determine which OAS is activated and achieves its maximum activity when bound to RNA that is longer than what one molecule of enzyme can interact with (Wang et al. 2020). Thus, many factors that can impact catalytically active OAS species are accountable for suppressing viral replication across species. Our understanding of which OAS species are most important for different viruses represents a significant gap in our ability to understand innate immune function against viruses and further represents a gap in our understanding of how best to design therapies and treatments for viral illness. In this study, we first sought to determine which OAS innate immune proteins were most likely to impact viral replication for *Alphaviruses* SINV and CHKV and *Flaviviruses* ZIKV and WNV.

Togaviruses are vector-borne positive-strand RNA viruses that are ~11kb-12kb in length. They typically lead to symptoms such as rash, fever, persistent arthritis, and encephalitis that can result in case mortality in severe cases. *Togavirus* members include those in the *Alphavirus* family, such as Sinbis virus (SINV) and Chikungunya Virus (CHKV), both used in this study. The *Alphavirus* life cycle, similar to that of the *Flavivirus* life cycle, begins with the attachment of the viral particle to a cell surface and subsequent

clathrin-mediated endocytosis of the viral particles (J. Y. S. Leung, Ng, and Chu 2011). After acidification of the vesicle, the nucleocapsid is released into the cytoplasm and disassembled to release the (+) strand viral RNA, which is further translated in the cytoplasm into the polyprotein. Viral proteinases are cleaved with different specificities. These enzymes copy (+) strands of the virus into full-length (-) and (+) strands, producing a dsRNA intermediate and subgenomic mRNA which can activate innate immune mechanisms, such as OAS proteins, among others (D. Liu et al. 2002). The synthesis of the viral RNA occurs at membranous structures that accumulate at the plasma membrane and then move to the cell interior. Subgenomic mRNA is further translated to viral proteins by ribosomes in the cytoplasm, and proteolytic cleavage ensues. Cleavage to liberate the capsid protein occurs, exposing the hydrophobic sequence of the viral protein PE2, which induces ribosomes to associate with the ER. Viral polyproteins begin to enter the secretory pathway, and glycoproteins are transported to the cell surface. Next, viral capsid proteins and (+) strand genomic RNA assemble to form nucleocapsids that migrate to the plasma membrane and are associated with viral glycoproteins. The nucleocapsid becomes enveloped by budding at this site, releasing the virus. Several *in vitro* studies indicate the importance of OAS3 in suppression of both SINV and CHKV replication (Li et al. 2016; Bréhin et al. 2009). Though the viral entry of *Alphaviruses* into cells has been characterized and further studied, the immune mechanisms needed to prevent *Alphavirus* infection are being further elucidated in this study.

The Flavivirus genus is a family of enveloped, positive-strand RNA viruses with genomes ~11kb in size that is accountable for causing severe disease in both humans and animals; they include members such as Zika (ZIKV) and West Nile (WNV), both of which

were used in this study (Halstead n.d.). *Flaviviruses* are vector-borne and often carried through bites from the mosquito *Aedes aegypti* (Muktar, Tamerat, and Shewafera 2016). Replication of *Flaviviridae* begins with binding to host receptors on the surface of a cell, followed by clathrin-mediated endocytosis of the viral particles. The fusion of viral and cellular membranes is triggered by the late endosome's low pH, allowing the release of the viral RNA into the cytoplasm (Brinton 2013). Further, the viral RNA is translated into a polyprotein that is cleaved into at least 10 proteins; these proteins recruit the viral genome into a replication complex, consisting of ER invaginations open to the cytoplasm. The genome length (-) strand of RNA is created and is further copied to produce a new (+) strand of RNA. This replication forms a dsRNA intermediate that can trigger multiple innate immune pathways to detect dsRNA (D. Liu et al. 2002). Virus assembly occurs at the ER surface when C protein dimers begin to associate with viral (+) RNA. The complex of protein and RNA buds into the ER membranes, which contain envelope (E), pre-membrane (pr), and membrane (M) proteins. Immature virus particles are then formed and further transported to the surface by the secretory pathways; during this process, they undergo maturation steps, including glycosylation of prM and E and furin-induced cleavage of the prM. Mature viral particles are transported to the cell surface and secreted by exocytosis. The *Flavivirus* replication cycle involves virus-induced changes in host cellular membranes, allowing the virus to replicate efficiently and separately from cellular immunological components. This may impact the magnitude that certain immune proteins impact viral replication (Rothan and Kumar 2019). Though we know that *Flaviviruses* utilize the molecular machinery of cells to replicate, little is known about what immunological factors contribute to the immune system's ability to fight *Flavivirus*

infection. Studies that have investigated the impact of genetic variations in certain innate immunological factors across populations discovered that alterations in OAS1 often correlate with severe *Flavivirus* disease in humans (Lim et al. 2009). Understanding which innate proteins are responsible for defense against *Flaviviruses* could provide helpful insight into potential therapeutics and treatments for those with a severe viral infection.

Since OAS family proteins and subsequent variations in humans at loci encoding these factors can broadly impact the degree of antiviral immune responses during viral infection, it is crucial to understand how these proteins can affect viral replication and whether the importance of OAS proteins can vary for different viruses. Knowledge gained from studies about how these proteins affect viral replication can be expanded on for the design of antiviral therapeutics. They can also serve as biomarkers to determine if people are predisposed to severe infections. Additionally, certain viruses in this study present mildly in the broad population but progress to severe diseases in some people. This circumstance could be due to genetic variations in the general population (Choi et al. 2015). For example, most people infected with West Nile Virus (WNV) present asymptomatically, but <1% will progress to severe infections resulting in encephalitis (Mostashari et al. 2001).

Here we utilized a Tetracycline-on (TET-ON) system to engineer several human A549-derived cell lines that express OAS isoforms based on the three enzymatically active OAS proteins in humans--OAS1, OAS2, and OAS3. Through plaque assay, we then determined which isoforms of OAS most impacted replication in these cells for SINV, CHKV, ZIKV, and WNV. We further sought to characterize whether or not the ability of these proteins to inhibit viral replication for specific viruses relied on RNase-L or not by

using a TET-ON RNase-L knockout (KO) cell line that also expressed OAS1, OAS2 and OAS3.

We found that although viruses belonging to the same viral families have similarities in viral replication, they still may be impacted differently by the overexpression of specific OAS proteins. Further, we found that only in OAS3 overexpressing cells were viral replication ablated, whereas in OAS1 and OAS2 over-expressing cells, some viral suppression was still evident, even in RNase-L-KO cells. These results indicate that OAS1 and OAS2 proteins may utilize alternative mechanisms to suppress viral replication independent of RNase-L. This research and future investigations based on these results may have aid not only in our understanding of how innate immune elements impact viral replication but also may pave the way for improvements in anti-viral therapies and screenings used to determine the risk of progressive or severe viral infection with SINV, CHKV, ZIKV, and WNV.

Methods:

Viruses

WNV Kunjin strain (Hall, Scherret, and Mackenzie 2001), CHKV (Taschuk et al. 2020), and ZIKV-PR (Y. Liu et al. 2018) were obtained from Sara Cherry, University of Pennsylvania, Philadelphia and were propagated in either Vero or BHK-21 cells; SINV-GW strain was obtained from the lab of Mark Heisen and was propagated in BHK-21 cells.

Cell Lines

A549 HiLO cells were obtained from Susan Weiss's lab at the University of Pennsylvania. Vero E6 cells used in plaque assays in this study were obtained from ATCC. All A549 HiLo cells were maintained in a growth phase at 37 °C in RPMI 10% FBS 1% P/S. Cell lines were discarded after passage 25 of cells to ensure that genes of interest were maintained, and new stocks were thawed and used for experiments. Vero cells used for plaque assays were maintained in the growth phase at 37 °C in DMEM 10% FBS 1% P/S.

Antibodies

A mouse α-Flag M2 antibody (1:1,000; Sigma-Aldrich) was used to detect OAS1 p42/p46, OAS2 p69, OAS3 p100, proteins containing a FLAG tag at the N terminal end of each protein. A mouse α-RNase-L antibody (1:1000; Sigma-Aldrich) was used to detect RNase-L presence in samples. Secondary antibodies used included α-mouse antibody conjugated with HRP (1:5,000; Thermo Fisher) for western blot analysis and α-mouse conjugated with AlexaFlur488 for Immunofluorescent imaging (IFA).

Construction of HiLo OAS expressing Cell Lines Using the TET-ON System:

OAS-expressing cells used in this experiment were created using the High-Efficiency low-background (HiLo) system described by *Khandelia et al*. (Khandelia, Yap, and Makeyev 2011). To construct these cells, we had HiLo cells given to us by Susan Weiss's lab that contain a lentiviral vector cassette containing the human EF-1α promoter and a blasticidin resistance gene (Bsd), which is flanked by Cre recombinase-specific sites *Lox2272* and

LoxP **(Fig. 1).** A donor plasmid that contained the gene of interest (either OAS1-42, OAS1-46, OAS2, OAS3 or GFP) based on pRD-RIPE **(Fig. 1)** included a *Lox2272* and *Lox-P* floxed Pur resistance gene. The RIPE cassette on this plasmid contains a constitutively active reverse tetracycline transactivator gene (rtTA3). The A549 acceptor cell line was transfected with a mixture of pRD1 and the pCAGGS-Cre plasmid that encoded wildtype Cre recombinase. Transfected cells were treated with puromycin (Pur) in RPMI 10% FBS. Media was changed every two days to ensure that Pur degradation did not occur, and cell selection continued. Colonies typically appeared within 14d following Pur selection.

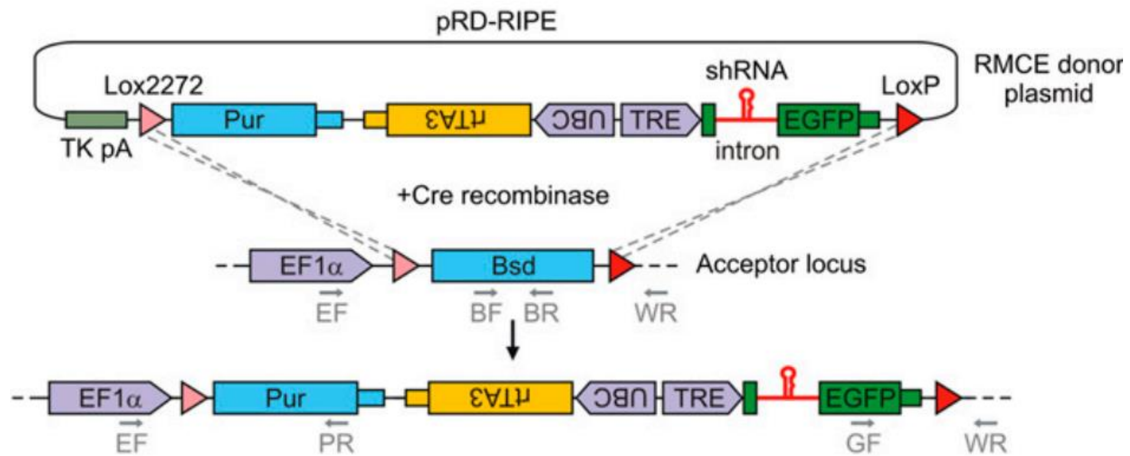


Figure 1: Diagram of the HILO-RMCE Reaction Using the pRD-RIPE Donor Plasmid. Both the pRD-RIPE plasmid and the HiLo locus contain *Lox 2272* and *LoxP* sites that allow for recombination with the acceptor locus when Cre recombinase is added to the reaction. This reaction allows for the insertion of a desired gene into the HiLo system and controls the capacity in which specific genes are expressed. *(Khandelia et al, PNAS, 2011)*

Western Immunoblotting:

Constructed A549 HiLo cells were grown to confluency in six-well plates and mock-treated with DMSO or Doxycycline for 24 hours. Cells were then harvested, washed in PBS, and lysed with cell lysis buffer (CLS) with protease inhibitor. Cells were scrapped with a 200 µL pipette tip, using a new tip for each well. Plates were placed on ice for 20 minutes; cells were resuspended. Cell lysates were next transferred to a 1.5 mL microcentrifuge tube and centrifuged at 4°C. Supernatants were mixed with 4× Laemmli buffer, boiled at 95 °C for 5 min, and run on a 4–12% gradient of pre-made SDS gels (Biorad). Proteins were transferred to PVDF membranes using an electroblotting system. Membranes were treated in 5% nonfat milk in Tris·HCl buffer saline with 0.5% Tween-20 (TBST). Membranes were washed three times in TBST and incubated in α-FLAG primary Ab overnight at 4°C. Membranes were again washed three times in TBST and then incubated with secondary antibodies for 1 hr at room temperature. Membranes were washed thrice with TBST and incubated in SuperSignal West Dura Extended Duration substrate (Thermo Fisher). The signal was detected using an AMERSHAM ImageQuant 800. Proper expression of GFP in samples was completed using IFA.

Immunofluorescent Imaging (IFA)

Cells were first fixed via incubation in 4% formaldehyde and subsequent incubation at room temperature for 20 minutes. Formaldehyde was aspirated off of cells, and cells were washed twice with PBS. Cells were then permeablized by incubation in 0.1% Triton X-100 at room temperature for 10 minutes. These cells were washed with PBS twice and incubated with primary antibody mouse α-Flag M2 antibody (1:1,000; Sigma-Aldrich) in

2% BSA with 0.05% sodium azide and incubated overnight. Cells were further washed with 1X PBS or 5 minutes 3 times. Secondary antibody incubations were completed using α-mouse AlexaFlur 488 in 2% BSA with 0.05% sodium azide for one hour at room temperature. Cells were rinsed with 1X PBS for 5 mins 3 times, and further nucleus staining was completed using DAPI stain in 2% BSA with 0.05% sodium azide for 10 minutes. Cells were rinsed further with PBS and imaged on an Evos Fluorescent microscope (Life Technologies).

Viral Kinetics

Viral kinetic assays were performed to determine the number of days post-infection (DPI) required to reach peak viral titer for viruses to ensure that actual infections would yield countable plates at specific MOIs. To complete these, A549-GFP cells not induced with doxycycline were seeded in a 24-well plate. Cells were grown to confluency and infected with the appropriate MOI of each virus, detailed in **Table 1**. At least two replicates of each assay were completed as shown in **Figure 2,** to determine how many days were needed post-infection to reach peak viral titer for each virus. A total of 200 μL were harvested on 2, 3, 4, 5, 6, and 7 DPI. Further, plaque assays were completed, as detailed below.

Doxycycline Induction and Infection of A549 Cells:

A549 HiLo Cells, and A549 RNase-L -KO HiLo Cells, each expressing GFP, OAS1-p42, OAS1-P46, OAS2, or OAS3 under a tetracycline-on (TET-ON) promoter, were seeded in two 24-well plates for each replicate following the schematic detailed below in **Figure 3** for A549 HiLo cells, and **Figure 4** for experiments involving RNase-L -KO HiLo Cells,

Cells were seeded in RPMI 10% FBS. When confluency was reached, mock cells (M) and cells that were meant to be treated only after viral infection with doxycycline (AV) were incubated in a working solution (WS) of DMSO by adding 10uL of DMSO stock to 10mLs of RPMI 10% FBS 1% P/S. Cells to be treated before viral infection were incubated with a working solution of doxycycline by adding 10uL of doxycycline stock to 10mLs of RPMI 10% FBS 1% P/S. Each well was incubated with 500uL of WS appropriate for the respective wells. Cells were incubated at 37°C overnight for 24 hours before virus infection. Cells were infected for one hour with a virus at MOI specified in **Table 1**, 24 hours after doxycycline induction. Cell plates were rocked every 15 minutes. After 1 hour, viral media was removed from cell wells, and each well was washed with PBS. PBS was removed, and warm RPMI 10% FBS 1% P/S media was added to each well. Wells labeled BV and AV were given WS of Doxycycline, while M wells were given WS of DMSO.

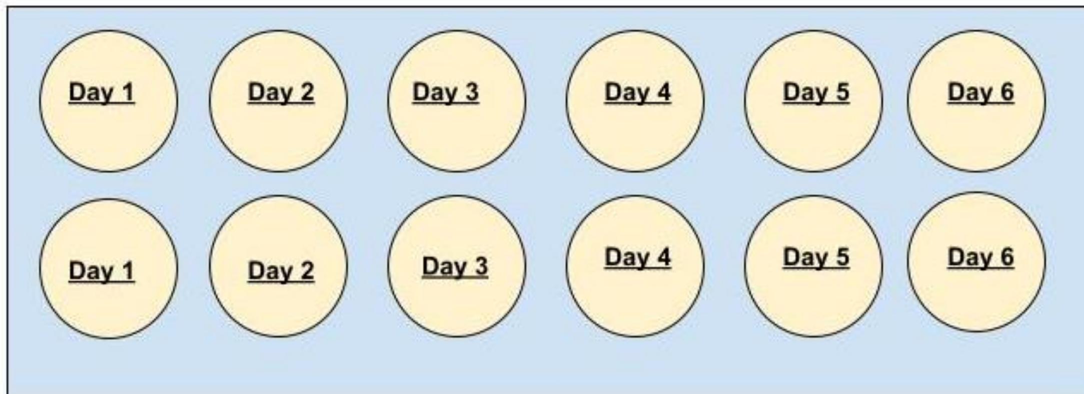


Figure 2: Schematic of A549 Cell Seeding for Viral Growth Kinetics. Each well was seeded on the same day, infected at confluency, and incubated without doxycycline. Approximately 200uL of supernatant was harvested on the days indicated in the schematic. Each well was completed in duplicate.

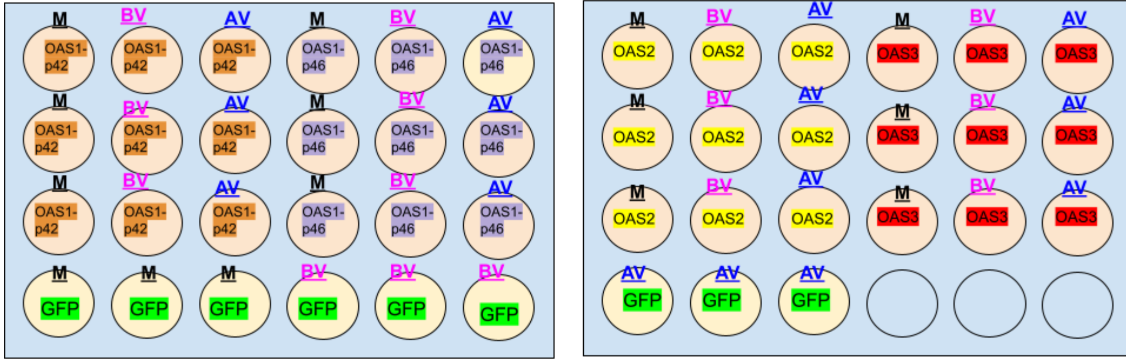


Figure 3: Schematic of A549 Cell Seeding in 24 Well Plates. Each replicate was seeded on the same day and incubated with either doxycycline or DMSO when cells reached equivalent confluency. A549 cells expressed OAS-1-42, OAS1-46, OAS2, OAS3, or GFP under a tetracycline-induced promoter. Cells were further infected after 24 hours post-induction with doxycycline. Mock (M)= DMSO only, before and after virus infection; Before Virus (BV)= Doxycycline, before and after virus infection; After Virus (AV)= Doxycycline, after virus infection.

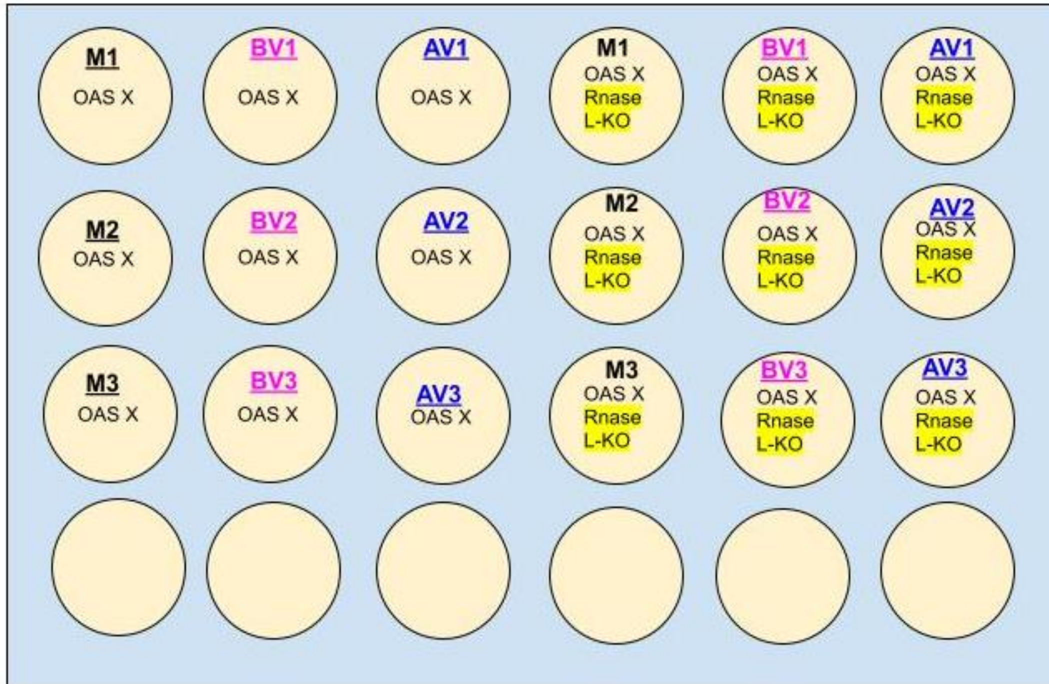


Figure 4: Schematic of A549 Cell Seeding in 24 Well Plates for RNase-L-KO Cell Experiments. Each replicate was seeded on the same day and incubated with either doxycycline or DMSO when cells reached confluency. A549 cells expressed either OAS-1-42, OAS1-46, OAS2, OAS3, or GFP under a tetracycline-induced promoter. OASX represents the OAS protein which was found to inhibit viral replication of specific viruses. Cells were further infected after 24 hours post-induction with doxycycline. Mock (M)= DMSO only, before and after virus infection; Before Virus (BV)= Doxycycline, before and after virus infection; After Virus (AV)= Doxycycline, after virus infection

Plaque Assays:

All viruses used in this study were diluted in DMEM, and 200uL of each dilution was added to confluent Vero cells in six-well plates. Virally infected plates were incubated for 1h at 37 °C and were rocked every 15 minutes. Cells were overlaid with 3mLs per well of warm plaque assay media (PAM) made of 2X DMEM, FCS, 8% $NaHCO_3$, HEPES, L-Glutamine, Fungizone, and 0.7% Agar. Viral plaques were stained with Crystal Violet at days post-infection (DPI), specified in **Table 1.**

Statistical Analysis:

All statistical analysis for this study was completed using GraphPad Prism. P values reported here were determined through an unpaired T-test and F-tests on GraphPad Prism software.

Table 1: Viral Infection of A549 cells and Plaque Assay Vero cells				
Virus	Virus Family	MOI for A549 Infection	DPI for harvest in A549 Cells	DPI for Plaque Assay staining and viewing
ZIKV	*Flaviviridae*	0.1 (225uL/40mL)	3	5-6
WNV-KUJV	*Flaviviridae*	1 (100uL/20mL)	3	5-6
SINV	*Alphaviridae*	0.5 (20uL/20mL)	2	2.5
CHKV	*Alphaviridae*	0.1 (40uL/20mL)	2	3

Table 2: Viral Titers of Viruses used in Infections		
Virus	Virus Family	Titer in PFU/mL
ZIKV	*Flaviviridae*	2.25*10^7
WNV-KUJV	*Flaviviridae*	1.03* 10^8
SINV	*Alphaviridae*	5.0*10^8
CHKV	*Alphaviridae*	2.3*10^7

Results:

To verify the expression of OAS isoforms OAS1-42, OAS1-46, OAS2, and OAS3, as well as Green Fluorescent Protein (GFP) as a control, in our TET-ON cells, cells were cultured in a 24-well cell culture plate and incubated without and with Doxycycline when confluency was reached**.** Cell protein was measured and ran on an SDS-PAGE gel. All OAS isoforms had a FLAG-tag (sequence DYKDDDDK) inserted at the N terminus of the protein sequence. After running each sample on a Western Blot, OAS isoforms were visible at the appropriate kDa when probed with α-FLAG **(Fig. 5).**

TET-ON cells express OAS1-42, OAS1-46, OAS2, and OAS3 when induced with Doxycycline.

Constructed OAS cells were seeded in a 24-well plate, with some samples induced with Dox while others were not. After protein samples were made and run on a 14% SDS-PAGE gel, cellular expression of OAS proteins was tested using an anti-FLAG Ab linked to HRP. Only when induced with Doxycycline did the proteins express at the correct sizes OAS1-42 at 42kDa, OAS1-46 at ~46kDa, OAS2 at ~71 kDa, OAS3 at ~100kDa **(Fig. 5).**

A

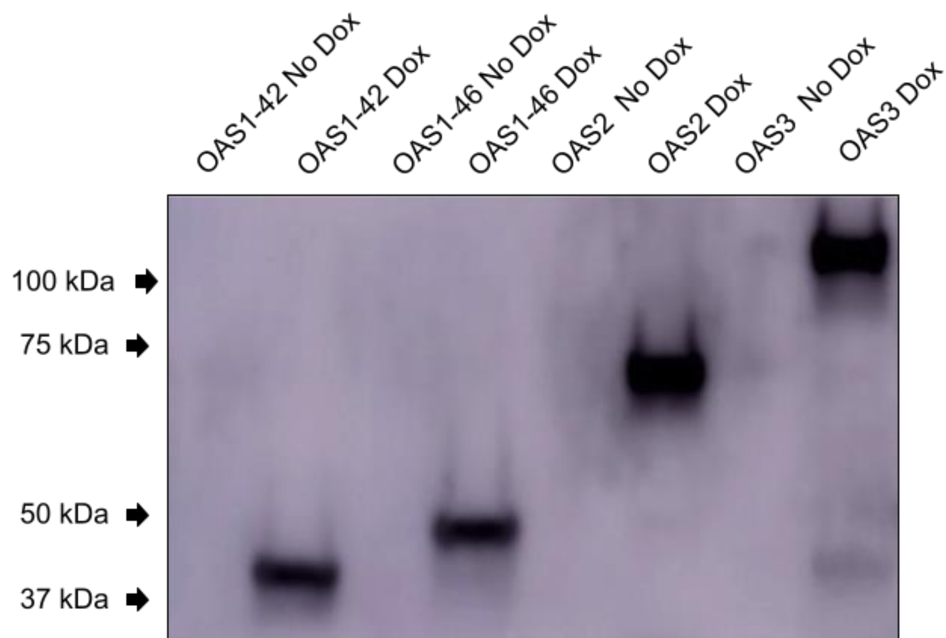
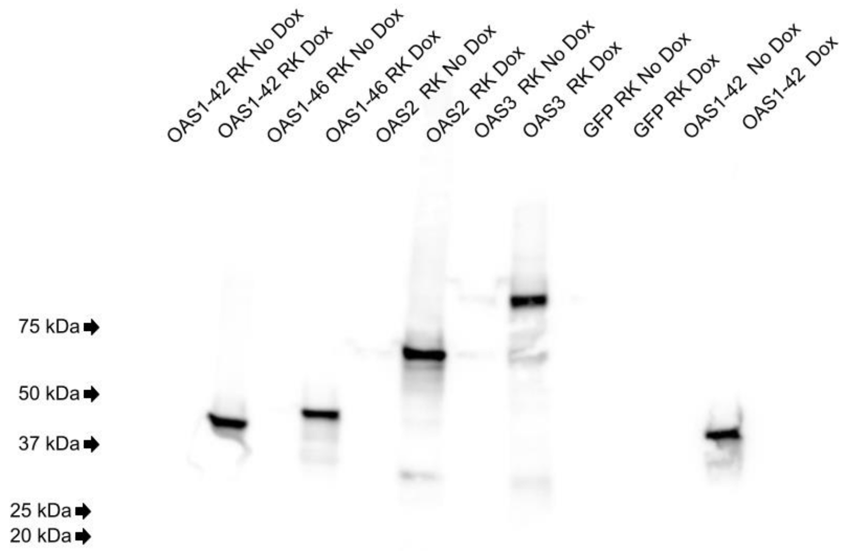


Figure 5: Western Blot of OAS Cell Verification: Western Blot of Doxycycline-induced samples vs. non-induced samples probed with α-FLAG, No Dox= No Doxycycline, Dox= Doxycycline.

Constructed RNase-L- KO cells expressed OAS1-42, OAS1-46, OAS2, and OAS3 when induced with Doxycycline and Do Not Express RNase-L.

Constructed OAS cells were seeded in a 24-well plate; one sample well for each OAS isoform-expressing cell line was induced with Doxycycline, while an adjacent well containing the same cell line was instead only incubated in an equal amount of DMSO, which the Doxycycline stocks we use are dissolved in. After protein samples were made and run on a 14% SDS-PAGE gel, cellular expression of OAS proteins was tested using an anti-FLAG Ab linked to HRP. Only when induced with Doxycycline did the proteins express at the correct sizes OAS1-42 at 42kDa, OAS1-46 at ~46kDa, OAS2 at ~71 kDa, OAS3 at ~100kDa **(Fig. 6A).** Additionally, all cell samples that came from RNase-L-KO cells were verified to lack RNase-L-KO both with and without Doxycycline induction and compared to WT cells **(Fig. 6B).**

A



B

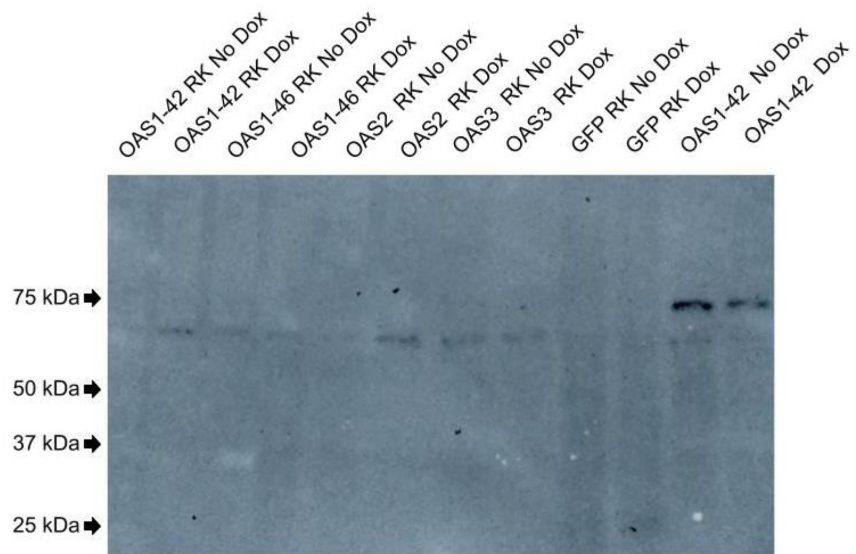


Figure 6: Western Blot of RNase-L KO Cell Verification: A) Schematic of Cells induced with Doxycycline vs. Cells not induced with Doxycycline. Cells were induced with Dox for 24 hours before cell harvest. B) Western Blot of Doxycycline-induced samples vs. non-induced samples probed with α-FLAG, RK=RNase-L-KO, No Dox= No Doxycycline, Dox= Doxycycline.

TET-ON cells express OAS proteins in various parts of the cell within A549 cells when induced with Doxycycline.

All cells were fixed, permeabilized with 0.1% TritonX in PBS, and stained with mouse α-FLAG. Cells were further stained with anti-murine secondary Abs conjugated to AlexaFlur 488. Upon fluorescent imaging, OAS1-42, OAS2, and OAS3 expressing A549 cells and A549 RNase-L -KO cells showed the presence of FLAG-tagged proteins in the cytosol of cells. OAS1-46 expressing A549 cells and A549 RNase-L -KO cells revealed the presence of FLAG-tagged proteins closer to the center of the cell, with less presence of the proteins in the cell's cytosol **(Fig.7).** These findings are consistent with previous studies that indicate that OAS1-46 can localize to endomembrane compartments and mitochondria within a cell rather than to the cytosol (Wickenhagen et al. 2021).

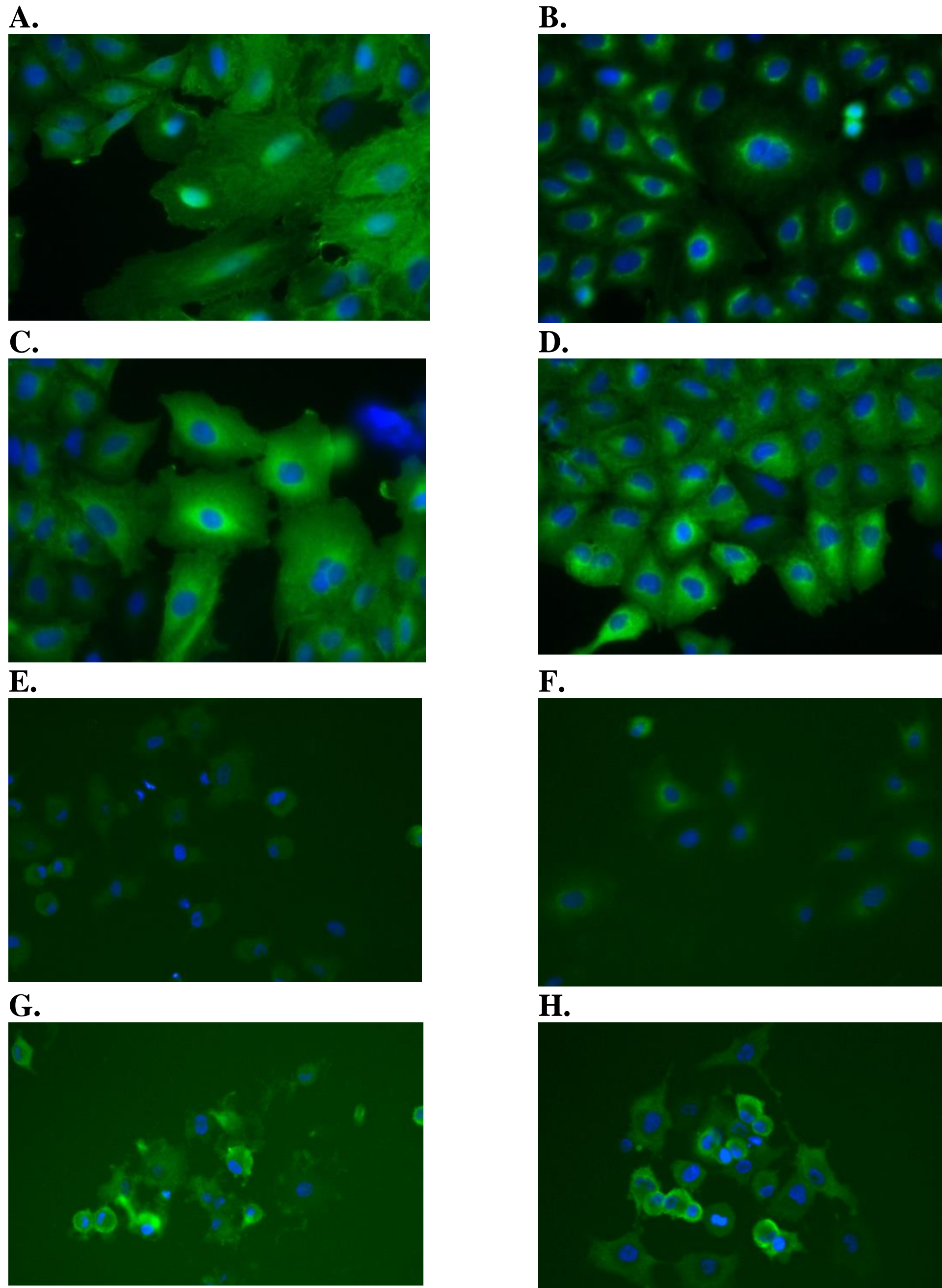


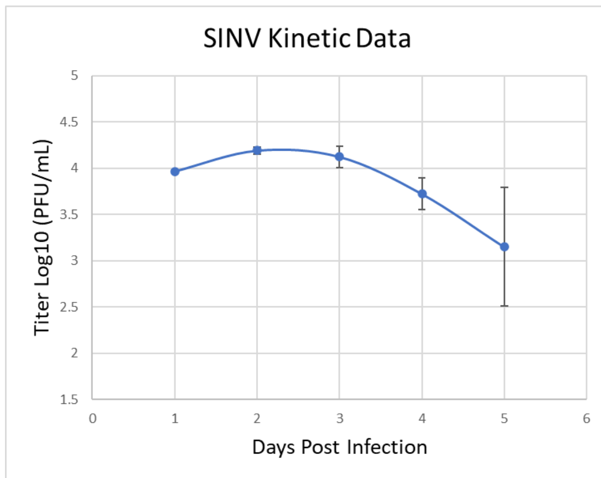
Figure 7: Immunofluorescent images of TET-ON Cells Induced With Doxycycline (Dox).: Green= 3XFLAG-OAS, Blue= DAPI **A.)** TET-ON Cells induced with Dox, expressing OAS1-42 **B.)** TET-ON Cells induced with Dox, expressing OAS1-46 **C.)** TET-ON Cells induced with Dox, expressing OAS2 **D.)** TET-ON Cells induced with Dox, expressing OAS3**, E.)** RNase-L-KO TET-ON Cells induced with Dox, expressing OAS1-42 **F.)** RNase-L-KO TET-ON Cells induced with Dox, expressing OAS1-46 **G.)** RNase-L-KO TET-ON Cells induced with Dox, expressing OAS2 **H.)** RNase-L-KO TET-ON Cells induced with Dox, expressing OAS3.

The day peak viral titer secreted from A549 cells post-infection occurred for each virus used in this study was determined to ensure that when OAS-expressing cells were infected with virus, virus samples would be harvested on a day that would allow us to wholly and accurately determine if suppression of viral replication was occurring through the presence of viral plaques taken from each sample on the plate.

Alphaviruses SINV and CHKV show peak viral titer released from A549 cells at MOI of 0.5, and 0.1 occurs between Day 2 and Day 3 post-infection

The peak viral titer of SINV released from A549 cells infected at an MOI of 0.5 occurred on Day 2 post-infection **(Fig. 8A),** though plaques were detectable at a 50-fold dilution on Day 1post infection. The peak viral titer of CHKV released from A549 cells infected at an MOI of 0.1 occurred on Day 2 post-infection **(Fig. 8B),** though plaques were detectable at a 50-fold dilution on Day 1 post-infection.

A.



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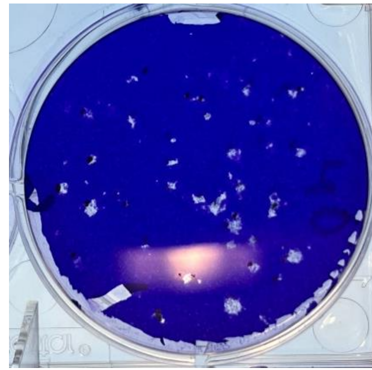

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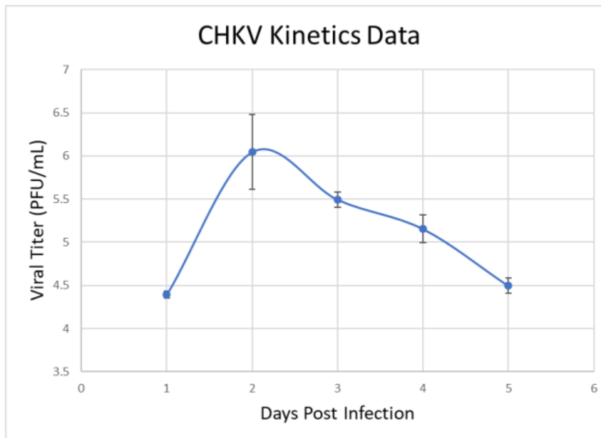

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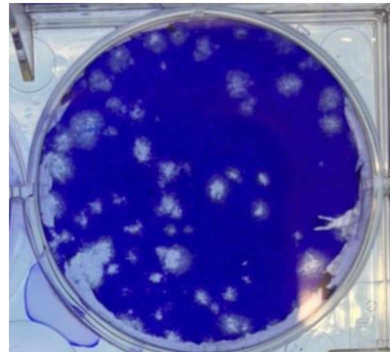
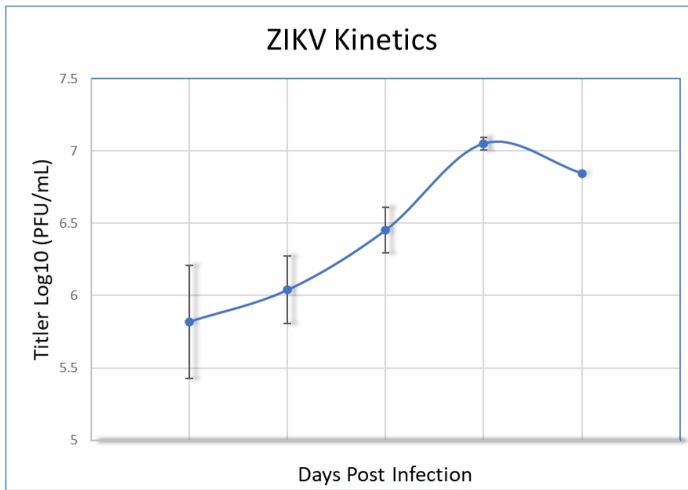


Figure 8: *Alphavirus* Viral Replication Kinetics: A.) Sinbis Virus (SINV) Kinetic Viral Titer plotted in days vs. total virus. **B.)** Plaques formed by SINV. **C.)** Chikungunya Virus (CHKV) Kinetic Viral Titer plotted in days vs. total virus **D.)** Plaques formed by CHKV.

Flaviviruses ZIKV and WNV show peak viral titer released from A549 cells at MOI of 0.1, and 1 occurs on Day 5 and Day 3, respectively, post-infection

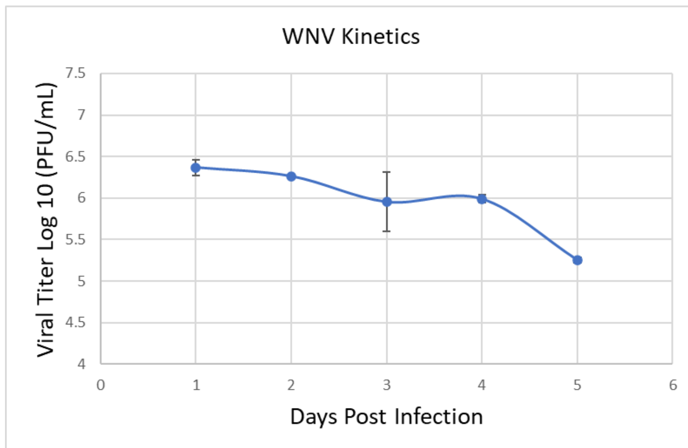
The peak viral titer of ZIKV released from A549 cells infected at an MOI of 0.1 occurred on Day 5 post-infection **(Fig. 9A),** though plaques were detectable at a 50-fold dilution on Day 1 post-infection. The peak viral titer of WNV released from A549 cells infected at an MOI of 1 occurred on Day 2 post-infection **(Fig. 9C),** though plaques were detectable at a 50-fold dilution on Day 1 post-infection.

A.



B.

C.



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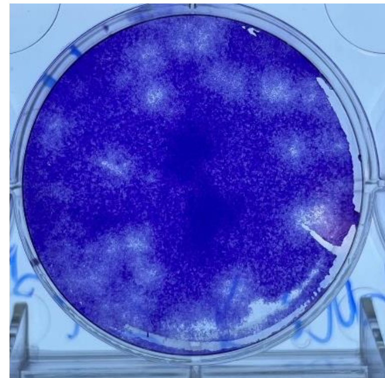


Figure 9: *Flavivirus* Viral Replication Kinetics: A) Zika Virus Kinetic Viral Titer plotted in days vs. total virus **B)** Example of plaques formed by Zika Virus **C)** West Nile Virus (WNV) Virus Kinetic Viral Titer plotted in days vs. total virus. **D)** Example of plaques formed by West Nile Virus (WNV).

Sinbis Virus (SINV) Replication Shows a Mean 12-fold Reduction When OAS3 is Overexpressed in A549 Cells Compared to Mock Treated Groups.

Infection of A549 TET-ON cells with SINV was completed following the infection scheme detailed in **Figure 3**. Results showed that over-expression of OAS3 via doxycycline induction of TET-ON cells resulted in a mean 12.03-fold reduction in virus replication compared to mock-treated groups. Our first, second, and third replicates of SINV infection showed only a significant impact on viral replication in OAS3 over-expressing cells with p= 0.000061, p= 0.000859, and p= 0.000520, respectively **(Fig. 10).** All other TET-ON cells did not differ significantly when comparing cells treated with doxycycline before and after virus infection (Dox B+A) with Mock-treated groups.

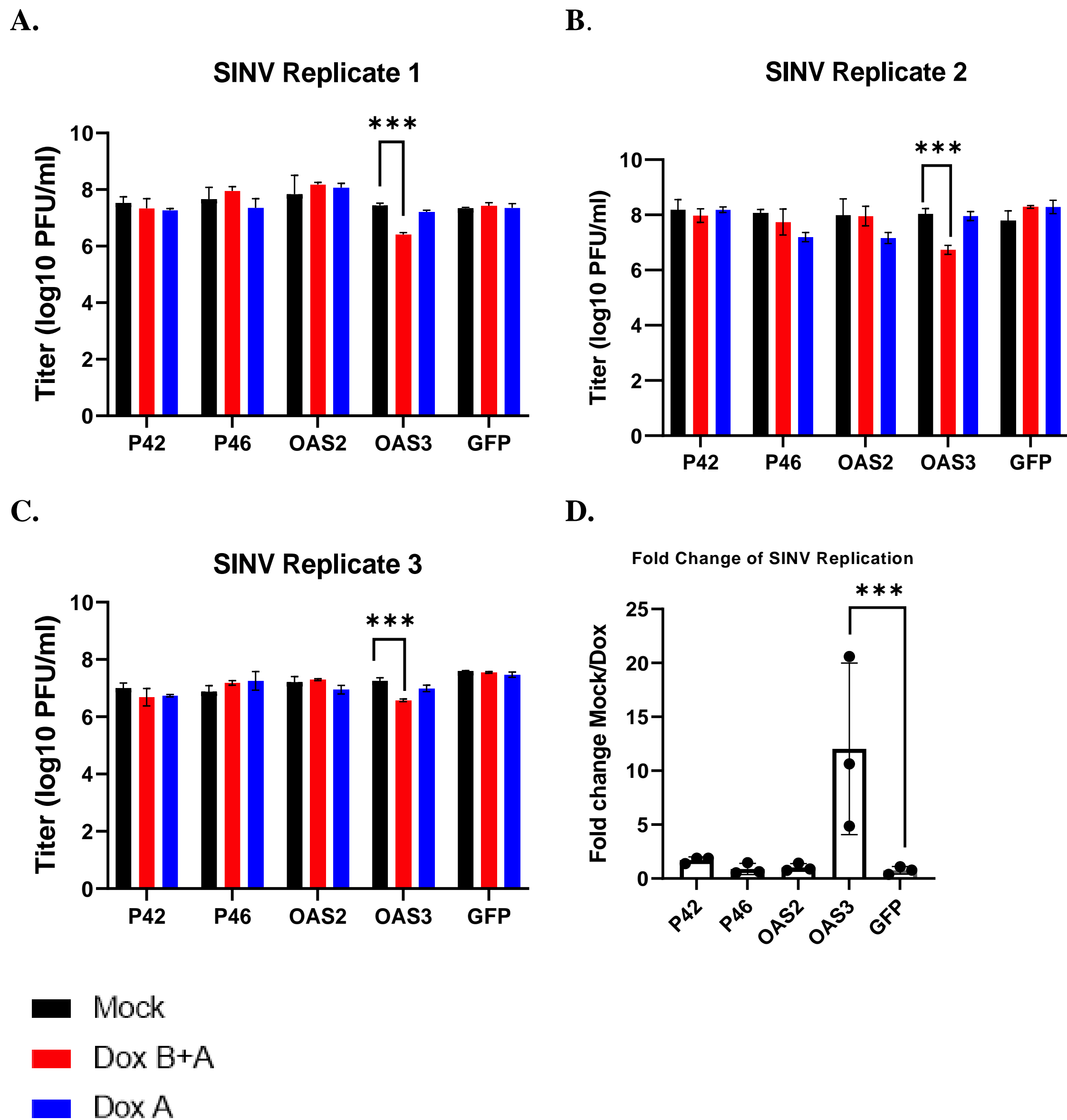


Figure 10: OAS3 Overexpression Inhibits SINV Replication: A.) Initial Replicate of SINV infected OAS expressing cells. **B.)** Second Replicate of SINV infected OAS expressing cells. **C.)** Third replicate of SINV OAS expressing cells. D.) Calculated Fold Change of SINV replicates. *** =p value <0.001; ** = p value <0.01; * =p value <0.05

Chikungunya Virus (CHKV) Replication Shows a Mean 69-fold and 23-fold Reduction when OAS1-42 and OAS3 are Overexpressed in A549 Cells Compared to Mock Treated Groups.

Infection of A549 TET-ON cells with CHKV was completed following the infection scheme detailed in **Figure 3**. Results showed that over-expression of OAS1-42 via doxycycline induction of TET-ON cells resulted in the most significant reduction of viral replication in all three replicates, with a mean 69.45-fold reduction in virus replication compared to mock-treated groups. Our first, second, and third replicates of CHKV infection showed a significant impact on viral replication in OAS1-42 over-expressing cells with p= 0.000862, p= 0.000363, and p= 0.000026, respectively **(Fig. 11).** Results also showed that over-expression of OAS3 via doxycycline induction of TET-ON cells resulted in the reduction of viral replication in all three replicates, with a mean 22.83-fold reduction in virus replication compared to mock-treated groups. Our first, second, and third replicates of CHKV infection showed a significant impact on viral replication in OAS3 over-expressing cells with 0.000866, p= 0.000020, and p= 0.000290, respectively **(Fig. 11).** All other TET-ON cells did not differ significantly when comparing cells treated with doxycycline before and after virus infection (Dox B+A) with Mock-treated groups.

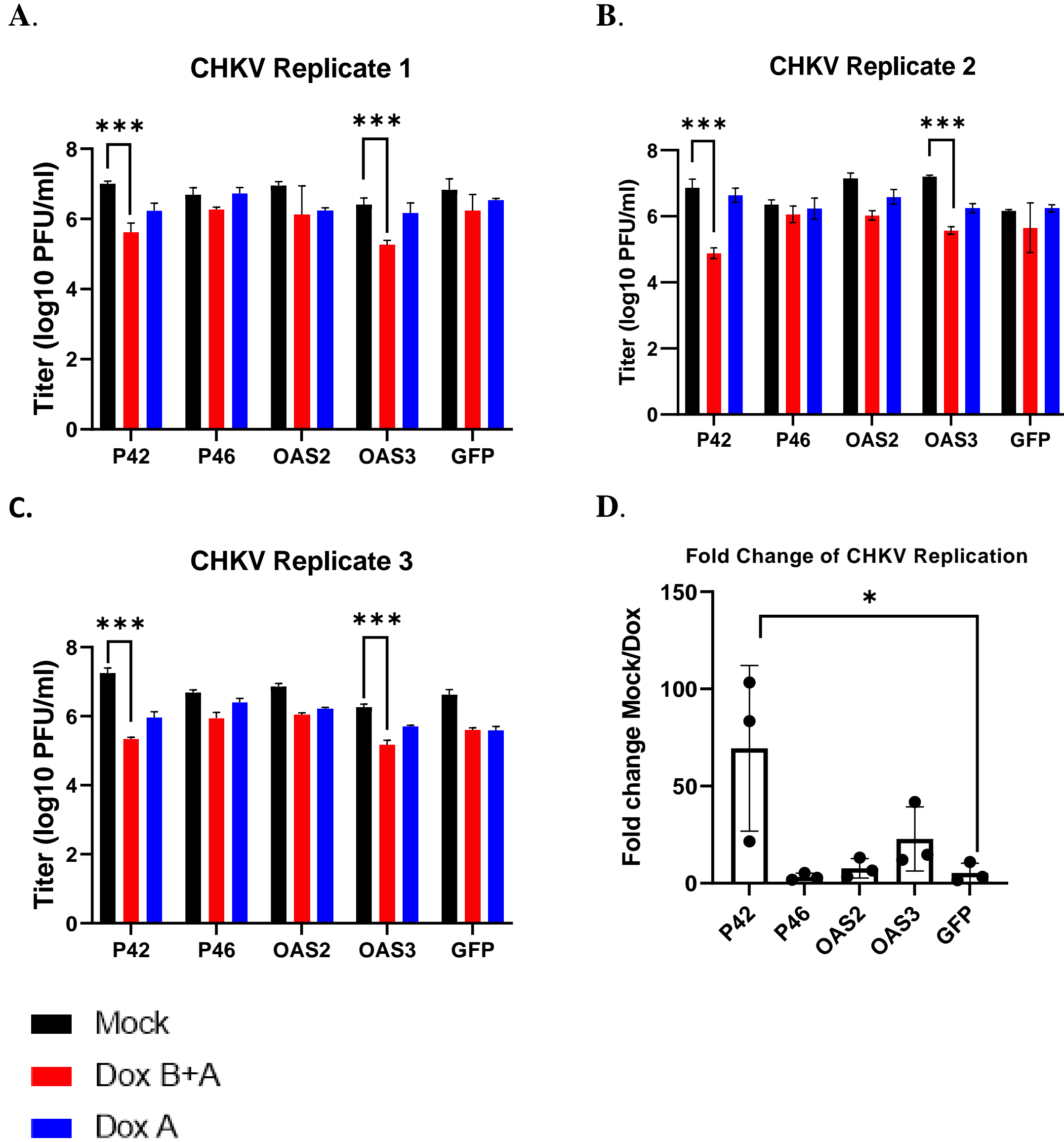


Figure 11: OAS1-42 and OAS3 Overexpression Inhibits CHKV Replication. A.) Initial Replicate of CHKV infected OAS expressing cells. **B.)** Second Replicate of CHKV infected OAS expressing cells. **C.)** Third replicate of CHKV OAS expressing cells. **D.)** Calculated Fold Change of CHKV replicates. *** =p value <0.001; ** = p value <0.01; * =p value <0.05

Zika Virus (ZIKV) Replication Shows a Mean 21-fold Reduction when OAS1-42 is Overexpressed in A549 Cells Compared to Mock Treated Groups.

Infection of A549 TET-ON cells with ZIKV was completed following the infection scheme detailed in **Figure 3**. Results showed that over-expression of OAS1-42 via doxycycline induction of TET-ON cells resulted in the greatest reduction of viral replication in all three replicates, with a mean 20.62-fold reduction in virus replication compared to mock-treated groups. Our first, second, and third replicates of ZIKV infection showed a significant impact on viral replication in OAS1-42 over-expressing cells with p= 0.000862, p= 0.000232, and p= 0.000169, respectively **(Fig. 12).** All other TET-ON cells did not differ significantly when comparing cells treated with doxycycline before and after virus infection (Dox B+A) with Mock-treated groups.

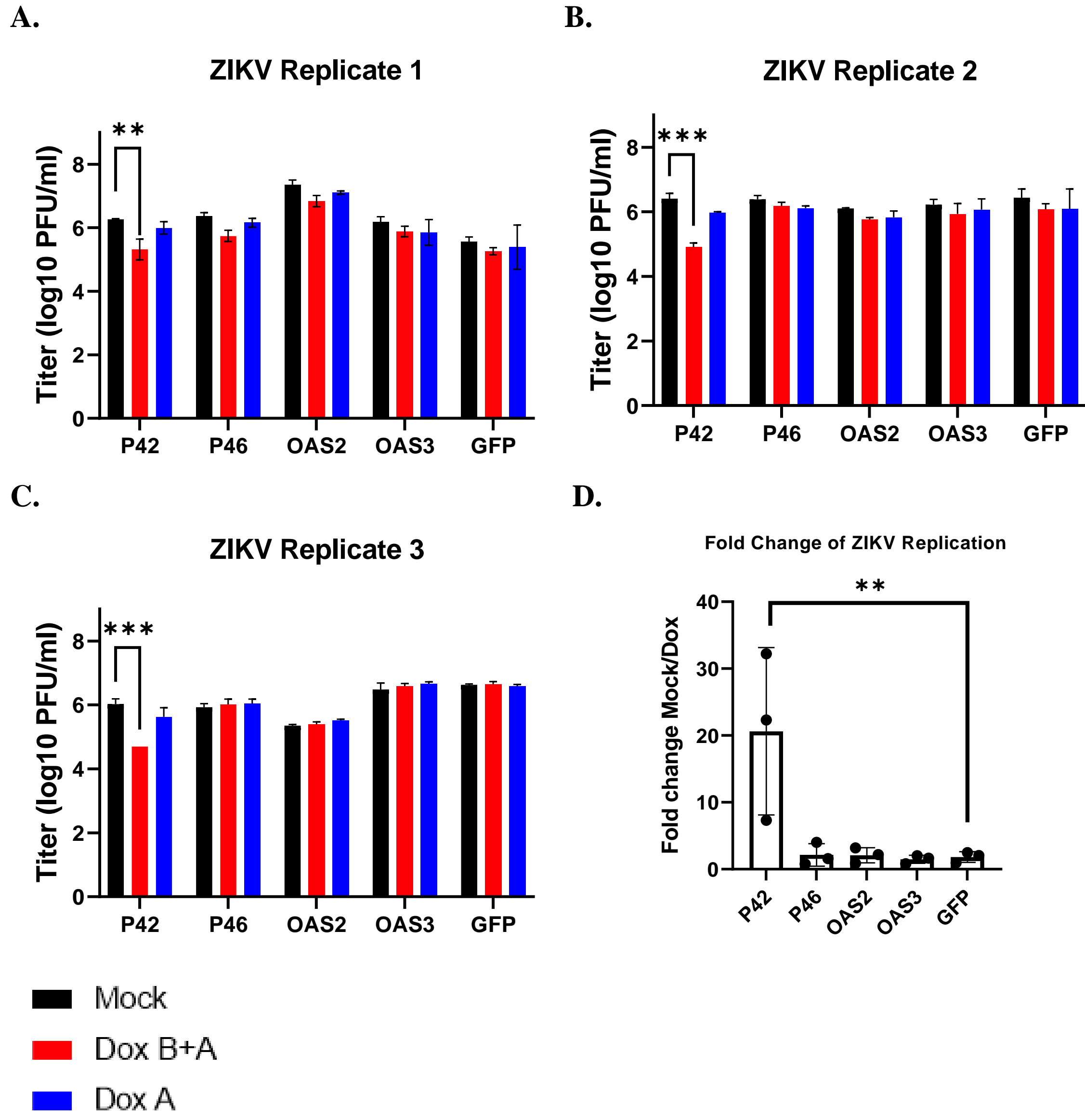


Figure 12: OAS1-42 Overexpression Inhibits ZIKV Replication. A.) Initial Replicate of ZINV infected OAS expressing cells. **B.)** Second Replicate of ZINV infected OAS expressing cells. **C.)** Third replicate of ZINV OAS expressing cells. **D.)** Calculated Fold Change of ZINV replicates. *** =p value <0.001; ** = p value <0.01; * =p value <0.05

West Nile Virus (WNV) Replication is significantly reduced when OAS2 and OAS3 are Overexpressed in A549 Cells Compared to Mock Treated Groups.

Infection of A549 TET-ON cells with ZIKV was completed following the infection scheme detailed in **Figure 3**. Results showed that over-expression of OAS2 and OAS3 via doxycycline induction of TET-ON cells resulted in a decrease in virus replication compared to mock-treated groups. Our first, second, and third replicates of WNV infection showed a significant impact on viral replication in OAS2 over-expressing cells with a mean 9.38-fold reduction in viral replication with p= 0.000044, p= 0.009500, and p= 0.004458, respectively **(Fig. 13).** Additionally, our first, second, and third replicates of WNV infection also showed a significant impact on viral replication in OAS3 over-expressing cells with a mean 5.85-fold reduction in viral replication with p= 0.000046, p= 0.016383, and p= 0.176894 respectively **(Fig. 13).** Lastly, our first, second and third replicates of WNV infection also showed a reduction in GFP over-expressing cells with a mean 3.15-fold reduction between replicates 2 and 3 of WNV. However, the viral replication decrease for both replicates was not significant **(Fig 13).** Our first replicate showed a significant 22.22 fold reduction in GFP overexpressing cells, which we believed to be an error, and so did not include in our fold reduction calculations **(Fig. 13).** All other TET-ON cells did not differ significantly when comparing cells treated with doxycycline before and after virus infection (Dox B+A) with Mock-treated groups.

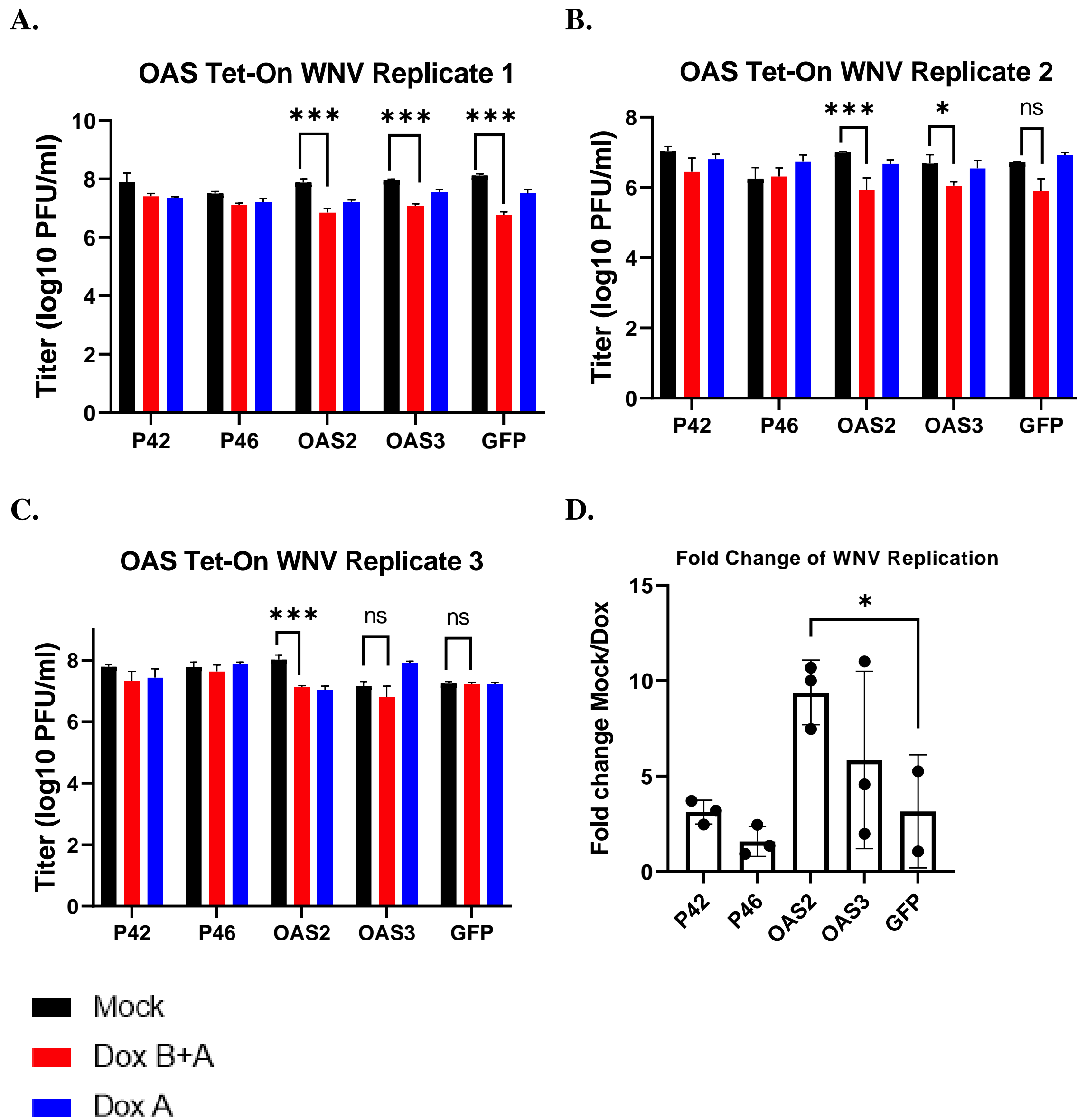


Figure 13: OAS2 and OAS3 Overexpression Inhibits WNV Replication. A.) Initial Replicate of WNV infected OAS expressing cells. **B.)** Second Replicate of WNV-infected OAS-expressing cells. **C.)** Third replicate of WNV OAS expressing cells. **D.)** Calculated Fold Change of WNV replicates. *** =p value <0.001; ** = p value <0.01; * =p value <0.05

The ability for OAS3 overexpression to suppress SINV Replication is ablated in RNase-L -KO A549 *cells.*

Infection of A549 TET-ON cells and A549 RNase-L- KO TET-ON cells with SINV was completed following the infection scheme detailed in **Figure 4**. Results showed that over-expression of OAS3 via doxycycline induction of WT TET-ON cells resulted in a mean 11.56-fold reduction in virus replication compared to mock-treated groups. Our first, second, and third replicates of SINV infection showed a reduction in OAS3 over-expressing cells with p= 0.003280, p= 0.036337, and p= 0.003301, respectively **(Fig. 14).** While in each replicate over-expression of OAS3 via doxycycline induction of RNase-L-KO TET-ON did not have a significant impact on SINV viral replication. Our first, second, and third replicates showed no significant reduction in SINV viral replication in OA3 overexpressing RNase-L-KO cells with p= 0.181013, p= 0.657751, and p= 0.118960, respectively.

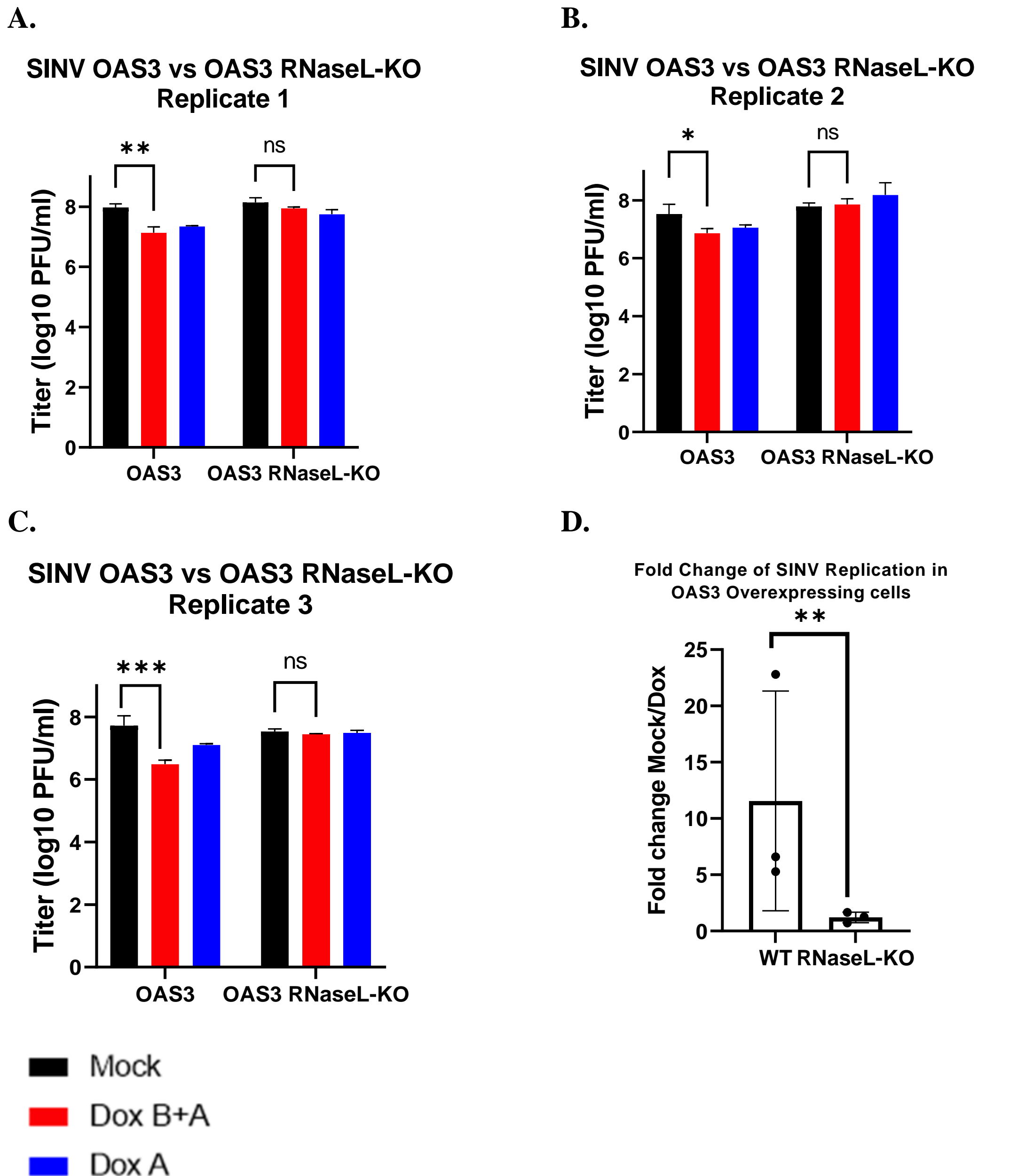


Figure 14: The Ability for OAS3 Overexpression to Suppress SINV Replication is Ablated in RNAse-L -KO Cells: A.) Initial Replicate of SINV infected OAS3 vs. OAS3 expressing RNase-L -KO cells. **B.)** Second Replicate of SINV infected OAS3 vs. OAS3 expressing RNase-L -KO cells. **C.)** Third replicate of SINV infected OAS3 vs. OAS3 expressing RNase-L -KO cells. **D.)** Calculated Fold Change of SINV replicates. *** =p value <0.001; ** = p value <0.01; * =p value <0.05

The ability for OAS1-42 overexpression to suppress CHKV Replication is ablated in RNase-L -KO A549 *cells.*

Infection of A549 TET-ON cells and A549 RNase-L- KO TET-ON cells with CHKV was completed following the infection scheme detailed in **Figure 4**. Results showed that over-expression of OAS1-42 via doxycycline induction of WT TET-ON cells resulted in a mean 18.10-fold reduction in virus replication compared to mock-treated groups. Our first, second, and third replicates of CHKV infection showed a decrease in OAS1-42 over-expressing cells with p= 0.002513, p= 0.019259and p= 0.353648, respectively **(Fig. 15).** While in each replicate over-expression of OAS1-42 via doxycycline induction of RNase-L-KO TET-ON did not have a significant impact on CHKV viral replication. Our first, second, and third replicates showed no significant reduction in CHKV viral replication in OAS1-42 overexpressing RNase-L-KO cells with p=0.247214, p= 0.416778, and p= 0.719098, respectively.

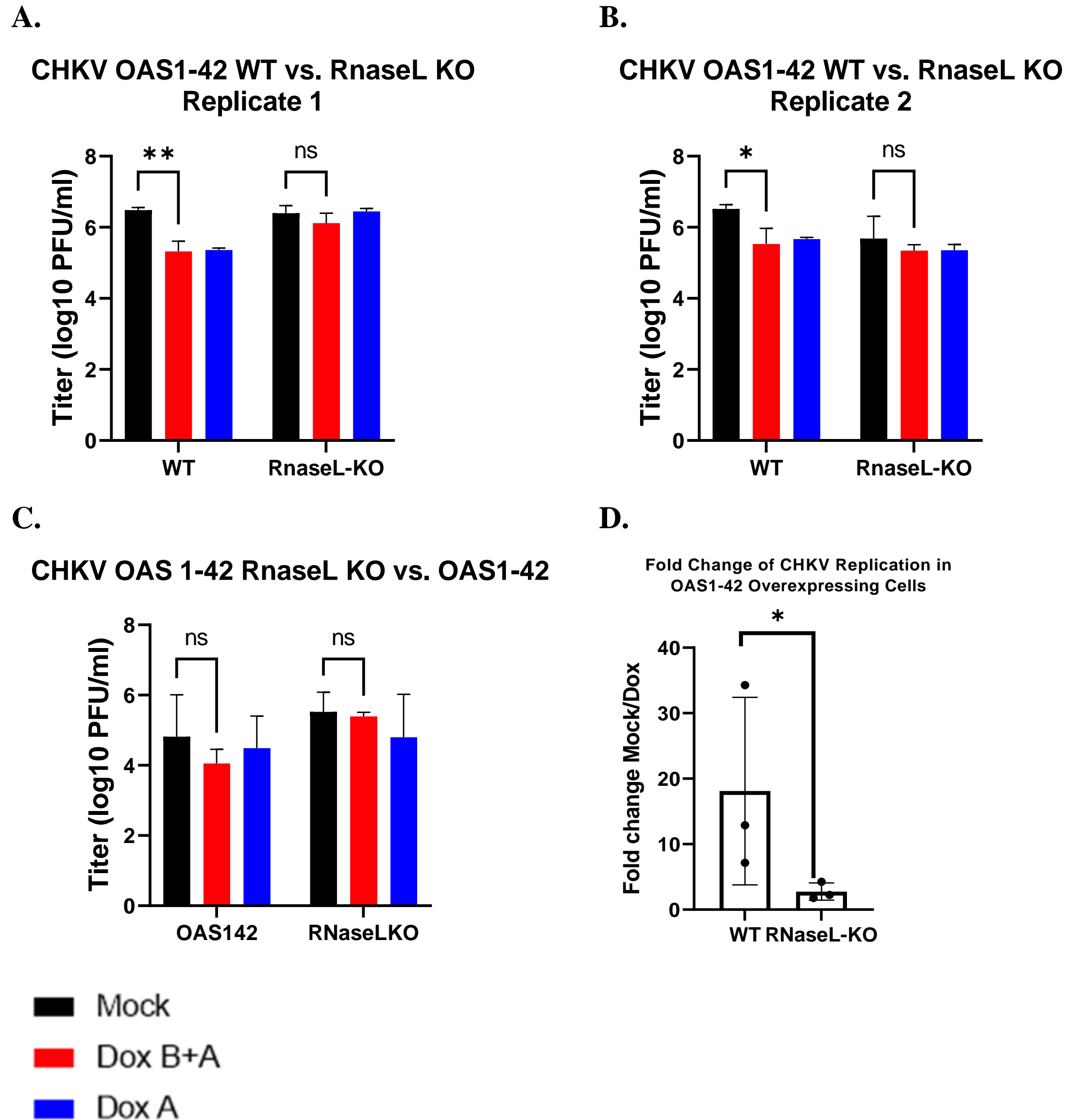


Figure 15: The Ability for OAS1-42 Overexpression to Suppress CHKV Replication is Ablated in RNase-L -KO Cells: A.) Initial Replicate of CHKV infected OAS1-42 vs. OAS1-42 expressing RNase-L -KO cells. **B.)** Second Replicate of CHKV infected OAS1-42 vs. OAS1-42 expressing RNase-L -KO cells. **C.)** Third replicate of CHKV infected OAS1-42 vs. OAS1-42 expressing RNase-L -KO cells. **D.)** Calculated Fold Change of CHKV replicates. *** =p value <0.001; ** = p value <0.01; * =p value <0.05

The ability for OAS3 overexpression to suppress CHKV Replication is ablated in RNase-L -KO A549 *cells.*

Infection of A549 TET-ON cells and A549 RNase-L- KO TET-ON cells with SINV was completed following the infection scheme detailed in **Figure 4**. Results showed that over-expression of OAS3 via doxycycline induction of WT TET-ON cells resulted in a mean 11.56-fold reduction in virus replication compared to mock-treated groups. Our first and second replicates of SINV infection showed a reduction in OAS3 over-expressing cells with p= 0.000085, and p= 0.000032, respectively **(Fig. 14).** While in each replicate over-expression of OAS3 via doxycycline induction of RNase-L-KO TET-ON did not have a significant impact on SINV viral replication. Our first and second replicates showed no significant reduction in SINV viral replication in OA3 overexpressing RNase-L-KO cells with p= 0.056842 and p=0.136339, respectively.

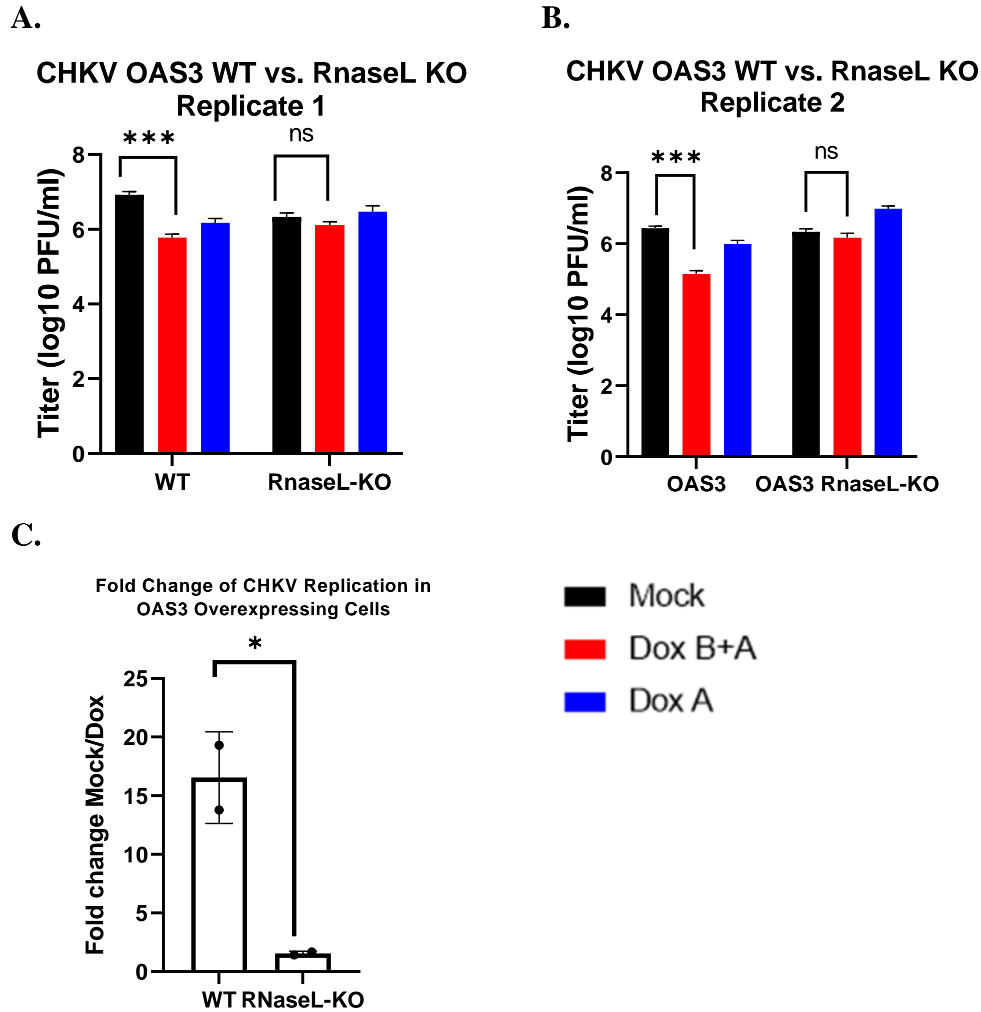


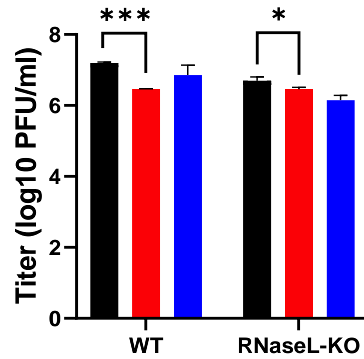
Figure 16: The Ability for OAS3 Overexpression to Suppress CHKV Replication is Ablated in RNase-L -KO Cells: A.) Initial Replicate of CHKV infected OAS3 vs. OAS3 expressing RNase-L -KO cells. **B.)** Second Replicate of CHKV infected OAS3 vs. OAS3 expressing RNase-L -KO cells. **C.)** Calculated Fold Change of CHKV replicates. *** =p value <0.001; ** = p value <0.01; * =p value <0.05

The ability for OAS1-42 overexpression to suppress ZIKV Replication is reduced but not ablated in RNase-L -KO A549 *cells.*

Infection of A549 TET-ON cells and A549 RNase-L- KO TET-ON cells with ZIKV was completed following the infection scheme detailed in **Figure 4**. Results showed that over-expression of OAS1-42 via doxycycline induction of WT TET-ON cells resulted in a 5.43-fold reduction in virus replication compared to mock-treated groups. Our ZIKV infection showed a reduction in OAS1-42 over-expressing cells with p= 0.000897 **(Fig. 17).** There was a slight but significant reduction in ZIKV viral replication in OAS1-42 overexpressing RNase-L-KO cells with p= 0.023719 (**Fig. 17**).

A.

ZIKV OAS142 WT vs. RNaseL KO Replicate 1



B.

Fold Change of ZIKV Replication in OAS1-42 Overexpressing cells

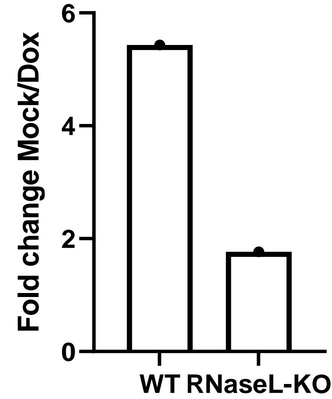


Figure 17: The Ability for OAS1-42 Overexpression to Suppress ZIKV Replication is Reduced But Not Fully Ablated in RNase-L -KO Cells: A.) Initial Replicate of ZIKV infected OAS1-42 vs. OAS1-42 expressing RNase-L -KO cells. **B.)** Calculated Fold Change of ZIKV replicates. *** =p value <0.001; ** = p value <0.01; * =p value <0.05

The ability for OAS2 overexpression to suppress WNV Replication is reduced but not fully ablated in RNase-L -KO cells.

Infection of A549 TET-ON cells and A549 RNase-L- KO TET-ON cells with WNV was completed following the infection scheme detailed in **Figure 4**. Results showed that over-expression of OAS2 via doxycycline induction of WT TET-ON cells resulted in a mean 6.15-fold reduction in virus replication compared to mock-treated groups. Our first, second, and third replicates of WNV infection showed a reduction in OAS2 over-expressing cells with $p= 0.057840$, $p= 0.005529$, and $p= 0.000197$, respectively **(Fig. 18).** While in each replicate over-expression of OAS2 via doxycycline induction of RNase-L-KO TET-ON did not have a significant impact on WNV viral replication. Our first, second, and third replicates showed a significant reduction in WNV viral replication in OAS2 overexpressing RNase-L-KO cells with $p= 0.057840$, $p= 0.035064$, and $p= 0.212273$, respectively.

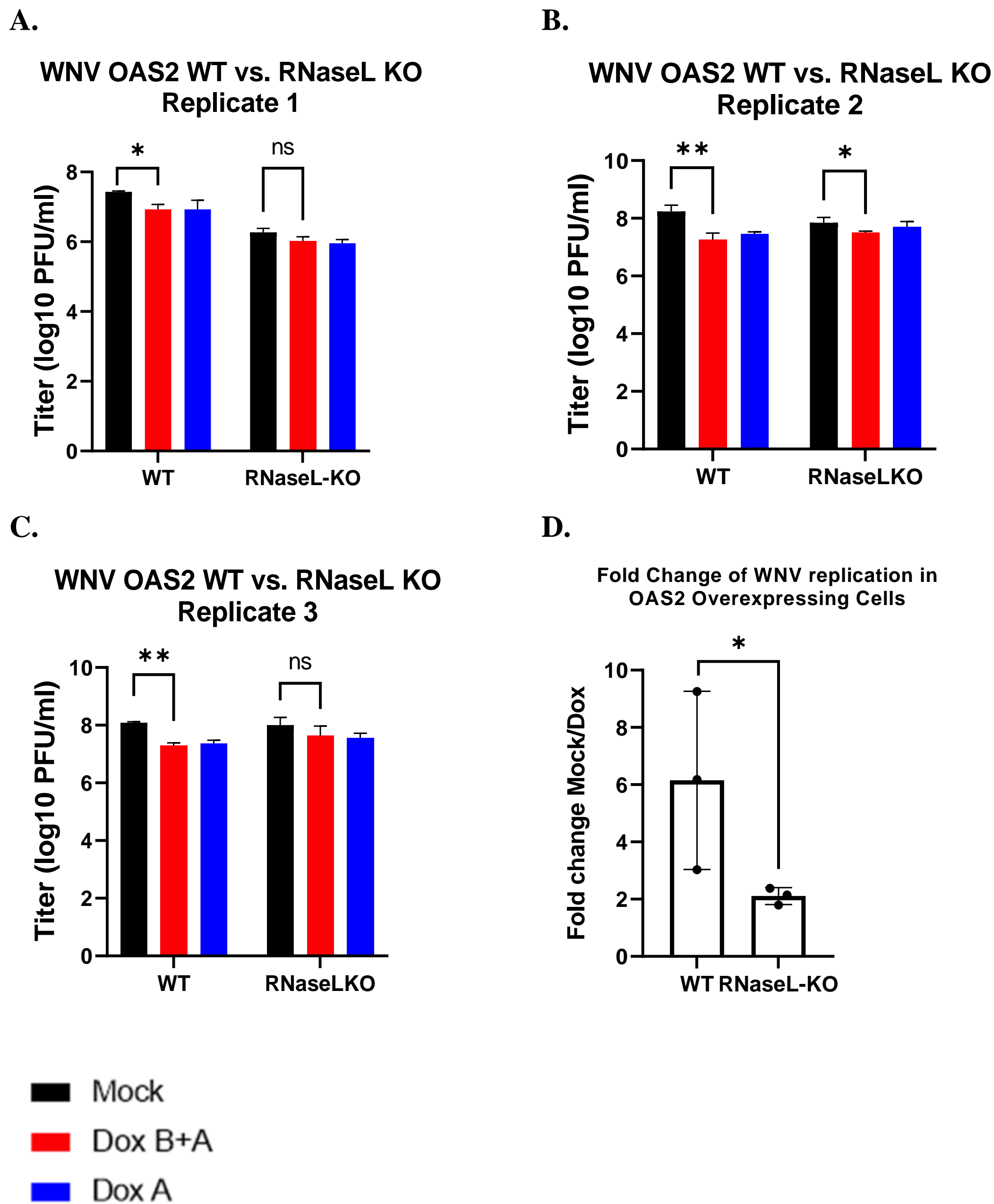


Figure 18: The Ability for OAS2 Overexpression to Suppress WNV Replication is Ablated in RNase-L -KO Cells: A.) Initial Replicate of WNV-infected OAS2 vs. OAS2 expressing RNase-L -KO cells**. B.)** Second Replicate of WNV infected OAS2 vs. OAS2 expressing RNase-L -KO cells. **C.)** Third replicate of WNV infected OAS2 vs. OAS2 expressing RNase-L -KO cells. **D.)** Calculated Fold Change of WNV replicates. *** =p value <0.001; ** = p value <0.01; * =p value <0.05

Discussion

The 2',5'-oligoadenylate synthetase (OAS)-RNase-L pathway.is a major innate immune defense pathway against viral infections. It is primarily induced by IFN secretion produced in response to viral infection by adjacent cells (Koyama et al. 2008). Upon IFN binding to the IFN-R on the cell surface, phosphorylation of STAT proteins by the Janus kinases (JAK) results in the dimerization of STAT and translocation of STAT protein to the nucleus of the cell. The cell is then transitioned into an antiviral state via the transcription of copious amounts of ISGs, which are further translated to combat viral infection (Schoggins 2014). Among these ISGs is the OAS family of proteins, which plays a vital role in this process; after OAS proteins are translated, they subsequently bind mainly cytoplasmic dsRNA in a length-dependent manner (Wang et al. 2020; Eskildsen et al. 2003). The binding of these OAS proteins to dsRNA causes synthesizing of 2-5A, utilizing ATP as a substrate, which acts as a potent activator of RNase-L. RNase-L activation results in nonspecific cleavage of cytoplasmic RNA, aiding in the termination of viral replication within the cell and ultimate clearance of viral infection (Eskildsen et al. 2003).

Mutations in OAS proteins in humans and other mammals have extensively been correlated with heightened vulnerability to specific viral infections (Soveg et al. 2021; Lim et al. 2009; Wickenhagen et al. 2021), indicating that certain fully functioning OAS proteins may be more significant in safeguarding against select viruses. Despite a wealth of clinical evidence suggesting heightened susceptibility or severity of infection associated with mutations in specific OAS proteins for certain viruses, research gaps remain in our understanding of which OAS proteins are most crucial in protecting against most viral infections. Furthermore, underlying reasons why these proteins may be more important

than others in combating specific viral infection remains elusive. In addition to better prediction of which patients may be at risk for severe illness from certain viruses, understanding which OAS proteins are most important for protection against certain viruses could aid in the development of prophylactic antiviral strategies as well as therapeutic interventions for those persons who are at a higher risk of severe viral illness after being infected with particular viruses. Moreover, gaining a deeper comprehension of the interdependence between specific OAS proteins and RNase-L can reveal further insights into the intricate antiviral mechanisms that occur within a cell, of which a better understanding would allow more progress in the development of antiviral strategies.

Not only do OAS proteins exhibit tissue-specific expression patterns resulting in variations of specific isoform concentrations across cell types, but OAS proteins also occupy various cellular compartments based on their isoform (J. Liu, Qian, and Cao 2016; Hovanessian 2007; Perry et al. 2005). While the majority of OAS proteins reside in the cytoplasm, enabling them to bind cytoplasmic viral RNA effectively, specific isoforms of OAS can localize to other cellular compartments. For instance, specific OAS isoforms have been identified in distinct cellular compartments such as the ER membrane (OAS1) and even the nucleus (OAS3) (Soveg et al. 2021; Malaguarnera, Nunnari, and Di Rosa 2016). This multifaceted distribution of OAS proteins is believed to aid in the suppression of viral replication at different points in the viral replication process and helps to defend against viruses that may utilize certain cellular compartments more often than others. Recent evidence suggests that the importance of specific OAS proteins regarding their ability to inhibit specific virus replication may be associated with the cellular localization of viral replication within a cell (Wickenhagen et al. 2021; Soveg et al. 2021). For instance,

prenylated OAS1-46 has been shown to localize to endomembrane systems, which SARS-CoV-2 utilizes during its replication process. Previous reports showed that less severe cases of COVID-19 were associated with patients containing alleles with a common splice-acceptor single nucleotide polymorphism in OAS1 (Rs10774671) were associated with less severe COVID-19 and that these people could express a prenylated form of OAS1 (p46) (Wickenhagen et al. 2021).

Recognizing that cellular localization of OAS proteins has previously been correlated with their importance in impeding viral replication for certain viruses, we hypothesized that the capacity of OAS proteins to inhibit viral replication, when overexpressed, would be consistent across viruses within the same family. The logic behind this hypothesis stems from the notion that viruses within the same family have analogous viral replication strategies and utilize similar organelles and cellular compartments to replicate. However, our results indicate that this is not fully the case. Here we show that even within the same family, the capacity for overexpression of specific OAS proteins to inhibit viral replication can include various isoforms of OAS and can vary among viruses even within the same virus family, indicating that there may be factors besides cellular localization of viral replication that influence the importance of specific OAS isoforms in impeding viral replication.

Further, we aimed to investigate the underlying mechanism behind the inhibition of viral replication for distinct OAS isoforms; specifically, we analyzed whether or not suppression of viral replication via overexpression of OAS isoforms relied on RNase-L. We show here that though suppression of viral replication achieved by overexpression of OAS3 was nullified in RNase-L-KO cells, overexpression of OAS1 and OAS2 isoforms in

RNase-L-KO cells resulted in reduced but not entirely eliminated viral replication. These results suggest that though suppression of viral replication via OAS1 and OAS2 does depend, at least partially, on RNase-L, there may be alternative mechanisms of viral suppression utilized by OAS1 and OAS2 isoforms that have yet to be elucidated.

We showed that when OAS3 is overexpressed in A549 cells before and after viral infection, there is a mean 12.03-fold decrease in viral replication when compared to mock-treated groups **(Fig. 10)**. These findings are consistent with previous reports that showed OAS3 is significant for suppressing SINV replication in cell culture experiments (Li et al. 2016). Further, OAS3 overexpression in A549 cells also reduced CHKV replication, with a mean 22.83-fold decrease in viral replication compared to mock-treated groups (**Fig. 11**). This is also consistent with real-world *in vitro* data that demonstrated that when HeLa cells express a truncated form of OAS3, they were less resistant to infection with CHKV (Bréhin et al. 2009). Some studies have shown that this inhibition can be overcome by mutations in CHKV that render it immune to OAS3 antiviral effects. Interestingly, our results here show that CHKV virus replication was reduced when OAS1-42 was overexpressed, though this did not appear to be the case when SINV infection was present in the same cell line. Both CHKV and SINV are primarily arthropod-borne viruses and are *Alphaviruses* within the family *Togaviridae*. The differences in the impact of specific OAS proteins on viral replication indicates that reliance on OAS proteins is unlikely to be based solely on the cellular localization of viral replication because both SINV and CHKV utilize similar virus replication strategies and similar cellular organelles during the viral replication process (J. Y. S. Leung, Ng, and Chu 2011; Holmes et al. 2020). Other differences beyond cellular

localization during the viral entry and replication process between SINV and CHKV may contribute to the differences in the impact of certain OAS proteins on viral replication.

For instance, SINV and CHKV differ in the architecture of their genome and proteome, resulting in differences in transcribed genes and eventually translated non-structural proteins. Both the polyprotein of SINV and CHKV gives rise to the four nonstructural proteins (nsP1-4) essential in virus replication and five structural proteins that makup the viral particle. Each of these proteins can be made after they are translated from transcribed RNA, which varies in length. Previous studies have shown that the ability of OAS proteins to bind dsRNA is length-dependent (Wang et al. 2020). Thus, it is possible that if viruses, even within the same family, produce dsRNA during their replication cycle that, even if it has a similar function or is made in the same areas of the cell, is different in length, they may resultantly be more likely to bind certain isoforms of OAS that better bind the length of dsRNA produced by those viruses. Additionally, differences in the type of cells that viruses infect can contribute to the types of cells that a particular virus can enter, and different cells have different concentrations of OAS isoforms (Shuvojit Banerjee et al. 2014). Thus, it is possible that if a certain cell type has a naturally higher concentration of OAS isoforms than other cells, that isotype may make a larger impact on viral replication for viruses that typically infect those cells.

Our findings also show differences in the impact of OAS protein overexpression and viral replication when comparing ZIKV and WNV, both of which are members of the *Flavivirus* family of viruses. We demonstrate here that when OAS1-42 is overexpressed in ZIKV infecte4d A549 cells before and after viral infection, there is a mean 20.62-fold reduction in virus replication compared to mock-treated groups **(Fig. 12)**. There is currently

no reliable pre-clinical or clinical data that supports or contrasts these findings of the importance of OAS1-42 in the suppression of ZIKV replication. Further, results showed that over-expression of OAS2, OAS3, and GFP via doxycycline induction of WNV-infected TET-ON cells reduced virus replication at least approximately 10-fold compared to mock-treated groups (**Fig. 13**). Note that in real-world pre-clinical mouse data and clinical human patient data, OAS1 mutations were associated with a greater susceptibility to infection (Lim et al. 2009; Mashimo et al. 2002). Note that there was some decrease in viral replication in WNV-infected cells for which OAS1-42 and OAS1-46 were also overexpressed. However, compared to mock-treated groups, these did not display statistical significance when comparing groups treated with doxycycline before and after viral infection. It is possible that differences in which OAS proteins impact viral replication may be distinct from those that impact susceptibility to infection. Additionally, these experiments were completed in overexpression models. Though they may be directly translatable to cells within the body that express similar levels of OAS proteins as those expressed in this experiment, it is also possible that cells that are naturally infected by viruses used in this study do not contain translatable amounts of OAS proteins, therefore, even if these results provide information as to what protein can inhibit viral replication in the largest capacity and are relevant for the design of therapeutic interventions, they may not be reflective of which OAS proteins are most important during a particular infection.

Additionally, it is interesting that GFP overexpression slightly inhibits viral replication of WNV. Though GFP, to our knowledge, does not contain the ability to bind dsRNA or mimic OAS protein functions, it is possible that the mere expression of GFP interfered with the viral replication process of WNV in this study in ways that we currently

do not fully understand. Though a solid understanding of which OAS proteins are most important for the inhibition of viral replication is significant for understanding which patients may be most at risk for severe infection or illness from viruses used in this study, we still aimed to understand why these proteins inhibited viral replication in the ways that were seen in this work. Thus, we sought to determine whether the inhibition of viral replication posed by overexpression of OAS proteins relied on RNase-L.

Previous studies have shown that OAS3 plays a substantial role in 2'5' OAS synthesis and RNase-L activation, more so than OAS1 or OAS2 isoforms (Li et al. 2016). The impact of OAS proteins on the activation of RNase-L in this study was tested using an A549 cell line in which OAS1, OAS2, OAS3, or RNase-L were knocked out, and the subsequent ability for polyIC, a dsRNA analog, to activate degradation of cellular RNA was tested. It was discovered that RNA degradation was absent in both OAS3-KO and RNase-L-KO cell lines upon poly-IC transfection. Further, in the same cell lines, and upon SINV infection, OAS3-KO cells showed a large reduction in the amount of 2'5'A production, which is required to activate RNase-L, indicating that OAS1 and OAS2 are not required to activate RNase-L. This study further showed that OAS3 was the predominant antiviral OAS for several RNA viruses, evidenced by a significant increase in viral replication in OAS3-KO cells that were not present in OAS1-KO and OAS2-KO cells (Li et al. 2016). Studies like these posed the question as to whether viruses that show suppression of viral replication via OAS1 or OAS2 mediate this suppression of viral replication in part through RNase-L independent mechanisms that are still to be further elucidated. This study aimed to answer this question for *Alphavirsues* and *Flaviviruses* viruses used in this study.

Intriguingly, we observed that even in RNase-L -KO cells, overexpression of OAS proteins OAS1-42, OAS1-46, and OAS2 still displayed a reduction in viral replication, at least to some extent. In contrast, RNase-L-KO cells that overexpressed OAS3 showed an ablation in viral replication compared to cells that contained RNase-L, such as those results seen for SINV and CHKV-infected cells that overexpressed OAS3 before and after viral infection **(Fig. 14 and Fig 16).**

CHKV replication in cells that overexpressed OAS1-42 before and after viral infection showed a decrease in viral replication in WT cells, and though they still displayed a decrease in viral replication in RNase-L-KO cells, this decrease did not appear to be statistically significant **(Fig. 15).** ZIKV replication in cells that overexpressed OAS1-42 before and after viral infection also showed a decrease in WT cells, but in RNase-L-KO cells, the viral replication was reduced but not fully ablated **(Fig.17).** These results are suggestive of alternative mechanisms utilized by OAS1-42 able to suppress viral replication that does not rely on RNase-L. Additionally, there appear to be differing results for CHKV and ZIKV. While in CHKV-infected RNase-L-KO cells that overexpressed OAS1-42, a decrease in viral replication was not statistically significant, in ZIKV-infected RNase-L-KO cells that expressed OAS1-42, the reduction in viral replication did display statistical significance. This indicates that the extent to which these proteins can decrease viral replication in conjunction with RNase-L activation may depend on the type of virus being tested. Further, WNV-infected RNase-L-KO cells that overexpressed OAS2 before and after viral infection showed a decrease in their ability to suppress viral replication when compared to WNV-infected cells that contained WT RNase-L but still were able to suppress WNV viral replication **(Fig 18).**

These findings suggest the existence of alternative pathways in which OAS proteins OAS1-42 and OAS2 inhibit viral replication, bypassing the need for RNase-L. Though the findings from these investigations have provided us with initial data on what OAS enzyme is most important for halting the replication of certain viruses, it is essential to consider that these results were completed *in vitro*. In a living system, many more immunological factors may be present that can impact how well or how quickly a virus replicates. Additionally, these investigations' findings can be expanded by applying *in vivo* models. For example, the pathogenesis of particular viruses can be studied in mice's knockout (KO) models, where a specific gene coding for an enzyme of interest, such as an isoform of OAS, is removed. Further, RNase-L knockout mouse models have already been developed and help determine viral pathogenesis associated with viruses that utilize RNase-L to suppress viral replication. Future *in vitro* experiments could examine other portions of the 2' 5' Oligoadenylate synthetase (OAS) pathway to determine if those factors impact viral replication by upregulation of proteins or by removing those proteins from the cell line to determine if the function is affected in any way. These future studies could help to expand on the knowledge gained through experiments presented here. They could aid in a better understanding of OAS and RNase-L-mediated suppression of replication for certain RNA viruses, which ultimately could be applied clinically and in the production of anti-viral interventions.

CHAPTER 3

DISTINGUISHING CIRCULATING ANTIBODY REPERTOIRES OF RESPONDERS VS. NON-RESPONDERS TO IMMUNE CHECKPOINT BLOCKADE (ICB) FOR METASTATIC OSTEOSARCOMA (MOS) IN BALB/C MICE

Abstract

Osteosarcoma is the most common childhood bone malignancy, with a sharp decline in survival rates upon metastasis. We have previously demonstrated immune checkpoint blockade (ICB) of α-CTLA-4/α-PD-L1 given to mice inoculated with a K7M2 metastatic osteosarcoma (mOS) cell line resulted in ~50% survival with complete tumor clearance. Differences in response rates to ICB are common among patients with the same malignancy. However, unlike inbred lab mice, human patients have multiple variance factors, causing the discrepancy in ICB response to remain poorly understood. Prior to inoculation with mOS or ICB, our previous findings unveiled that blood from mice displays distinct differences in circulating Abs between responders vs. non-responders, suggesting the presence or absence of particular Abs influences ICB efficacy. The genesis of antibody production varies from subject to subject, as the specificity of Abs is not encoded in germline DNA; instead, its variation originates from somatic recombination and somatic hypermutation processes which are specific to each subject. For this reason, both human and murine subjects are likely to display distinctions in Ab repertoire, even if they come from the same genetic background. Here, we sought to replicate our previous results that showed differences in distinct Ab repertoires between responders and non-responders to ICB for mOS before inoculation with mOS or ICB treatment. Further, we investigated and

characterized other factors that could contribute to these observed differences based on Ab binding patterns.

Introduction

Osteosarcoma (OS) is the most common bone cancer in children, making up ~3% of total child cancer cases, with survival rates declining from ~70% to ~20% upon metastasis (Czarnecka et al. 2020). The incidence rate of osteosarcoma is currently at projected ~1,000 cases to be diagnosed this year, with the majority of these cases in children and adolescents ("Home | American Cancer Society - Cancer Facts & Statistics" n.d.). It is predicted that cases of adolescent OS will only increase in the coming years, as past trends have shown that little progress in the development of treatment for the disease has resulted in ongoing poor prognoses for patients, but increases in population and respective illness are expected to continue (Eyre et al. 2010). Traditional treatment methods for metastatic Osteosarcoma (mOS) involve surgery, radiation therapy, chemotherapy, or a combination of all three but have shown limited efficacy, encouraging interest in pioneering alternative treatment strategies (Lindsey, Markel, and Kleinerman 2017). Immune checkpoint blockade (ICB) that employs α-CTLA-4/α-PD-L1 has improved survival rates amongst various malignancies (Y. J. Park, Kuen, and Chung 2018). We have previously demonstrated that human mOS cells express PD-L1, a ligand for PD-1 on cytotoxic T lymphocytes (CTL), implying PD-L1 expression may limit CTL control of mOS (Lussier, O'Neill, et al. 2015). Similarly, murine mOS over express PD-L1 upon metastasis compared to primary tumors. Our previous results also showed that α-PD-L1 increases T cell killing of mOS cells, reduces tumor burden, and extends lifespan in mice

inoculated with mOS before treatment, resulting in prolonged survival but not complete eradication of mOS (Lussier, O'Neill, et al. 2015). Further, α-CTLA-4/ α-PD-L1 has improved progression-free survival among patients in both pre-clinical and clinical trials for several cancers (Y. J. Park, Kuen, and Chung 2018; Topalian et al. 2014; Postow et al. 2015; Wolchok et al. 2013).

When α-CTLA-4 and α-PD-L1 were co-administered to mice inoculated with mOS prior, ~50% who received the treatment survived and later exhibited immunity to the same mOS cells (Lussier, Johnson, et al. 2015). It is common for patients with the same malignancy to respond to immunotherapies differently, if at all (Sambi, Bagheri, and Szewczuk 2019). However, unlike inbred laboratory mice, human patients have differences in genetic makeup, environment, and other circumstances that make it difficult to identify why some patients respond inversely to treatment (Sambi, Bagheri, and Szewczuk 2019). However, in our model, genetically identical mice housed under identical conditions also display differences in ICB effectiveness. There is an urgent need to determine *why* ICB is effective in some individuals and not others to improve ICB effectiveness for mOS leading to better patient outcomes. Our long-term goal in this research is to determine what factors limit the efficacy of ICB for mOS, with the ultimate goal of translating these findings to improve life expectancy for many pediatric patients with mOS. This specific research aimed to examine biological differences in recipients of α-CTLA-4/α-PD-L1 ICB for mOS in a murine model by characterizing circulating Ab compositions between responders and nonresponders to ICB composed of α-CTLA-4 and α-PD-L1, to determine potential improvements in ICB efficacy for mOS.

The discrepancy among mice regarding their response to combination ICB therapy is curious, considering the mice used in the study are inbred Balb/c mice with similar/identical genetic makeup. They were also the same age and experienced the same living conditions. One possibility of variance is differences in circulating antibodies (Abs). Variance in circulating Abs can be present among genetically similar subjects for a number of reasons, mice and humans produce natural Abs formed during development without antigen induction (Holodick, Rodríguez-Zhurbenko, and Hernández 2017). In addition to natural Abs presence, circulating Abs can often be indicators of risk for or development of certain diseases. Recent studies have characterized Ab presence in sera using microarrays with >300,000 short peptides. The binding pattern of the Abs on the microarray is termed an "immunosignature." Analysis of immunosignatures has been shown to predict the identity of infectious diseases with >95% accuracy (Holodick, Rodríguez-Zhurbenko, and Hernández 2017). We initially wished to characterize the Ab response between ICB responders and non-responders in mOS-bearing mice but found that immunosignatures from pre-bleeds, prior even to tumor inoculation, could distinguish responders from non-responders before ICB treatment on a microarray of 10,000 unique peptides **(Fig. 19).**

Traditionally, immunosignatures have diagnostically predicted disease via the binding pattern of Abs in sera to the many peptides on these arrays, resulting in reproducible patterns for specific diseases (Restrepo, Stafford, and Johnston 2013; Helmink et al. 2019). However, this technology has not been used to identify treatment responders vs. non-responders. Here, we reproduced the model of responders and non-responders to α-CTLA-4/α-PD-L1. We then sent sera from pre-bleeds in these mice for immunosignature assays to be characterized according to the currently available library of

300,000 peptides. We now have created a "trained" immunosignature profile to support predictive ability in this mOS ICB model. By analyzing peptide sequences bound by Abs, we further identified potential targeted antigens that could indicate why some subjects respond to treatment, and others do not.

Predictive immunosignatures for determining the efficacy of ICB could prioritize patients likely to respond to therapy, while identification of Abs that facilitate or impede ICB could be translated to improve patient outcomes by aiding in the design of novel therapeutics.

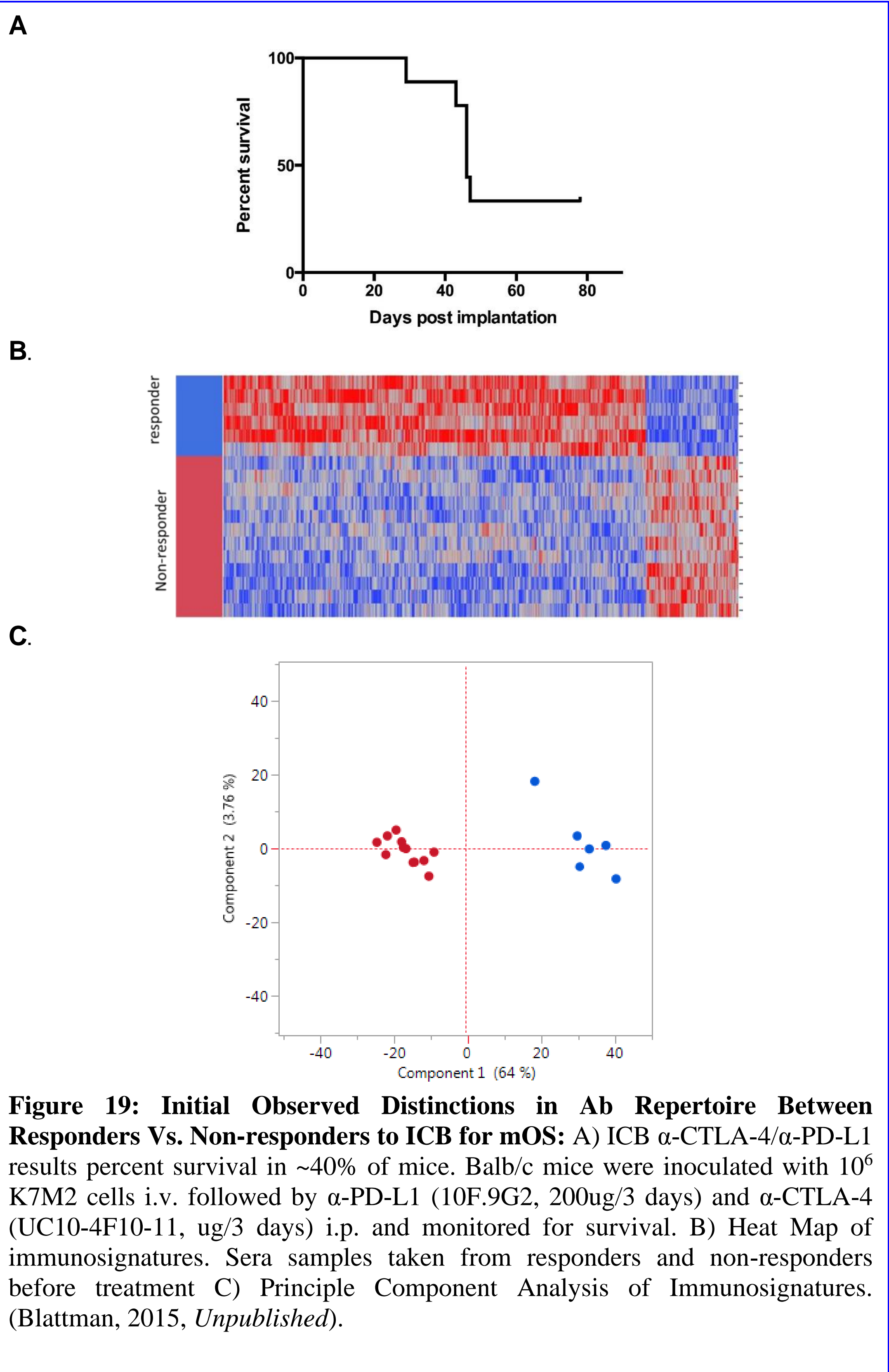


Figure 19: Initial Observed Distinctions in Ab Repertoire Between Responders Vs. Non-responders to ICB for mOS: A) ICB α-CTLA-4/α-PD-L1 results percent survival in ~40% of mice. Balb/c mice were inoculated with 10^6 K7M2 cells i.v. followed by α-PD-L1 (10F.9G2, 200ug/3 days) and α-CTLA-4 (UC10-4F10-11, ug/3 days) i.p. and monitored for survival. B) Heat Map of immunosignatures. Sera samples taken from responders and non-responders before treatment C) Principle Component Analysis of Immunosignatures. (Blattman, 2015, *Unpublished*).

Metfhods

In vivo Antibodies and Cell lines

The α-CTLA-4 monoclonal antibody was purified via saturated ammonium sulfate (SAS) precipitation from the UC10-4F10-11 hybridoma (ATCC, Manassas, VA). Hybridomas were maintained in Roswell Park Memorial Institute (RPMI) media and were initially cultured in 20% fetal bovine serum (FBS) but were weaned to lower levels of FBS media until 2.5% FBS was reached. After cells grew to confluency in 2.5% FBS RPMI, cells were allowed to die for one week by not adding fresh media to the cells, and the supernatant was harvested in preparation for SAS precipitation. The α-PD-L1 monoclonal antibody (clone 10F.9G2) for *all in vivo* blockade experiments was purchased from BioXCell (West Lebanon, NH). K7M2- Luc cells were a gift from Helman lab and were cultured in 10% fetal bovine serum (FBS), 1% of 100x penicillin, Streptavidin, and glutamine (PSG), in Dulbecco's Modified Eagle's medium (DMEM). K7M2- Luc cells were verified mycoplasma free before tumor inoculation.

Mice and generation of tumors

Twenty Balb/c/J mice were purchased from Jackson Laboratories (Bar Harbor, ME) and were housed in the Arizona State University Biodesign Institute under specific pathogen-free conditions. All experiments listed here were approved by the Institutional Animal Care and Use Committee (IACUC) and were conducted under the appropriate supervision. Metastatic osteosarcoma tumors were established in all 20 mice by injection of $2* 10^6$ either K7M2-Luc cells via lateral tail vein in 100 μL of phosphate-buffered saline (PBS).

After inoculation with K7M2-Luc cells, mice were monitored by weight and a clinical scoring system that evaluated breathing, ambulation, lethargy, and scruffiness (BALS).

Treatment and Bleed Schedule

Mice were treated with ICB composed of α-PD-L1 and α-CTLA-4. Mice were given five doses of α-PD-L1 monoclonal antibody (clone 10F.9G2) at 200 µg per dose, purchased from BioXCell (West Lebanon, NH), which occurred every three days beginning one day after tumor inoculation. Mice were also given three doses of α-CTLA-4 at 100 µg per dose of monoclonal Abs beginning one day after tumor inoculation. Mice were bled via submandibular bleeds three days before inoculation with K7M2 cells 11 days after K7M2 inoculation and 25 days after inoculation. Mice were later monitored for tumor progression and survival.

Immunosignature Assays

Immunosignature assays were prepared as described previously (Stafford et al. 2012) with a 300,000 peptide library. Arrays were scanned at 10-µm resolution at 647-nm wavelength by an Agilent C scanner for fluorescence readings. Microarrays were first incubated in a blocking buffer of PBS, 0.5% BSA, and 0.5% Tween for 1 hour. Serum samples taken from mice were added to the arrays at a 1:500 dilution into a sample buffer of 0.5% BSA and PBS for 1 hour at 25 °C. The serum samples served as the primary antibody. The primary antibody was then washed away with a blocking buffer, and peptide-bound antibodies were determined by secondary incubation with 5 nM AlexaFluor 647-conjugated α-mouse (Rockland Antibodies) which incubated for 1 hour at 25 °C

Factor Analysis

Factor Analysis was completed by Calviri. A total of 76 peptides from a t-test with a p-value less than 0.005 from t-tests were filtered using a LASSO (least absolute shrinkage estimator) and a Schwarts Bayesian (SBC) stop criterion. A total of 4 of the 10 samples were selected at random for training and 6 were used for testing. This was repeated 5 times. Logistic regression, least angle regression random forest and support vector machine (SVM) with an interior point polynomial degree 2 kernel were used. Factor analysis was conducted with maximum likelihood estimation and promax rotation and was based on eigenvalues greater than 1.0; two factors were extracted.

Identification of Peptide Homology Among Peptides With High Binding using Gapped Local Alignment of Motifs (GLAM2)

Peptides from **Table 3** were recorded in FASTA format and analyzed using Gapped Local Alignment of Motifs (GLAM2) (Frith et al. 2008). Sequences were aligned and subsequent frequency of each amino acid at specific peptide positions was recorded. GLAM2 attempts to find the best possible motifs 10 times then GLAM2 provides a score for each motif, higher scores indicate stronger motifs. A total of 63 peptide sequences were included in the final analysis while 11 were excluded since the sequences were less than 8 amino acids in length and GLAM2 only can attempt to align sequences 8 amino acids in length or longer (Frith et al. 2008). Sequences that were excluded from GLAM2 analysis have an asterisk (*) in front of the sequence number in **Table 3**. Additionally, the "GSG" linker which is included at the N terminus was not included in any sequence when analyzed using GLAM2.

Basic Local Alignment Search Tool (BLAST) of Sequence Motifs

Using NCBI BLAST, sequence motifs that were determined by running peptide sequences through GLAM2 were input using pBLAST which is specialized to find protein sequences with shared homology to an input sequence. Parameters were set to look only at standard databases, and non-redundant protein sequences. Algorithm was set to blastp (protein-protein BLAST). Only the possible combinations of peptide from Motif A were input into BLAST and analyzed.

Results

Balb/C mice inoculated with K7M2 and treated with α-CTLA-4 and α-PD-L1 showed 60% survival and complete tumor clearance.

In our study, mice treated with a combination of α-CTLA-4 and α-PD-L1 following the treatment schedule described showed 60% of mice (6 mice) survival after 150 days with no signs of metastasis, while 40% of mice (4 mice) succumbed to metastasis. This replicates past trends (Lussier, Johnson, et al. 2015). Control groups of mice that were only given PBS succumbed to metastasis by day 80 post-tumor inoculation **(Fig. 20).**

High Binding Peptides Identified in Responder Group

We initially sent ten sera samples to be arrayed. The rfu data was log base 2 transformed for analysis. The results show noteworthy mean differences between the average rfu of different sample types on average. Generally, the non-responder samples have a lower total array rfu than the responder samples. Satterthwaite corrected T-Tests were run on the 10 known samples to look for mean differences on a per peptide level. Seventy-four peptides were identified with p-values less than 0.05 **(Table 3).**

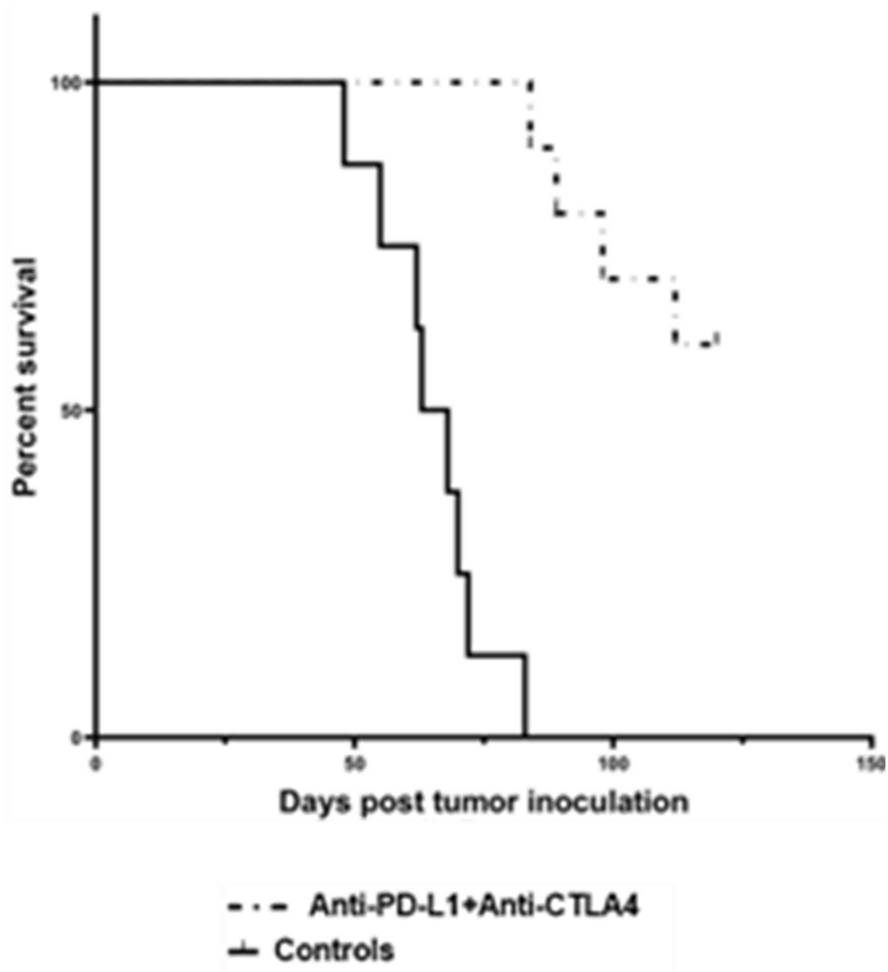


Figure 20: Survival Curve of Mice Treated with α-CTLA-4/α-PD-L1 for mOS: Mice treated with α-CTLA-4/ α-PD-L1 showed 60% survival, while mice treated with PBS controls all succumbed to metastasis by day 80 post-tumor inoculation.

Factor Analysis

Responder samples typically showed greater overall reactivity on arrays and accounted for most of the variance seen in the factor analysis. Overall, responders load more heavily on factor 1 while non-responders load more heavily on factor 2 **(Fig. 21).** One responder H3 loads more heavily on factor 2 than on factor 1, indicating a possible misclassification. Sample H4 loads on both factors and appears to be slightly higher on factor 1 and so is ostensibly indeterminate.

Sequence Alignment Using Gapped Local Alignment of Motifs (GLAM2)

A total of 5 gapped sequence alignments with GLAM2 scores 100 or higher were discovered after the initial run using GLAM2 **(Fig. 22).**

Table 3: Sequences Identified by immunosignature analysis to have distinct high binding in Responder samples

Sequence Number	*Peptide Sequence with High Binding in Responders*
>Sequence1	YLVVGWQSGGSG
*>Sequence2	PGGVNEDGSG
>Sequence3	ALQVNQVNDGGSG
>Sequence4	YGYRLFGGVSGGSG
>Sequence5	PLRWWVLFSGSG
>Sequence6	EQFNLQKGGSG
>Sequence7	FQAANQVSGGSG
>Sequence8	PAVKLELKVGGSG
>Sequence9	FRAKLLFDGSG
>Sequence10	AFFLYWHVGGSG
*>Sequence11	QQNSLSDGSG
>Sequence12	WSEVRWRFGGSG
>Sequence13	WPLRFSWVLGGSG
>Sequence14	DQQEHVLGGSG
>Sequence15	NLGEVRSGGSG
>Sequence16	FVALNKVGGSG
>Sequence17	PAGQANQAGGSG
*>Sequence18	HAAGSEGGSG
*>Sequence19	LKNEEDGSG
>Sequence20	WWWEWFEGGSG
>Sequence21	HQVVSSDGGSG
>Sequence22	YYVAANKFSGSG
>Sequence23	PNANDASRLFGSG
>Sequence24	NAWNLVFQFGGSG
>Sequence25	NVDVNQGLDGGSG
>Sequence26	QQKNLVLSGSG
>Sequence27	LYYHDGYQKSDGSG
>Sequence28	SRLASDGQGGSG
>Sequence29	EEHNGGSG
*>Sequence30	DVVLRSGGSG
>Sequence31	VNLVKLDGGSG
>Sequence32	WQLLHLRLGGSG
>Sequence33	QFAWLKLDLSGSG
>Sequence34	VLWAVLAGGSG
>Sequence35	FYGRLHLFSGSG
>Sequence36	FNLKFSWQDGSG
>Sequence37	NVDLAQHHDGSG
*>Sequence38	KQPLVGGSG
>Sequence39	FAWYVKFDGSG
>Sequence40	QQDQRFLGGSG
>Sequence41	FSVWHFHLFGSG
>Sequence42	PDNGVYQVSGSG

*>Sequence43	ESGDEGGSG
*>Sequence44	SNSFVSEGSG
>Sequence45	FSYSVGARLGGSG
>Sequence46	ESNLNLFSGGSG
>Sequence47	YQLQLVAKDGGSG
>Sequence48	SYQSQKDRSEGSG
>Sequence49	GLVANRVGGSG
>Sequence50	PESRLDDGGSG
>Sequence51	RQNNQVLEGSG
>Sequence52	QQRNALSEGSG
>Sequence53	LQSNNLGLGGSG
>Sequence54	SWLGWWQLSGSG
>Sequence55	YWSVVWKFDGSG
>Sequence56	VKLVDAFEGGSG
>Sequence57	RELQSNQRSGSG
>Sequence58	ERLKVSLEDGSG
>Sequence59	SANNQQVGGSG
>Sequence60	KVVGDRLDGGSG
>Sequence61	GQYNQQVDGSG
>Sequence62	LYAQYWWHGGSG
>Sequence63	EHNGKHDGGSG
>Sequence64	QHYNVKKVEGSG
>Sequence65	LQPLAGGSG
>Sequence66	LQLSVEKDGGSG
*>Sequence67	KQALVLSGSG
*>Sequence68	LNLAPNGGSG
>Sequence69	VEDQPARPHEGGSG
>Sequence70	KFSYNAQANDGSG
>Sequence71	WSGGYWKGQVFGSG
>Sequence72	SDEHQHGGSG
>Sequence73	KQSNGLSDGSG
*>Sequence74	WVWWGWGGSG

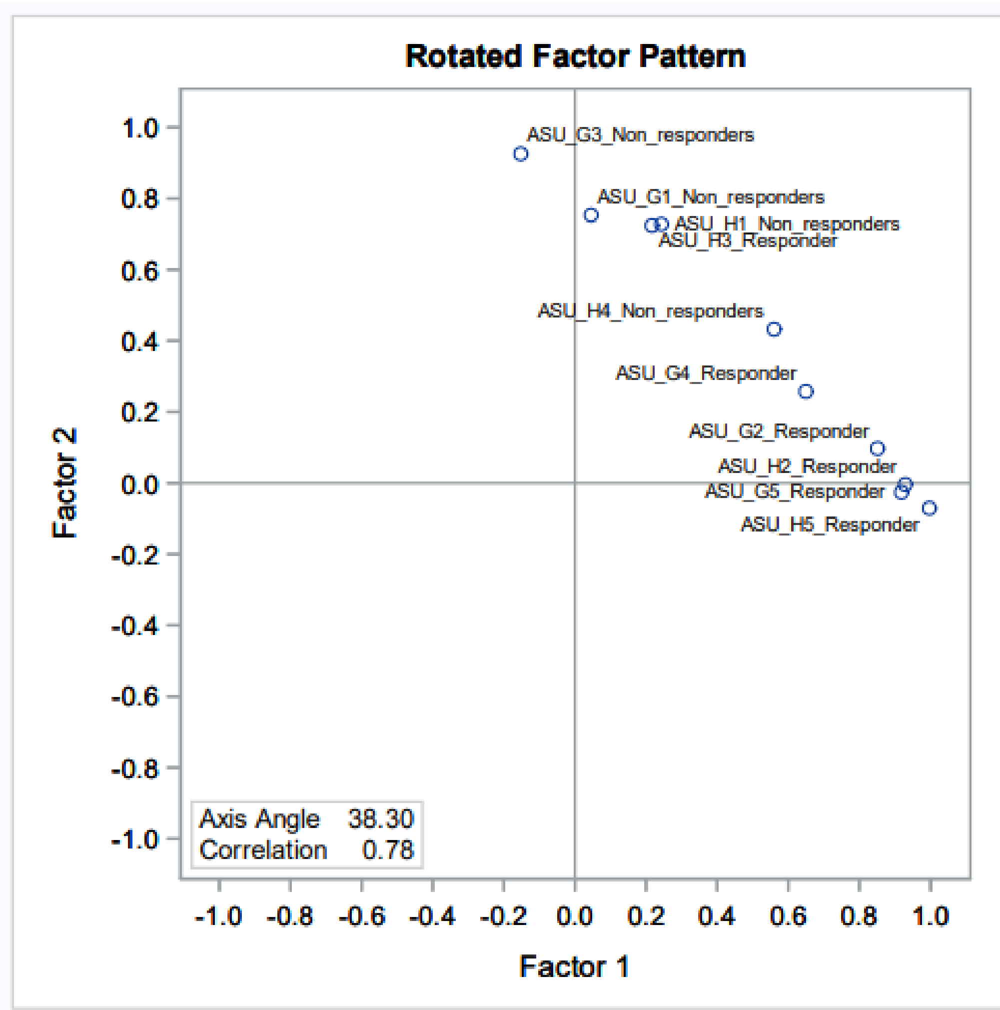


Figure 21: Factor Analysis of Immunosignature Responder and Non-Responder Samples on Factor 1 vs. Factor 2. Responders tend to load on Factor 1 more closely, while non-responder samples tend to load more closely on Factor 2.

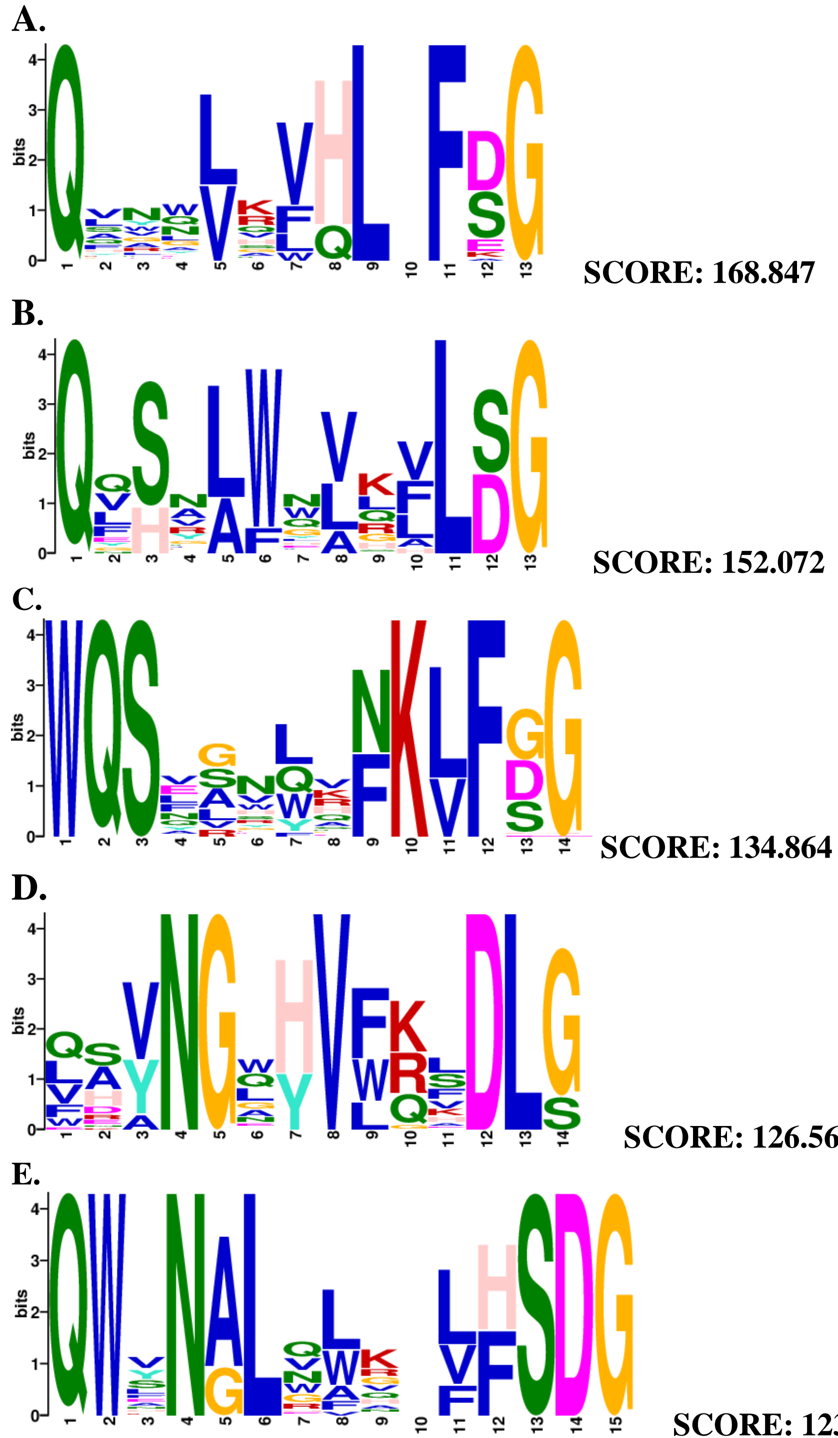


Figure 22: Motif Analysis of Peptides in Table 3. Sequences were aligned and frequency of each amino acid at the indicated positions was determined. The 5 top scoring replicates with a GLAM2 score of more than 120 are shown above. Larger peptides at a certain position indicate higher frequency of a peptide in that location among sequences.

Table 4: Regular Expression for Motifs in Figure 22		
Seq	**GLAM 2 Score**	**Expression Motif**
A	168.84	Q?VNW[LV]?K[FV]?[QH]?L?.?F?[SD]?G?
B	152.07	Q?[QV][HS]?N[AL]?W?N[LV]?K[FLV]?L?[SD]?G?
C	134.86	W?Q?S?.[AGS]?N[QLW]?.[FN]?K?[LV]?F?[SDG]G?
D	126.56	[LQV][AS]?[YV]?N?G?[QW][YH]?V?[LWF]?[QKR]?[LS]D?L?G?
E	123.01	Q?W?VN?[AG]?L?Q[LW]?K.?[FLV]?[HF]?S?D?G?

Discussion

This research represents a novel association of pre-existing immunosignature profiles as predictors of eventual ICB treatment efficacy for mOS. Immunosignatures have traditionally been used as diagnostic tests to determine the likelihood of certain diseases before the genesis of untreatable symptoms, such as those seen in Alzheimer's and certain cancers (Restrepo, Stafford, and Johnston 2013; Stafford et al. 2014b). Here, we have already shown the technology's ability to serve as a correlative test to determine whether a subject will respond to α-CTLA-4/α-PD-L1 even before inoculation with mOS **(Fig. 19)** and further showed that these results are reproducible **(Fig. 20).** Regarding factor analysis of our most recent data, responders mostly loaded on Factor 1, while non-responders loaded more strongly on Factor 2 **(Fig 21).** During our most recent data replication, one sample, H3, was a responder to ICB but, upon factor analysis, loaded more closely to Factor 2, which was more typical of non-responders. This result represents a potential margin of error in these immunosignatures recorded previously in immunosignature trials, which report a 10% margin of error in predictive modeling (Stafford et al. 2012, 2020). Since these assays rely on the integrity of Ab stability to determine the binding capacity of antibodies in a sample to a particular peptide, factors that decrease the integrity of Ab structure can impact the ability of these assays to predict phenotypes correctly.

For example, samples in this study underwent multiple freeze-thaw cycles due to the necessary transport of the samples, which could lead to the degradation of Abs in the sample and an overall decrease in reactivity, potentially skewing results. Additionally, the trial results were cut-off at 150 days post-tumor inoculation, and it is possible that an

extension of this trial would have led to H3 developing tumor burden. However, tumor burden was not evident after the trial period. These trials can be repeated with larger sample sizes in the future, which may negate outliers like H3, which skew results.

Factor analysis is a method used to relate a set of underlying observed "latent" variables. It was chosen to analyze the data set because it can explain covariance among observed random variables concerning latent factors without defining what those latent factors are. This system is helpful for our analysis since, at the time of data analysis, we could not determine the factors that differentiated the responder from non-reponder antibody profiles. This analysis differs from principal component analysis (PCA) in that PCA analyses assume no measurement error, random error to be expected within an experiment, or systemic error that could be due to issues with instrumentation or assays, which was likely present to some degree in the samples assayed. Additionally, PCA forces all variance to be common and does not allow for unique variance. Lastly, PCA is only orthogonal and does not allow for correlation or relationships among components; factor analysis allowed us to determine how similar and different samples in the analysis were.

Though immunosignatures have often been used to characterize Ab repertoires and classifications within samples, this technology has not yet been used to determine why differences in pre-existing immunosignatures exist among genetically identical mice and if they can then be altered, indicating potentially more positive outcomes for ICB-treated subjects. Analysis of bound peptide sequences and comparison to protein databanks could explain observed Ab discrepancies. Findings from this work can shift current research paradigms because they model a novel way to determine *why* ICB therapy will or will not be effective on a subject rather than merely determining *if* it will be effective. Such findings

could be widely applied to other sarcoma immunotherapy regimens through the same methods.

The epitopes on both the immunosignature arrays used in this study and microarrays used to verify the results' reproduction were randomly generated and therefore do not necessarily represent proteins found in nature. If there exists shared sequence homology between an epitope on an array and a protein present in nature, differences in protein folding and binding to other proteins that may be present due to the presence of other charged amino acids in a natural protein may not be present in the peptides on the microarray. For this reason, an Ab that might bind to the conformation of a particular peptide on a microarray may not attach to that same sequence when it is present and in a different folding conformation in its natural form. For these reasons, information about which peptide Abs in this study bind on microarrays should be analyzed; knowing that they may or may not represent immune reactions to natural proteins, they continue to serve as a valuable tool to identify and characterize circulating Ab populations in different subjects.

Immunosignatures and protein microarrays that seek to characterize circulating Ab repertoires have many applications that extend past identification of potential responders and non-responders to cancer immunotherapy. Recent studies have also used them to diagnose cancer and other diseases, such as Alzheimer's, on the basis that during the development of these diseases, circulating antibody repertoire may be altered (Stafford et al. 2014b; Restrepo, Stafford, and Johnston 2013). If immunosignatures based on positive and negative patient responses to treatment were characterized, future applications could include monitoring cancer development and presence after immunotherapy treatment to determine which treatments are effective for a patient.

CHAPTER 4

THE IMPACT OF MICROBIOME DYSBIOSIS ON T CELL FUNCTION WITHIN THE TUMOR MICROENVIRONMENT (TME)

Abstract

Insights into the effect of the microbiome's composition on immune cell function have recently been discerned and further characterized. Microbiome dysbiosis can result in functional alterations across immune cells, including those required for innate and adaptive immune responses to malignancies and immunotherapy treatment. Dysbiosis can yield changes in or elimination of metabolite secretions, such as short-chain fatty acids (SCFAs), from certain bacterial species that are believed to impact proper immune cell function. Such alterations within the tumor microenvironment (TME) can significantly affect T cell function and survival necessary for eliminating cancerous cells. Understanding these effects is essential to improve the immune system's ability to fight malignancies and the subsequent efficacy of immunotherapies that rely on T cells. In this review, we assess typical T cell response to malignancies, classify the known impact of the microbiome and particular metabolites on T cells, discuss how dysbiosis can affect their function in the TME then further describe the impact of the microbiome on T cell-based immunotherapy treatment, with an emphasis on recent developments in the field. Understanding the impact of dysbiosis on T cell function within the TME can carry substantial implications for the design of immunotherapy treatments and further our understanding of factors that could impact how the immune system combats malignancies.

Introduction

Microbial species inhabit nearly every organ of the human body; their significance has recently been established in proper health and immune cell function, with potential impacts in the tumor microenvironment (TME) through the presence or absence of microbial-derived metabolites such as short-chain fatty acids (SCFAs), that can impact T cell functioning. The human microbiome comprises a complex network of various organisms, including those of bacterial, archaeal, fungal, viral, and protozoan populations, many of which are capable of symbiotic or pathogenic manifestations on the host, particularly when oscillation in microbial composition occur (Riiser 2015). In a healthy host, microbial populations typically outnumber human cells; current studies have estimated the number of bacteria alone in the human body is roughly the same as that of human cells (Sender, Fuchs, and Milo 2016). Technological advancements such as metagenomic sequencing and sophisticated data analysis have allowed scientists to characterize the abundance and diversity of the human microbiome, classifications that have allowed researchers to elucidate the potential mechanisms by which these species impact health and disease (Freilich et al. 2009; Qin et al. 2010; Methé et al. 2012). Studies support the necessity of a diverse, stable, and balanced microbiome to maintain general health and proper immunity to disease, with negative impacts during microbiome dysbiosis (Tuddenham and Sears 2015). Microbial dysbiosis is an imbalance in the composition of microbial communities within a host resulting in perturbations from normal cellular and organ function, illness, or reduced treatment efficacy for an infection or disease (Petersen and Round 2014). The etiology of dysbiosis is diverse and includes pathologies resulting from inflammation, infection, diet, genetics, and antibiotic administration (Willing et al. 2010; Claesson et al.

2012; Pham and Lawley 2014). Current studies have also demonstrated the impact of host-microbiome interactions during dysbiosis on cancer progression and treatment, including disease presence and chronic inflammation (Czesnikiewicz-Guzik and Müller 2018). Dysbiosis is believed to impact the regular functioning of immune cells caused by modifications in microbial-produced metabolites needed for proper performance (Arpaia et al. 2013; Luu et al. 2019). Such alterations in immune cells can impact not only how we respond to pathogenic infections but also the immune response to neoplastic events.

The degree of T cell tumor site infiltration and proper function within tumor sites can significantly affect tumor progression or regression (Al-Shibli et al. 2008). Therefore, an alteration in T cell function can cause a massive change in the efficacy of how T cells respond within the TME and, ultimately, their ability to clear malignancies. Understanding the mechanistic impact of microbial composition on T cells and their function within the TME is essential if we hope to understand and improve treatment strategies for malignancies. Here, we review canonical T-cell responses to malignancies, the impacts of the microbiome in the context of its typical and dysbiotic state on T-cell signaling and further how this can change T-cell function within the TME, recent discoveries in the field, and the potential that research stemming from these investigations can have on how we design and administer cancer therapies.

Conventional Anti-tumoral Activity of T-cells

Adaptive immune cells, such as T cells, play a considerable role in the antitumor immune response, with the presence of tumor-infiltrating T lymphocytes exhibiting positive

prognostic value across a wide range of cancers (L. Zhang et al. 2003; R. C. Taylor et al. 2007). To understand how microbiome dysbiosis can affect the ability of T cells to react to malignancies, we must first understand conventional α-tumoral T-cell mechanisms.

Typical CD4 T Cell Signaling and Function in Response to Malignancies:

CD4 T cells are directly and peripherally involved in the antitumor immune response through effects on innate and adaptive immune cells such as macrophages and T cells. Individually, CD4 T cells can be cytotoxic to incipient and progressive malignancies, acting directly on unhealthy cells that present neoepitopes to eliminate them through differentiation into Th1 cells and ensuing perforin/granzyme B-dependent killing or target cell elimination via ligation of Fas/FasL (or other TNF/TNFR family) death receptors **(Fig. 24 A**) (K. U. Lundin et al. 2004). Peripherally, CD4 T cells can also secrete IL-2 and IFN-γ, which have been found to elicit M1 macrophages to inhibit tumor propagation via secretion of nitric oxide synthase (iNOS), reactive oxygen species (ROS), and IL-12, resulting in direct and indirect clearance of tumor cells. Additionally, M1 macrophages can engulf malignant cells to eliminate them **(Fig. 24 B)** (X. Zhou et al. 2021). These M1 mechanisms are productive during MHC class II negativity, which can result from the loss of MHC Class II trans-activator (CIITA) expression during carcinogenesis (Haabeth et al. 2014). Additionally, CD4 T cells are necessary to support continued CD8 memory T cell survival and function (Janssen et al. 2003). They secrete cytokines, such as INFγ and IL-

2, that can act on CD8 T cells to improve the anticancer immune response **(Fig. 24 C)** (Ossendorp et al. 1998).

Typical CD8 T Cell Signaling and Function in Response To Malignancies:

The presence of CD8 T cells within the TME has been associated with improved tumor clearance and overall prognosis, often characterized by the concomitant presence of pro-inflammatory cytokines such as Type I IFN (Trujillo et al. 2018). The mechanisms by which CD8 T cells within the TME are believed to eliminate cancerous cells are via ligation of the Fas ligand (Fas L) on T cells with the Fas receptor (FasR) on target cells or by the perforin/ granzyme B pathway, with a preference for FasL/FasR pathway for cancerous cells **(Fig. 24 D)** (Chávez-Galán et al. 2009). Though these mechanisms can aid in the clearance of tumors, tumor-infiltrating lymphocytes often display upregulation of inhibitory markers, such as PD-1 and CTLA-4, which bind PD-L1 and CD80/CD86 respectively, which can halt α-tumoral effector functions and result in reduced effector cytokine production (Ahmadzadeh et al. 2009) .

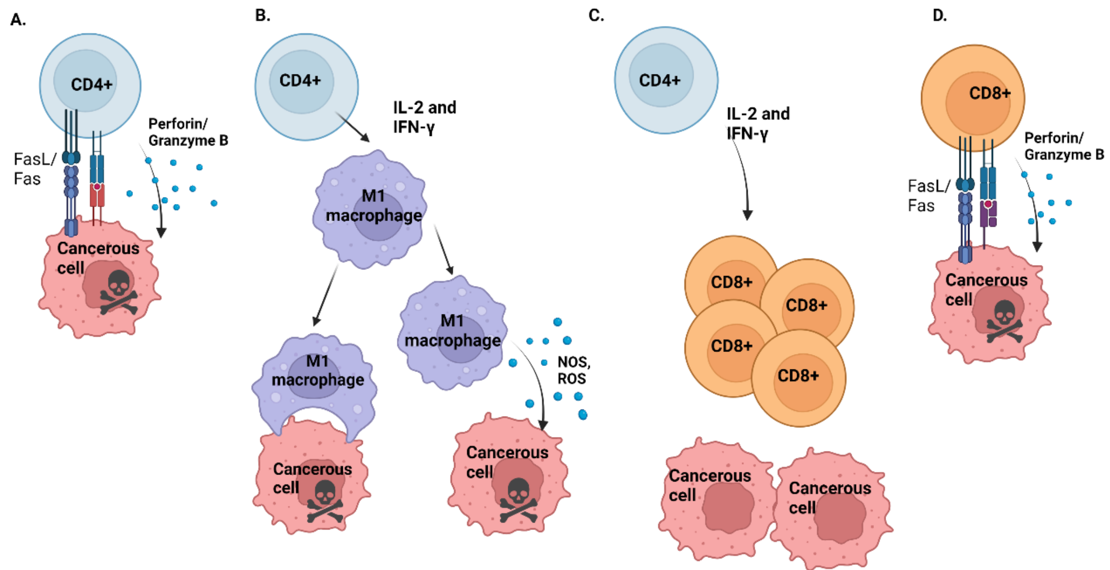


Figure 23: Conventional Mechanisms of T Cell Anti-Tumoral Activity. A). Th1 differentiated CD4 T cell killing of cancerous cells that present neoepitopes on MHC II via secretion of perforin and granzyme B. **B).** CD4 T cells release cytokines IL-2 and IFN-γ to recruit M1 macrophages, activated M1 Macrophages can kill cancerous cells either by direct phagocytosis or release of NOS or ROS, which results in tumor cell death, and continue to secrete IL-12, among other cytokines which recruits T cells to the site of the tumor. **C).** Activated CD4 T cell release of cytokines such as IL-2 and IFN-γ which recruits CD8 T cells to the site of tumor growth. **D).** CD8 T cells directly kill cancerous cells that present neoepitopes via MHC I via the Fas/FasL and via the release of perforin/ granzyme B, resulting in tumor cell killing. *Image Created with BioRender.com*

Molecular Impact of the Microbiome on Anti-tumoral Activity of T-cells

There is evidence that microbial species within the mucosal tissue of a host and within the tumor microbiome contribute to patient tumor immune response variations, with correlations between metabolic functions of microbes present within the TME and clinical patient presentation (Nejman et al. 2020). These trends have been documented for several pancreatic, bone, and breast cancers (Riquelme et al. 2019; Nejman et al. 2020; Sagarika Banerjee et al. 2021). Alteration of the host microbiome can change host cell function, including that of innate and adaptive immune cells (Thaiss et al. 2016; Russo et al. 2016). The mechanisms underlying these cellular changes are still being investigated but have been better characterized in recent studies and are believed to be primarily associated with alterations in metabolite secretions by microbial species that subsequently impact immune cell function (W. Yang et al. 2020; Luu et al. 2019). In particular, microbes produce fermentation products known as short-chain fatty acids (SCFAs); these free fatty acids contain short aliphatic carbon chains and are composed of less than six carbons. Typically when referring to SCFAs, the following are included: formic acid (C1), acetic acid (C2), propionic acid (C3), butyric acid (C4), and valeric acid (C5) (J. Tan et al. 2014). SCFAs are predominantly water-soluble, and therefore easily transported throughout the body, and are believed to play a significant role in the differentiation, function, and regulation of T cells and other immune cells through the promotion of pro- or α-inflammatory cytokines needed for particular effector functions (Arpaia et al. 2013; Ryu et al. 2022). Thus, alterations in microbiome composition and consequent changes in microbial metabolite secretion may disrupt T cells' conventional effector functions against malignancies.

Tumor-specific Microbiome Associations with Malignancies:

Microbial composition within the TME varies across cancers and further differs from adjacent healthy tissues, even in solid tumors with no direct contact with the external environment (Nejman et al. 2020). Several cancers, including breast, lung, ovarian, colorectal, melanoma, brain, prostate, and bone, have exhibited the presence of specific microbial species contributing to a dysbiotic state within tumor tissue (Thompson et al. 2017; Costantini et al. 2018; Greathouse et al. 2018; Apostolou et al. 2011; Castellarin et al. 2012; Sfanos et al. 2008; Nejman et al. 2020). These tumor-specific microbial populations can sometimes vary for different cancer types within the same organ systems (Sagarika Banerjee et al. 2018). Considering the known impact of microbial changes on immune cell function, changes within the TME regarding tissue-specific microbial populations may initiate changes in T cell function within neoplasms (Nejman et al. 2020). Intriguingly, *Rotter-Maskowitz et al.* recently identified correlations between intratumoral bacterial presence and predicted clinical presentation and response to anticancer treatment (Nejman et al. 2020). Similar concepts can be traced back to William Coley in the late 19th century, who showed that injecting killed bacterial species into tumor tissue resulted in tumor regression, which we now believe is due to adjuvant effects via activation of innate immune receptors and engagement of subsequent immune responses within the tumor (Kopenhaver, Carlson, and Snook 2020). Further investigations are needed to determine if bacterial presence in cancer impacts cancer progression via changes in immune cell function or if metabolites preferred by certain bacteria that are present as a result of cancer

progression provide a niche for those found in specific tumor tissues, the presence of particular bacterial species in tumor tissue compared to healthy tissue indicates a potential avenue for understanding better what factors impact cancer progression within the TME (Thompson et al. 2017).

Gut Microbiome Associations with Malignancies:

Investigations during cancer progression and treatment suggest certain gut microbial presence outside the TME correlate with systemic inflammatory processes that affect the TME, such as the upregulation of pro-inflammatory cytokines such as tumor necrosis factor (TNF) caused by increased immune cell responses, resulting in more significant tumor regression (Iida et al. n.d.). These systemic alterations could be associated with secreted metabolites from specific bacteria, noting that metagenomic studies concluded that the enrichment of anabolic pathways resulting from cellular metabolism and differences in pro-inflammatory cytokines caused by some bacteria's presence affect tumor response (Gopalakrishnan et al. 2018). Additionally, several studies suggest that gut microbiome composition plays a role in cancer progression at mucosal sites and in tumors not confined to mucosal tissue, which may be partly due to metabolites produced by microbes in the gut (Zhuang et al. 2019; Sánchez-Alcoholado et al. 2020).

Transport of Microbial-Derived Metabolites to T Immune Cells

Metabolites can be produced by gut-specific or organ-specific bacteria and passively or actively transported to other locations impacting organ and cellular function at local and

peripheral sites (Kamp and Hamilton 2006; Cummings et al. 1987). For this reason changes in microbiome composition will also impact metabolite presence. For instance, during dysbiosis, certain SCFAs or other metabolites may be reduced in quantity if the bacteria that produce them are no longer present. Active diffusion of metabolites in T cells can occur via membrane transporters, including MCT1 (monocarboxylate transporter-1/Slc16a1) and SMCT1 (sodium-coupled monocarboxylate transporter-1/Slc5 a8) (J. Park et al. 2014). Once inside the cell, SCFAs are known to act via G-protein-coupled receptor (GPCR) signaling, inhibition of histone deacetylase (HDAC), production of acetyl-CoA, and further changes in the metabolism of the cell resulting in increased or decreased functionality (C. H. Kim 2021). Evidence shows that SCFA presence activates mTOR, and STAT3 in T cells via GPCR41, GPCR43, GPCR109a resulting in Blimp-1 expression, which triggers the expression of many downstream signaling cascades (Y. Zhao et al. 2018). Studies in germ-free mice suggest that changes in microbiota can directly impact the expression of toll-like receptors (TLRs) (A. Lundin et al. 2008) and can affect antigen-presenting cell presence, T cell differentiation, and systemic immunity (Rangan and Mondino 2022).

Molecular Effects of Dysbiosis on CD4 T Cell Signaling and Function:

Recent studies showed C2, C3, and C4 metabolites, which can be produced by microbiota, could exhibit immunomodulatory functions by altering CD4 T cell differentiation in a concentration-dependent manner, as high concentrations of C2 and C3 drove expression of

IL-17A, IL-17F, RORα, RORγt, T-bet, and IFN-γ, cytokines associated with Th17 and Th1 profiles **(Fig. 25 A.)** (J. Park et al. 2014). More specifically, the natural killer group 2, member D (NKG2D) ligand system is a central immunomodulatory system in which immune cells recognize NKG2DL on stressed or infected cells through NKG2DR, present on immune cells such as CD4 T cells, CD8 T cells, and NK cells, activating effector functions (Groh et al. 2003). C3 produced by *propionibacteria* during cellular metabolism can induce the expression of NKG2D ligands MICA/B on both activated T lymphocytes and cancer cells in an intracellular calcium-dependent manner, allowing for proper immune effector function and potential prophylaxis of malignant cell**s (Fig. 25 B.)** (Andresen et al. 2009). This finding also implies the absence of C3 during the elimination of microbial populations that produce it can limit the degree of NKG2D ligands and, therefore, limit subsequent NKG2D/NKG2D interactions, resulting in a reduced overall T cell effector function within the TME. Further, C4 presence was shown to drive Treg differentiation in a concentration-dependent manner both *in vitro* and *in vivo* **(Fig. 25 C)** (Kespohl et al. 2017; Furusawa et al. 2013). Additional studies have indicated issues with the development of regulatory T cells (Tregs) in antibiotic-treated mice, indicating a requirement for healthy intestinal microbiota to achieve even normal Treg development (H. Han, Yan, and King 2021).

Molecular Impact of Dysbiosis on CD8 T Cell Signaling and Function:

In pre-clinical mouse models, microbial dysbiosis induced by maternal antibiotic treatment found that the offspring had altered CD8+ T Cell receptor signaling apparent by an inability to sustain interferon-gamma (IFN-γ) production *in vivo* after vaccination and *in vitro* upon

T cell receptor (TCR) stimulation. Resultantly, these cells did not maintain protein tyrosine phosphorylation and Erk1/2 activation, which are necessary for the proper functioning of CD8+ T Cells (Brownlie and Zamoyska 2013). Further, SCFA presence was shown to enhance the function of cytotoxic T lymphocytes through an increased function of mTOR post-treatment of T cells with pentanoate and C4 and driving supplementary inhibition of class I histone deacetylase activity. mTOR typically drives differentiation into Th1, Th2, and Th17 but is also a critical regulator in CD8 T cell differentiation through the regulation of cytolytic effector molecules (Finlay et al. 2012; C. Liu et al. 2015). Effector molecules such as CD25, IFN-γ, and TNF-α were then elevated in these treated cells, demonstrating enhanced cytotoxic activity and potential for pentanoate and C4 as supplements to cancer treatment for some malignancies **(Fig. 25 D)** (Schiweck et al. 2022).

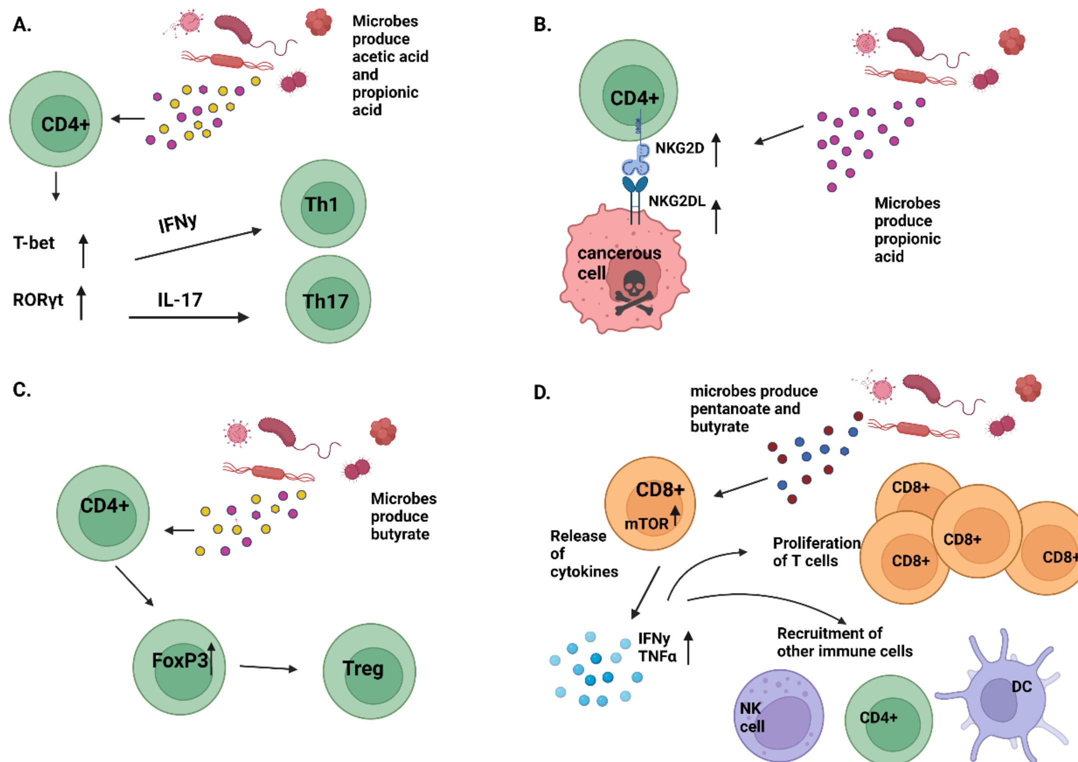


Figure 24: Impact of Microbial Secreted Metabolites on T Cell Function. A). Bacterial produced acetic acid (C2) and propionic acid (C3) can result in upregulation of T-bet and RORγt causing upregulation of IFNγ and IL-17 and subsequent differentiation into Th1 and Th17 T cells respectively**. B).** Microbial Produced propionic acid (C3) has been shown to result in upregulation of NKG2D and NKG2DL on CD4 T cells, CD8 T cells and NK cells, contributing to an α-tumoral environment and death of cancer cells. **C)**. Microbial produced butyrate has been shown to cause differentiation of T-regulatory cells (Tregs) in a concentration dependent manner. **D)**. Microbial produced pentanoate and butyrate have been shown to contribute to increased CD8 T cell effector functions. *Image Created with BioRender.com*

Elimination of Microbial Species by Antibiotics and the Resulting Effect on T Cell Populations

Considering the impact of microbial species on T-cell function, clinicians should be cognizant of the immunopharmacological behavior of antibiotics regarding microbial species-specific elimination based on antibiotic type. For instance, neomycin, which predominantly eradicates facultative gram-negative species, and vancomycin which predominantly eradicates gram-positive species, have both been associated with the reduced expansion of T cells in mouse models during antibiotic administration (Duan et al. 2010; Cheng et al. 2017). Cocktails of ampicillin, vancomycin, neomycin and metronidazole (AVNM) have been associated with lower immune function and decreased concentrations of bacterial metabolites C3 and C4 in mice (Ubeda and Pamer 2012). γδ T cells are a part of the Th17 subset and are a source of the pro-inflammatory cytokine IL-17. Antibiotics have also been shown to modulate γδ T cell populations, with variations depending on the antibiotic type used and species of bacteria eliminated (Duan et al. 2010). Understanding which antibiotics eliminate microbial populations may be critical when designing patient treatment regimens to reduce the chances of anomalous immune cell function.

Interactions of Dysbiosis on T Cell-based Immunotherapy in the TME

Both CD4 and CD8 T cells play an essential role in the clearance of malignancies. The impact of microbiome dysbiosis on immune cell function generates a challenging dilemma, considering that many immunotherapies in pre-clinical and clinical use, such as immune checkpoint blockade and adoptive cellular therapies, are T-cell-based.

Impact of Dysbiosis on T Cell Dependent Immune Checkpoint Blockade:

It has been documented that specific microbes in the gut can impact ICB immunotherapy approaches across several cancers (McCulloch et al. 2022). For instance, α-CTLA-4 treatment for melanoma relies on the presence of *Bacteroides* species; additionally, the treatment showed no effect on germ-free and antibiotic-treated mice (Vétizou et al. 2015). *Bacteroides* species can be propionogenic, having the capacity to generate the SCFA C3. Therefore, the absence of C3 can alter the proper function of T cells, which α-CTLA-4 treatment relies on (Louis, Hold, and Flint 2014). In a separate study, the introduction and restoration of propionogenic bacteria during antibiotic-induced dysbiosis resulted in the restoration of C3 levels, indicating that the re-establishment of propionogenic bacteria could counteract decreases in SCFAs required for the efficacy of particular immunotherapy (El Hage et al. 2019). In mice and humans, high C4 concentrations in the blood were associated with resistance to α-CTLA-4 therapies, evidenced by restrained upregulation of B7 on T cells (Coutzac et al. 2020). Similar results of reliance of immunotherapy efficacy on bacterial presence were seen in α-PD-L1 treatment for melanoma, which depended on the presence of *Bifidobacterium* (Sivan et al. 2015). *Bifidobacterium* species produce SCFAs C2, C3, and C4 SCFAs that contribute to immune cell function (Louis, Hold, and Flint 2014). Strikingly, more recent investigations have shown fecal transplants from ICB responders to non-responders for melanoma treatment saw that more than one-third of human patients previously unresponsive to treatment become responsive after transplants (Davar et al. 2021b).

Impact of Dysbiosis on Adoptive T-Cell Therapy:

Recent studies in mice have further shown that differences in gut microbiome composition and dysbiosis due to antibiotic administration could alter the efficacy of adoptive T-cell cancer treatments. However, these changes in response to treatment are likely species-specific since they vary based on the type of antibiotic administered. In fact, some mice treated with vancomycin displayed an increase in CD8α+ dendritic cells (DCs) with supplemental decreases in tumor burden in an IL-12-dependent manner. At the same time, alternative antibiotics did not produce the same effect (Uribe-Herranz et al. 2018). Additional studies have indicated that severe cytokine release syndrome during CAR- T cell therapy is associated with particular microbiome alterations and a higher abundance of *Bifidobacterium*, *Leuconostoc*, *Stenotrophomonas*, and *Staphylococcus* and that desired responses may require specific gut microbial presence (Hu et al. 2022; M. Smith et al. 2022). These results demonstrate that microbiome composition can also impact T-cell therapies whose efficacy relies on proper immune cell function.

The Impact of Diet on T cell Based Cancer Therapies

It is well known that diet can influence the establishment of microbial communities within a host (David et al. 2013). Since we now know that microbial community composition can impact T cell function, investigations regarding how diet can impact cancer treatments that rely on T cell function have been recently investigated. In mice, the western diet of high fat, high carbohydrate, and low fiber diet can decrease downstream production of short-chain fatty acids (SCFA), which originate from microbiota; this could impact T cell function and efficacy of treatments that rely on T cells. (Statovci et al. 2017). Other findings

have shown that ketogenic diets can increase antitumor immunosurveillance by reducing PD-L1 expression on tumor cells in a malignant glioma model. (Lussier et al. 2016). Additionally, when placed on a ketogenic diet, mice have displayed enhancement of the anticancer effects of PD-1 blockade. (Ferrere et al. 2021). More recent studies have shown that when fucoidan, a polysaccharide naturally derived from brown algae, was co-administered with ICB treatment, it significantly improved the antitumoral activity of PD-1 antibodies in a murine melanoma model *in vivo* through consistent activation of tumor-infiltrating CD8+ T cells. (J. Yang et al. 2021). Recent studies also indicate that calorie restriction can increase the antitumoral ability of T cells (Pietrocola and Kroemer 2019), an approach that, when applied to a murine triple-negative breast cancer model, augmented radiation efficacy (Saleh et al. 2013). Further, clinical trials analyzing melanoma patients showed that patients who consumed a high-fiber diet were five times more likely to respond to PD-1 therapy (Spencer et al. 2019). Modulation of diet serves as a potential interventional strategy that can be used to enhance T cell-based immunotherapies therapies in the future.

Discussion

With the onset of technological advancements such as molecular sequencing and sophisticated Metabolic-network modeling, insights into how microbiome composition impacts health, disease, and immune cell function disease, cancer, and immune cell function have been recently better illuminated. Further insights into cohesion between microbiome composition and proper immune cell function may be a helpful resource that will eventually allow scientists to regulate the immune response to and clearance of

malignancies. Further, broadening our understanding specifically of T cell function in the TME concerning microbiome composition may improve our understanding of how best to administer current antineoplastic drugs and therapies.

There also exists potential to exploit the dysbiotic microbiome's impact on immune cell function as an augmentation to α-tumoral therapies for some cancers that originate in common lymphoid progenitors (CLP). For instance, patients with cutaneous T-cell lymphoma (CTCL) reported decreased overall tumor burden when treated with an aggressive antibiotic regimen. Upon immunohistochemistry analysis, samples displayed a decrease in interleukin-2 high-affinity receptors in T cells at these sites, indicating a decline in mechanisms that allow for T cell proliferation (Lindahl et al. 2019). Similar regimens have also been pursued Mucosal Associated Lymphoid Tissue (MALT) lymphomas, improving 5- year survival rates (Ferreri et al. 2018). These incidents demonstrate the importance of identifying how antibiotics can affect immune cells, and the specific malignant cells clinicians might target during treatment.

Though it is evident that the composition of the microbiome and microbial dysbiosis can impact immune cell function and subsequent immune response to malignancy, there still exists research gaps that must be pursued if we hope to mitigate adverse effects of microbial dysbiosis and immune system dysfunction. Further research is needed into what comprises the "optimal" microbiome and whether this composition differs for particular diseases and malignancies. Current data indicate a requirement for the presence of specific microbial species' therapeutic efficiency (Sivan et al. 2015), but this also tends to vary from cancer to cancer, making it difficult to classify what an optimal microbiome for patients might look like. For this reason, a more significant effort is needed

to determine what microbes may offer benefits during cancer treatment and what causes these microbiomes to be beneficial or harmful in particular organ systems. Since both gut and tumor-specific microbial composition can potentially permute disease progression, identifying what comprises microbial populations both locally and systemically across specific cancers may serve as a helpful resource for future diagnostic approaches. In fact, several microbial signatures between blood and tissues have already been identified across cancers, indicating a potential diagnostic approach to cancer treatment that may soon be available (Poore et al. 2020).

Further research into how the dysbiotic state of the microbiome can impact T cell function can lead to potentially better treatments for cancer patients through the modulation of microbes present in the host. Knowledge pertaining to which bacterial communities and their associated mechanisms are needed for proper immune cell function would allow physicians to be cognizant of the implications associated with specific antibiotic use and subsequent species-specific elimination of bacterial communities in conjunction with ICB treatments. Overall, expanding our knowledge about the microbiomes' interconnection with the immune system and T-cell function during cancer progression and treatment can improve our knowledge of how to best design and administer cancer treatments and ultimately improve patient outcomes.

CHAPTER 5

THE IMPACT OF MICROBIOME MODULATIONS ON T CELL-MEDIATED IMMUNOTHERAPY EFFICACY FOR METASTATIC OSTEOSARCOMA (MOS)

Abstract

Osteosarcoma is the most common childhood bone malignancy, with a sharp decline in survival rates upon metastasis. We have previously demonstrated immune checkpoint blockade (ICB) of α-CTLA-4/α-PD-L1 to mice inoculated with a K7M2 metastatic osteosarcoma (mOS) cell line resulted in ~50% survival with not only complete tumor clearance but also resistance to the same K7M2 cell line upon later inoculation. Differences in response rates to ICB treatment are common among patients with the same malignancy across various cancers. However, unlike inbred lab mice, human patients have diversity in genetic makeup and other factors, making it difficult to understand what factors impact ICB efficacy. Recent studies have discovered that ICB's effectiveness can be associated with or even reliant on microbiome composition. Additional findings have shown that antibiotic treatment can significantly reduce CD3+ T cell presence in the lungs of saline-treated mice with viral infection compared to those not treated with antibiotics. These factors could be significant in the design of mOS treatments, as mOS frequently metastasize to the lungs, and antibiotic treatments are often needed for patients on ICB who are likely to develop nosocomial infections. For these reasons, we question whether microbiome composition impacts ICB for mOS and if alteration of the microbiome can cause subsequent alteration of T cell-mediated control of mOS. Our central hypothesis is that microbiome dysbiosis can alter T cell-mediated ICB efficacy for mOS. We first attempted to answer our research question by analyzing the microbiome composition of

these subjects before inoculation with mOS or ICB. We then altered the microbiome composition of our mice via antibiotic administration to determine if treatment outcomes would change. Microbiome composition and subsequent ICB efficacy for mOS have yet to be analyzed outside of this research but could significantly impact treatment outcomes if understood.

Introduction

Recent studies have demonstrated the impact of host-microbiome interactions on patient health, including disease presence and chronic inflammation (Czesnikiewicz-Guzik and Müller 2018; D. Kim, Zeng, and Núñez 2017). Further investigations in patients during cancer progression and treatment suggest the presence or absence of certain types of microbes in the gut could affect systemic pro- or anti-inflammatory processes, such as the upregulation of tumor necrosis factor (TNF), which could result in greater tumor regression (Iida et al. 2013). Additionally, the presence of specific microbes in the gut can also impact ICB immunotherapy approaches. For instance, α-CTLA-4 treatment for melanoma has been found to rely on the presence of *Bacteroides* species. At the same time, the therapy showed no effect on germ-free and antibiotic-treated mice (Vétizou et al. 2015). Similar results, depending on the presence of *Bifidobacterium,* were seen in the α-PD-L1 treatment of melanoma (Sivan et al. 2015). Across several cancers, it has been shown that when broad-spectrum antibiotics are administered during the first 6 weeks of ICB therapy, there is significantly reduced efficacy of treatment, a time frame that is important since the first 6 weeks of ICB treatment can largely impact the generation of initial T cell responses caused by ICB (Khan et al. 2021).

Strikingly, more recent investigations have shown fecal transplants from ICB responders to non-responders for the treatment of melanoma saw greater than one-third of human patients who previously were unresponsive to treatment became responsive after transplants, supporting a possible influence by the microbiome in ICB responsiveness and increasing curiosity to determine its role in the treatment across cancers (Routy et al. 2018b). Discord in the microbiome has been recorded even in inbred lab mice. Though the variation in microbiome composition is more significant among different strains than within the same strain, factors such as birthing conditions, cage-specific conditions, and the degree of stress experienced can cause a microbiome change composition (Laukens et al. 2016; Friswell et al. 2010; Moloney et al. 2014).

Several studies have aimed to determine why microbiome composition can impact ICB responses and various factors have been determined to contribute to changes in ICB efficacy posed by alterations in immune cell function, some of which have been discussed in **Chapter 4.** Metagenomic studies concluded that enrichment of anabolic pathways and differences in pro-inflammatory cytokines caused by some bacteria's presence results in disparities seen in tumor response and serve as one possible reason differences in patients' microbiomes are associated with variance in ICB efficacy and why the elimination of these microbial population can alter the ability for (Gopalakrishnan et al. 2018). As previously mentioned, bacteria contained in the microbiome of a host also have the capacity to impact the presence of metabolites that further affect immune cell function, including short-chain fatty acids (SCFAs), which are often products of microbe fermentation processes (DiPalma and Blattman 2023). Alterations in the presence of these microbes and subsequent changes

in metabolite presence can impact both innate and adaptive immune cell functions (Thaiss et al. 2016; McCoy, Burkhard, and Geuking 2019).

Primarily, influences on innate immune cell function posted by microbiome dysbiosis can progress to complex diseases, as the first line of defense against pathogenic infection is compromised. Additionally, since the innate immune system is responsible for informing the adaptive immune system of pathogen presence, disruptions in it can further alter how adaptive immune responses are constructed, dampening the overall adaptive immune response as well (Thaiss et al. 2016). In fact, cytokines released by innate immune cells, such as IFN, are needed to maintain central dendritic cells (cDCs) in their basal state. Alterations in microbiota can alter secreted IFN by plasmacytoid DCs, signals needed by cDCs to prime T cells in the formation of a proper immune response. This circumstance is likely why in a recent study, T cells in germ-free mice were not able to initiate responses to direct stimulation with CD40 antibodies and also demonstrated reduced inflammatory cytokine profiles in comparison to mice that contained a "model microbiota," typically found under specific pathogen-free conditions (Schaupp et al. 2020). Further, colonization of the same germ-free mice with the "model microbiota" restored T cells' ability to respond after α-CD40 treatment (Schaupp et al. 2020).

Additionally, recent studies have demonstrated that microbiome composition can impact memory T cell formation, which can be essential for sustained immunity against malignant calls after ICB treatment. Dysbiosis in the microbiome can change how memory T cells progress by causing reductions in the magnitude of memory T cell generation when in its dysbiotic state (Sallusto et al. 1999). In fact, mice housed in specific pathogen-free (SPF) conditions had fewer T memory cells than wild-type mice (Beura et al. 2016). This

reduction in T cell memory could be due to several factors, including the fact that dysbiosis results in alterations of innate immune cells that inform adaptive cells and also that adaptive immune cells may have issues in their ability to home to specific sites that require a response.

For instance, a recent study demonstrated that antibiotic-treated mice infected with gammaherpesvirus displayed fewer CD3+ lung infiltrating T cells than mice not treated with antibiotics but still infected with the same virus. Mice that were not treated with antibiotics also showed better outcomes than mice that were (Yaron et al. 2020). These results indicate that not only can microbiome dysbiosis result in reductions in the ability for immune cells to respond to a particular stimulus but they also impact the ability for immune cells to home to a site of infection even after a response is generated.

These studies and those previously mentioned indicate a reliance on the microbiome for ICB efficacy, likely due to alterations in immune cell function and a potential effect on immune cells that can impact T-cell responses, memory, and homing.

Characterizing microbiome composition in responders and non-responders to mOS has not been previously done but could provide novel insights that could be utilized to improve ICB treatment. We sought to investigate how microbiome composition impacted α-CTLA-4/α-PD-L1 treatment efficacy and T-cell control of mOS. Findings from this work can shift current research paradigms and make a societal impact because they model a new way to determine why ICB therapy will or will not be effective on a subject rather than merely determining if it will be effective. Such findings could be widely applied to sarcoma immunotherapy regimens through the same methods.

Methods

Antibiotic Preparations and Administration

The antibiotic cocktail used in this study was composed of ampicillin, vancomycin, neomycin, and metronidazole (AVNM). Initially, 100g of ampicillin purchased from Alfa Aesar (Ward Hill, MA), 0.35g of vancomycin hydrochloride purchased from G Biosciences (Saint Louis, MO), and 100g of Neomycin purchased from BioBasic (Markham ON, Canada) were mixed in 1 L of reverse osmosis water, yielding AVN. The reverse osmosis water used in this study is typically used for mice in the ASU vivarium to drink. Next, 0.1g of Metronidazole, purchased from Alfa Aesar (Ward Hill, MA), was mixed with 1L of reverse osmosis water. Next, 100 mL of 0.1g/L Metronidazole was mixed with 900 mL of AVN, and 25g of grape Koolaid was added to the final mixture, yielding AVNM. Approximately 250 mLs of AVNM was placed in the water bottles of mice who were planned to be on antibiotic treatment. AVNM was changed every three days to ensure mold did not grow in the water. Mice were initially weighed every day for two weeks after the start of antibiotic treatment. They would be euthanized if any mouse dropped below 15% of their starting weight, taken immediately before the first antibiotic treatment. Mice in this trial that were given antibiotic treatments were maintained on AVNM for the entire trial to ensure continuous microbiome depletion.

In vivo Antibodies and Cell lines

The α-CTLA-4 monoclonal antibody was purified via saturated ammonium sulfate (SAS) precipitation from the UC10-4F10-11 hybridoma (ATCC, Manassas, VA). Hybridomas were maintained in Roswell Park Memorial Institute (RPMI) media and were initially cultured in 20% fetal bovine serum (FBS) but were weaned to lower levels of FBS media until 2.5% FBS was reached. After cells grew to confluency in 2.5% FBS RPMI, cells were allowed to die for one week, and the supernatant was harvested in preparation for SAS precipitation. The α-PD-L1 monoclonal antibody (clone 10F.9G2) for *all in vivo* blockade experiments was purchased from BioXCell (West Lebanon, NH). K7M2- Luc cells were a gift from Helman lab and were cultured in 10% fetal bovine serum (FBS), 1% of 100x penicillin, Streptavidin, and glutamine (PSG), in Dulbecco's Modified Eagle's medium (DMEM).

Mice and generation of tumors

Balb/c/J mice were purchased from Jackson Laboratories (Bar Harbor, ME) and were housed in the Arizona State University Biodesign Institute under specific pathogen-free conditions. All experiments listed here were approved by the Institutional Animal Care and Use Committee (IACUC) and were conducted under the appropriate supervision. Metastatic osteosarcoma tumors were established by injection of $1.5 * 10^6$ either K7M2 cells via lateral tail vein in 100 µL of phosphate-buffered saline (PBS). After inoculation with K7M2-Luc cells, mice were monitored by weight and a clinical scoring system that evaluated breathing, ambulation, lethargy, and scruffiness (BALS).

Treatment Schedule

For mice who were treated with ICB composed of α-PD-L1 and anit-CTLA-4. Mice were given five doses of α-PD-L1 monoclonal antibody (clone 10F.9G2) purchased from BioXCell (West Lebanon, NH), which occurred every three days beginning one day after tumor inoculation. Mice were also given three doses of α-CTLA-4 monoclonal antibody beginning one day after tumor inoculation.

Fecal Sample Collection and Processing

Fecal samples were collected from mice during multiple time points in the study, including before antibiotic administration (to determine pre-existing microbiome compositions), during antibiotic administration (to determine the depletion of microbiome compositions resulting from antibiotic administration), before tumor inoculation (to characterize microbiome composition at the time of mOS inoculation, and during lung harvest for T cell experiments (to verify that microbiome compositions of mice were depleted at the time T cell function was characterized. For each fecal sample, 2-5 pellets were collected from mice and immediately stored at -80°C. Samples were processed using ASU CORE facilities with Microbiome 16S rRNA gene amplicon sequencing, and analysis was completed in collaboration with Dr. Rosa Krajmalnik-Brown and Dr. Qiwen Cheng, who characterized pre-existing microbiomes of the mice in this study according to their alpha and beta diversity.

Spleen Harvest

Spleens were harvested prior to perfusion, were weighed and the percentage of body weight represented by the spleen was calculated. Splenocytes were processed using a 0.2µm cell strainer (Falcon) placed over a 50mL conical tube and the back of a 3mL syringe. Splenocytes were centrifuged at 1200 rpm for 5 minutes. The supernatant was removed, and red blood cells were lysed using ACK lysis buffer (Sigma) for 2 minutes at room temperature and quenched with 8mLs of RPMI. Cells were centrifuged and counted so that 10^6 cells per well were plated for flow staining.

Results

Mice Do Not Show Distinct Microbiome Differences on the Genus Level Between Responder and Non-Responder Groups Before Inoculation with K7M2 Cells or ICB.

Fecal samples were taken from all ten mice used in the initial trail referenced in **Figure 20** before they were inoculated with mOS and before they were given ICB treatment. Samples were sent for sequencing after determining whether mice responded to ICB or non-responders to ICB. 16S Ribosomal Unit sequencing analysis of the V3 region showed that mice did not have distinct differences in microbiome composition between responder and non-responder groups before inoculation with K7M2 or ICB on the genus level. *Muribaculaceae* is a family of bacteria within the order *Bacteroidales*, a gram-negative family of bacteria, and was moderately abundant among both responder and non-responder groups, without a preference for one group over the other. *Lachnospiraceae* is a family of obligate anaerobic bacteria known to generate SCFAs butyrate and acetate as products of polysaccharide fermentation(J. Zhang et al. 2019). *Lachnospiraceae* were moderately abundant in both responders and non-responders. Finally, *Lactobacillus* was relatively plentiful in both responders and non-responders. Overall, there was no statistical difference between the pre-existing microbiomes of mice in this study on the genus level **(Fig. 25).**

Alpha and Beta Diversity of Fecal Samples.

Fecal samples taken from responders and non-responders prior to inoculation with K7M2 or ICB did not show distinct differences in microbiome composition when analysis of Alpha and Beta diversity contained in samples was completed (Credit: Qiwen Cheng).

Microbiome composition differences were more apparent between mice cages than between responder vs non-responder sets **(Fig. 26).**

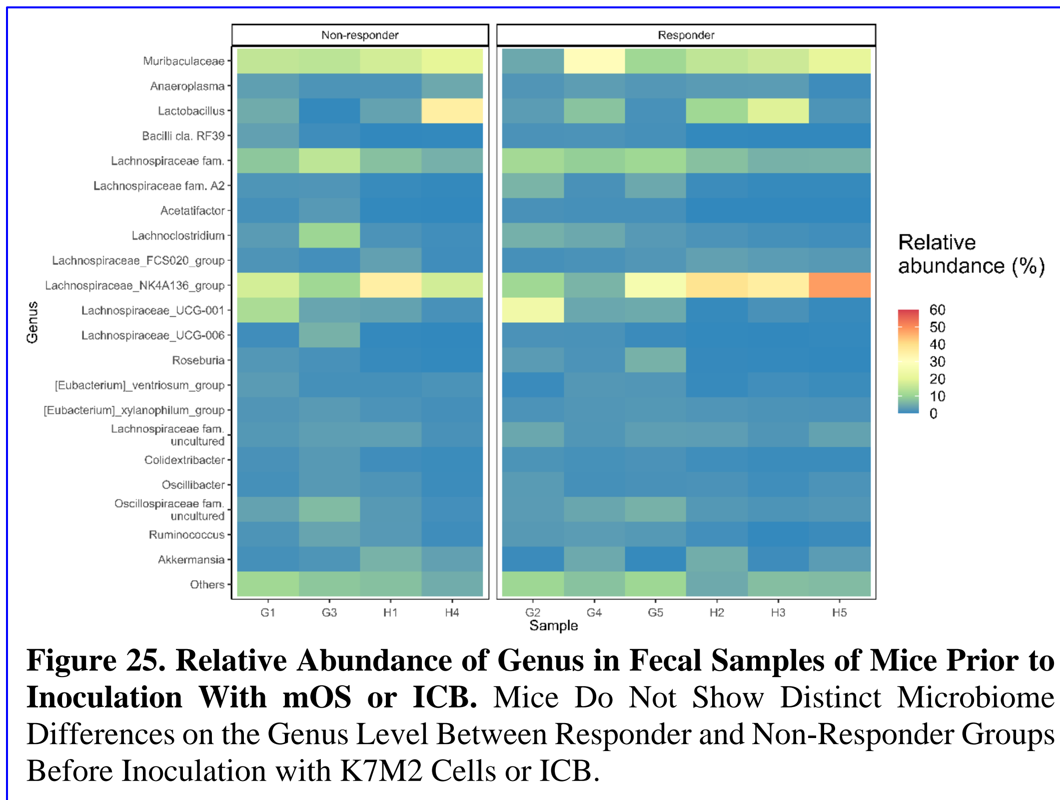


Figure 25. Relative Abundance of Genus in Fecal Samples of Mice Prior to Inoculation With mOS or ICB. Mice Do Not Show Distinct Microbiome Differences on the Genus Level Between Responder and Non-Responder Groups Before Inoculation with K7M2 Cells or ICB.

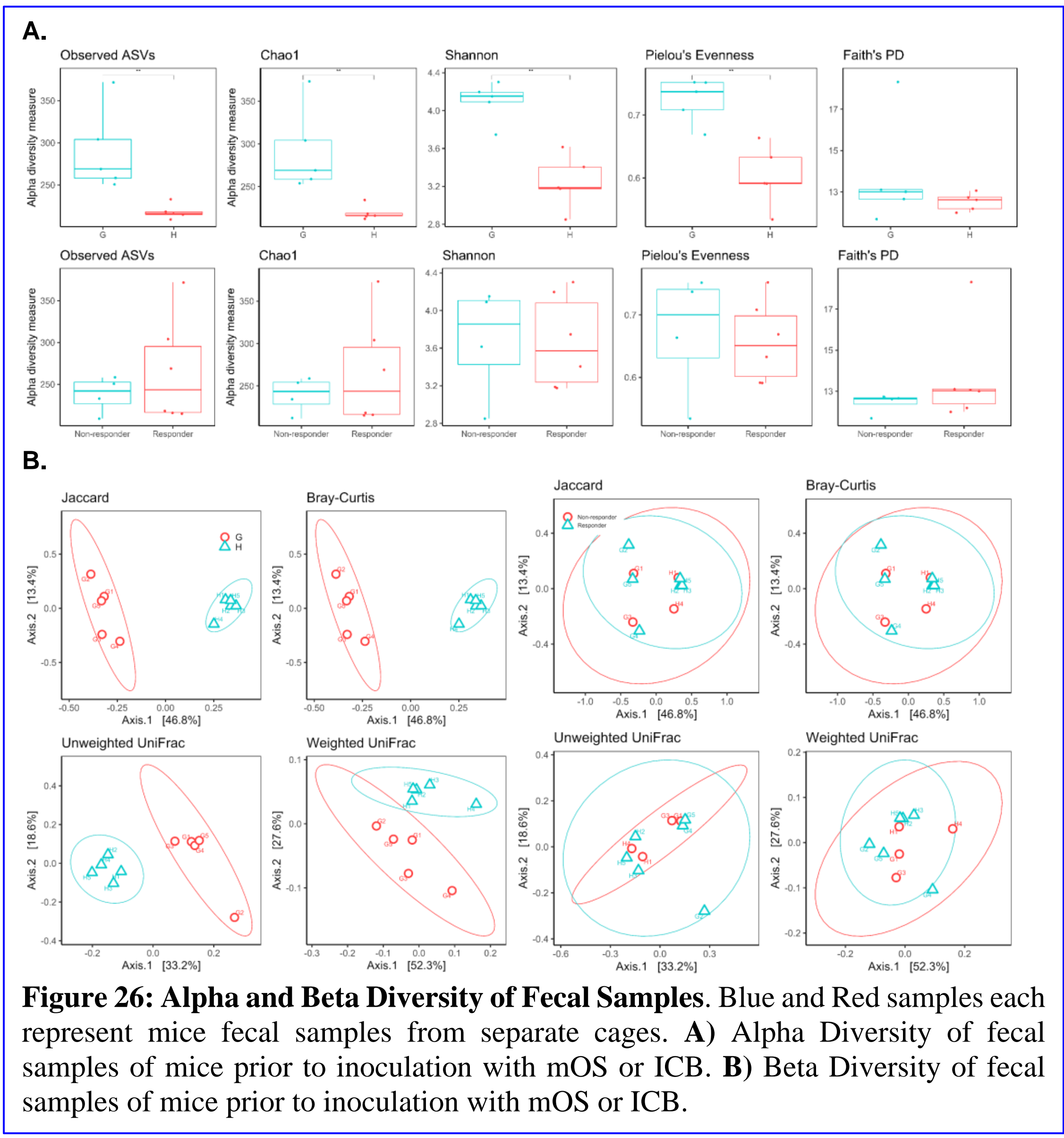
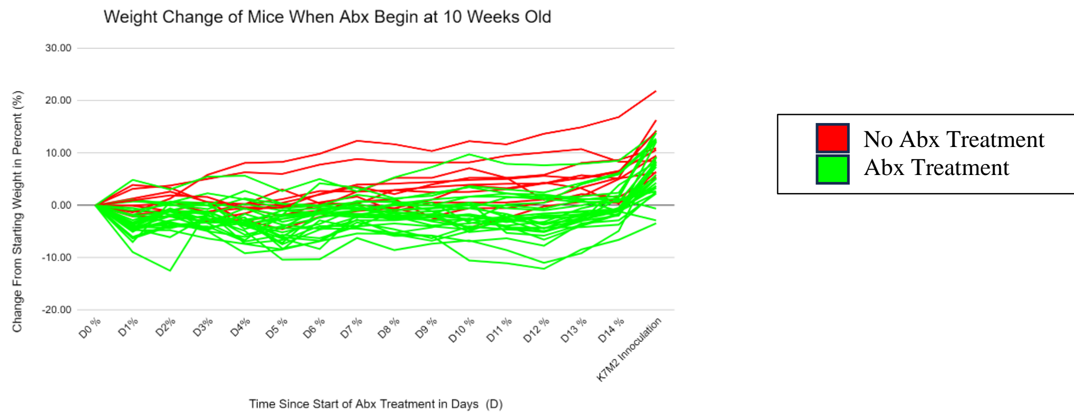


Figure 26: Alpha and Beta Diversity of Fecal Samples. Blue and Red samples each represent mice fecal samples from separate cages. **A)** Alpha Diversity of fecal samples of mice prior to inoculation with mOS or ICB. **B)** Beta Diversity of fecal samples of mice prior to inoculation with mOS or ICB.

The Impact of Antibiotic Treatment on Weight Varies Based on the Age of Mice

Mice that were treated with the AVNM antibiotic cocktail at an older age tended to lose weight more quickly within the first week of antibiotic treatment compared to mice that were treated with antibiotics beginning at weeks 4 and 10, but they also were able to recover more quickly than younger mice **(Fig. 27).** Additionally, Mice that were treated at 10 weeks of age, at which point they are considered fully adult (Catherine Hagan 2017), showed little variance in weight from their starting weight compared to other age groups tested, with the majority of the mice fluctuating no more than 5% from their initial starting weight within the first two weeks of treatment and by the time they were inoculated with K7M2.

A.



B.

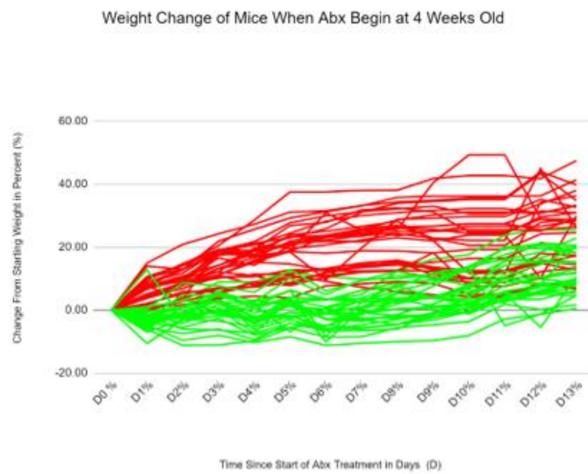


Figure 27: Comparison of Weight Change Within Mice Groups at Different Ages. Graphs are represented as Days post initial antibiotic (Abx) treatment vs. weight change: **A.)** Weight change of mice who were began on Abx at 10 weeks old **B.)** Weight change of mice were began on Abx at 4 weeks old.

Mice Inoculated with Mycoplasma Positive K7M2 and then Treated with Abx showed Better Survival Rates Than Mice Not Treated with Abx.

Both groups of mice pre-treated with AVNM Abx were given Abx for at least two weeks before mOS inoculation beginning at ten weeks of age. This included control mice who were treated with only PBS after inoculation with tumor and mice who were treated with α-PD-L1 and α-CTLA-4 after tumor inoculation. Both of these groups showed improved rates of survival compared to mice that were not treated with AVNM but were given the same treatment post mOS inoculation (**Fig. 28**). It is important to note that the K7M2 stocks these mice were given were determined to be contaminated with mycoplasma after testing.

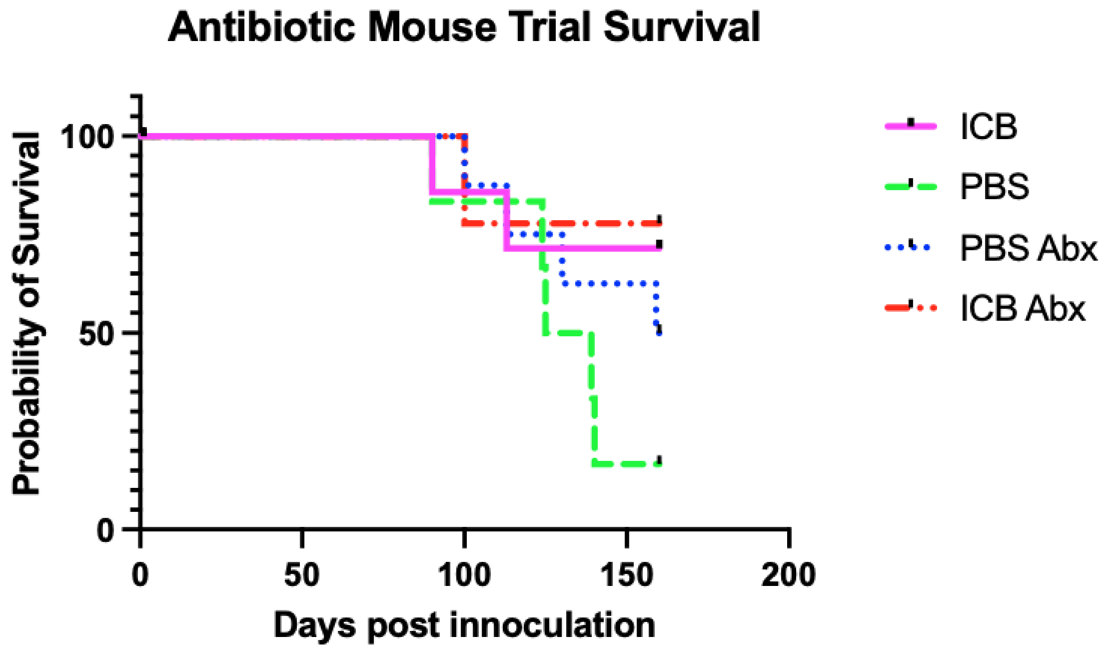
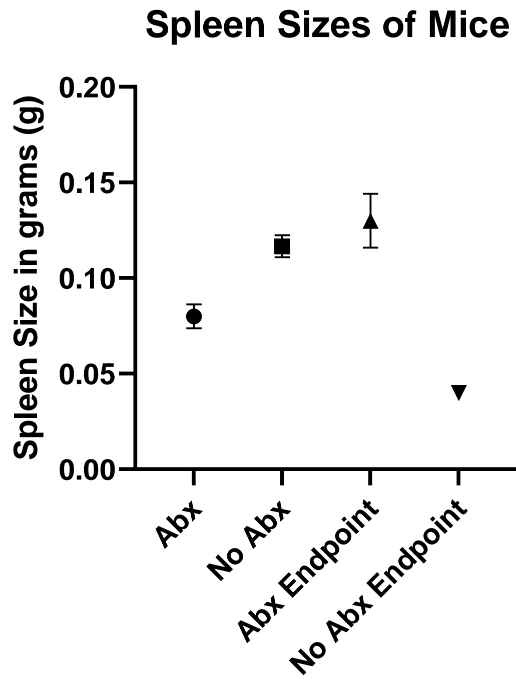


Figure 28: Survival Curve of Mice Inoculated With K7M2 With Without Abx: Mice appeared to have better survival outcomes when they were in the antibiotic-treated group vs. the non-antibiotic-treated group.

Splenomegaly was Present in Mice Treated with Abx Who Were at Endpoint Criteria Due to K7M2 Metastasis

Interestingly, mice that were at endpoint criteria after developing mOS metastasis and were also on Abx had variations in the size of their spleens based on the groups that they were in. Specifically, mice that were at endpoint criteria who were on the antibiotic cocktail for the entirety of the trial exhibited splenomegaly and had an average spleen size of 0.13g making up 0.787 % of their body weight (n= 2) **(Fig. 29).** When compared to mice that were not at endpoint criteria, these differences in spleen size are quite significant; mice that were not at endpoint criteria but were treated in Abx had a spleen size of 0.08g making up an average 0.333% of their body weight (n=6) **(Fig. 29).** A mouse that as not given Abx who were at endpoint titer showed had a spleen size of 0.04g making up an average 0.247% of their body weight (n=1) **(Fig. 29).** While mice that were not given Abx who were not at endpoint titer showed an average spleen size of 0.12g making up an average of 0.399% of their body weight (n=3) **(Fig. 29)**

A.



B.

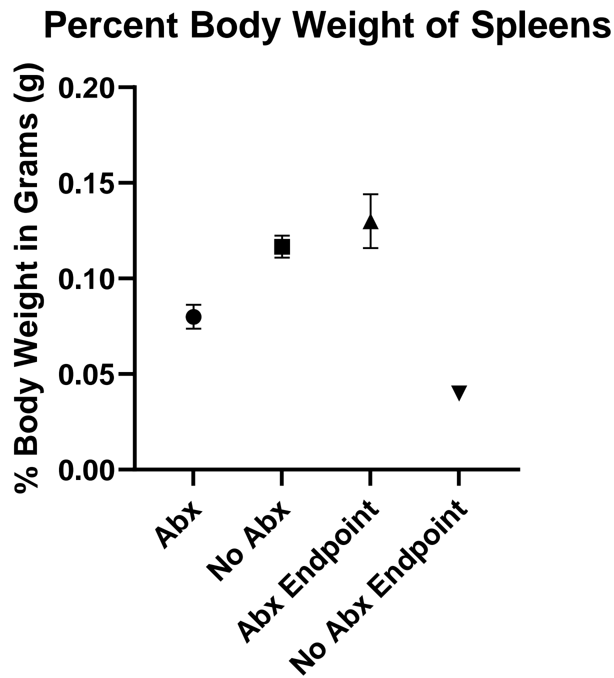


Figure 29: Sizes of Spleen Harvested From Mice A.) Spleen Sizes From Mice Treated with Abx vs. Without Abx **B.)** Percentage Body Weight of spleen in mice treated with Abx vs. Without Abx

Discussion

Though many studies support that the microbiome and variations in its composition profoundly impact treatment for some malignancies, investigations that characterize microbiome composition for most cancers have not been pursued (Mager et al. 2020; Vivarelli et al. 2019; Routy et al. 2018a; Almonte et al. 2021). For melanoma, studies have shown that ICB composed of either α-PD-1 or α-CTLA-4 can even rely on the presence of particular microbes to make any impact at all, with subjects showing little to no response when certain microbes are not present (Vétizou et al. 2015; Topalian et al. 2012). Further studies showed that alterations in microbiome composition resulted in subsequent changes in the presence of metabolites thought to be secreted by these microbes, causing inhibition of ICB and chemotherapeutic drug efficacy, which can be primarily caused by antibiotic administration (J. H. Yang et al. 2017). This result is a significant finding because specific metabolites can impact cellular immune function via changes in inflammatory processes and subsequent immune cell functioning altering treatment regimen efficacy of treatments that rely on cellular immune function (C. H. Kim and Betz 2018).

Under less controlled conditions, attenuation of ICB efficacy for combination PD-1/PD-L1 has been seen in human patients to treat various cancers when microbiome depletion has occurred. For instance, antibiotics were associated with attenuated efficacy of α-PD-1/ α-PD-L1 therapies in Chinese patients with advanced non-small-cell lung cancer (S. Zhao et al. 2019). Meta-analysis of 19 studies that involved antibiotic treatment during cancer treatments and possible effects on mainly PD-1 or CTLA-4 inhibitor therapies across human patients found that antibiotic usage significantly reduced the progression-free survival in patients treated with ICB among groups compared to those not

treated with antibiotics (Huang et al. 2019). These associations of ICB efficacy against melanoma and microbiome composition have inspired interest in understanding how microbiome manipulation and composition can influence cancer immunotherapy effectiveness for other cancers as well and inspired the investigations outlined here.

Characterizing microbiome composition and its impacts on responders and non-responders to mOS has not been previously done. Here, we investigated potential associations between microbiome composition and its effects on α-CTLA-4/α-PD-L1 treatment efficacy to control of mOS.

First, we took fecal samples from mice before they were inoculated with K7M2 cells or ICB and analyzed the composition of these samples on the genus level. We found that *Muribaculaceae*, a gram-negative member of *Bacteroidales,* was present in mouse fecal samples before inoculation with K7M2 and ICB at equal levels among responders and non-responders. *Bacteroidales* species have been cited for playing a negative role in ICB efficacy on several occasions due to decreased CTL response to cancer and diminished antigen presentation capacity (E. Kim, Ahn, and Park 2021). Studies have shown that an overabundance of *Bacteroidales* has been associated with shorter-term progression-free survival in ICB-treated patients (Rezasoltani et al. 2021). In a melanoma-bearing mouse model, over-abundance was associated with non-responsiveness to α-PD-L1 and lower CD8+ tumor-infiltrating lymphocytes (TILs) (Gong et al. 2019). Since *Bacteroidales* was present in both responders and non-responders before inoculation with tumor or ICB administration, it is unlikely that it was associated with non-responder outcomes **(Fig. 25)**. However, it is possible that selective antibiotic elimination of certain bacteria can alter ICB outcomes by allowing for the overgrowth of *Bacteroidales* species or other species that are

associated with negative ICB responsiveness. *Lachnospiraceae* was also present in our samples **(Fig. 25)** and is a family of obligate anaerobic bacteria. They can ferment diverse plant polysaccharides into SCFAs, such as butyrate and acetate (J. Zhang et al. 2019), which have been shown to impact ICB responses and T cell function within the TME (DiPalma and Blattman 2023). This family of bacteria was also present in mouse fecal samples before inoculation with ICB or K7M2, about equally between responders and non-responders. *Lactobacillus,* a gram-positive bacteria mainly present in several mammals' gut, was also detected in both responders and non-responders and has been associated with positive outcomes regarding ICB treatment **(Fig. 25)** (Bender et al. 2023).

Overall, there was no statistical difference between the pre-existing microbiomes of mice in this study on the genus level. The fact that *Bacteroidales, Lachnospiraceae, and Lactobacillus* **(Fig. 25)** were all moderately present in both NR and R fecal samples indicates that it was unlikely they were the cause of responsiveness or non-responsiveness to ICB. Further, differences in the composition of the microbiome were more significant between cages of mice rather than responder vs. non-responder profiles **(Fig. 26).** It is important to note that the sequencing of samples analyzed in these data was completed via 16S ribosomal unit sequencing and could only be clarified at the genus level. Future studies that wish to explore species-level variances among mice in our groups could utilize whole-genome sequencing, which has been shown to provide better resolution of species-level identification than 16S ribosomal unit sequencing (Ranjan et al. 2016). Better resolution of these samples on the species level could reveal differences that are not present in our current data. We further wished to determine if ICB efficacy could be altered by modulation of the microbiome rather than simply whether or not it was associated with

pre-existing microbiome compositions. For this reason, we attempted to alter the microbiome of mice in our study via the administration of a broad-spectrum antibiotic cocktail composed of ampicillin, vancomycin, neomycin, and metronidazole (AVNM), which has previously been shown to vastly reduce microbiome composition while ensuring that mice do not die from the intense elimination of essential microbial populations. This cocktail has previously been associated with reduced efficacy of immunotherapy (Ubeda and Pamer 2012).

We first found that the impact of antibiotic treatment composed of AVNM on mouse weight varies based on the age of mice at the start of antibiotic administration. For mice who were administered AVNM at an older age (~18 weeks), we noted that weight loss tended to occur quickly and to a greater extent when compared to mice that were treated with antibiotics at a younger age (~4-10 weeks). However, we also observed that older mice also acclimated more quickly to AVNM administration than younger mice and not only were observed to drink the water quickly but also were able to gain lost weight back within only a few days, with all mice recovering any lost weight by day 10 of AVNM administration. **(Fig. 27).** Mice that were treated at 10 weeks of age, at which point they are considered fully adult (Catherine Hagan 2017), showed little variance in weight from their starting weight compared to other age groups tested. All groups of mice, whether AVNM administration began at 4, 10, or 17 weeks, showed no more than -5% variance from their initial starting weight after two weeks of AVNM treatment and by the time they were inoculated with K7M2 cells. Thus, ensuring that a two-week period passes before beginning an experimental procedure that depletes the microbiome in Balb/c mice using AVNM is critical to ensure that outcomes caused by decreases in weight are minimized.

Though these findings do not establish an optimal age for administering AVNM treatment to Balb/c mice, they provide valuable insights into the influence of age on AVNM antibiotic tolerance in Balb/c mice. This contextual understanding allows researchers to optimize their experimental design while minimizing potential sources of error.

Though AVNM and other broad-spectrum antibiotic treatment regimens have been associated with reduced treatment efficacy, our initial mouse trial results demonstrated contradicting outcomes. The results of the mouse trial depicted in **Figure 28** may demonstrate a phenomenon similar to that of what was observed when Coley's toxin was administered directly into solid tumors (McCarthy 2006; Loughlin 2020). Testing of the K7M2 stocks used in this trial revealed that they were positive for mycoplasma. Mycoplasma lacks rigid cell walls, making them resistant to antibiotics that target bacterial cell walls, such as penicillin and streptomycin, which are widely used in cell culture (Young, Sung, and Masters 2010). However, the antibiotics used in this study, ampicillin, vancomycin, neomycin, and metronidazole, all function through various distinct mechanisms. Ampicillin is a beta-lactam antibiotic that primarily elicits bacteriocidal effects by inhibiting bacterial cell wall synthesis, resulting in cell death (Oates et al. 2010). Vancomycin is considered a glycopeptide antibiotic; it acts by binding to terminal D-alanyl-D-alanine (D-Ala-D-Ala) residues of the peptidoglycan precursors, thus, preventing the formation of the peptidoglycan chain and ultimately inhibiting bacterial cell wall synthesis (Hammes and Neuhaus 1974). Neomycin is an antibiotic that belongs to the aminoglycosides family. Neomycin inhibits bacterial reproduction by binding to the 30S subunit in the bacterial ribosome, disrupting the reading of mRNA, resulting in incomplete or non-functional proteins within the bacteria, impairing essential bacterial functions, and

causing the eventual death of the bacteria (Arya 2005). Finally, metronidazole interacts with ferredoxins, producing nitro radicals; these can cause DNA damage and inhibit protein synthesis within a bacterial cell.

Both neomycin and metronidazole have the potential to directly eliminate mycoplasma within a cell since neither of these antibiotics acts directly on cell wall components. Direct killing of mycoplasma may have resulted in the release of PRRs which could be primarily detected by innate immune elements, recruiting other immune cells to the site of cancer cells that contain them, thus, instigating an immune response and a higher rate of cancer cell clearance in mice treated with AVNM. Typical studies utilizing AVNM have demonstrated a reduced overall immune response and overall ICB treatment efficacy (Ubeda and Pamer 2012). This impact on ICB treatment is thought to be attributed to the antibiotic-specific elimination of certain species of bacteria needed to maintain effectiveness.

When analyzing organs from mice used in these studies, it was interesting to see that mice that were chronically treated with AVNM who also met endpoint criteria and were found to have pulmonary metastasis of K7M2, exhibited splenomegaly when compared to mice that were not treated with AVNM who met endpoint criteria **(Fig. 29).** Mice not treated with AVNM who met endpoint criteria and had pulmonary metastasis exhibited a large reduction in spleen size. This is significant because mice that did not have pulmonary metastasis but were chronically placed on AVNM antibiotics did not exhibit splenomegaly and also had spleen sizes similar to mice that were not treated with AVNM antibiotics, which also did not develop pulmonary metastasis **(Fig. 29).** Though reasons for the splenomegaly exhibited in mice treated with AVNM and who developed pulmonary

metastasis were not able to be investigated in this study, future investigations can seek to determine the reasons for this pathology. Additionally, more mice should be considered in groups that analyze mice who met endpoint criteria but who were not treated with Abx.

It may be possible that when a need for an immune response was present in the lung tissue, such as K7M2 metastasis, there was also a need for cells to respond to K7M2 cancer epitopes, resulting in a large increase in immune cell proliferation. However, decreases in overall innate and adaptive immune responses could have led to a subsequent decrease in the ability of immune cells to home to the metastasis present in the lungs. Previous studies have demonstrated that mice treated with broad-spectrum antibiotics during a viral infection displayed lower levels of CD3+ cells in the lungs compared to mice not treated with antibiotics (Yaron et al. 2020). The splenomegaly in this study may have been associated with a decrease in the ability for immune cells proliferating in the mice treated with AVNM to home to the site of metastasis. Future experiments could investigate the expression of mucosal homing receptors such as CCR9 and $\alpha_4\beta_7$ integrin, which are associated with mucosal homing (Manhas et al. 2022; Holechek et al. 2016), to determine if mice on AVNM are less likely to express these markers on their immune cells, potentially explaining why AVNM-treated mice exhibit splenomegaly, indicating they are likely making an immune response, though they also may be the first to succumb to metastasis.

Though the conclusions drawn from this study provide substantial implications for the potential impacts of microbiome composition on T cell function within the tumor microenvironment (TME) and further efficacy of cancer treatments that rely on the function of T cells, such as the ICB therapy referenced here, it is essential to note that limitations of this study must be considered when findings from this work are applied in a

clinical context. Our colleagues previously found that the chosen antibiotic mixture could deplete microbiome composition as much as possible without harming the strain of Balb/c mice used in the study. For this reason, we selected this antibiotic mixture but one should also note that our cancer model was completed in a specific-pathogen-free environment but has not been attempted in a germ-free mouse model. Further, this experiment was completed in only Balb/c mice; other mouse strains might have differences in baseline microbiome compositions than those seen in the Balb/c mice used in this study which could impact how ICB functions. This should be considered if findings from this research are ever applied in clinical settings. Additionally, Balb/c mice are genetically similar, though this allows for a controlled experiment; as described here, humans are significantly more genetically diverse and could have different responses to treatments due to factors that may not be as easily changed as microbiome composition.

Several future experiments can be drawn from these studies. For instance, *in vitro* analyses that seek to determine the impact of specific microbial-produced metabolites on mOS and immune cells could utilize the coincubation of K7M2 cells and immune cells taken from a mouse with an active K7M2 metastasis in the presence of specific SCFA metabolites that are typically produced by bacteria either in the gut or in the TME. Impact on surface marker expression of both cancerous cells as well as T immune cells could be observed. The information gained from these studies would allow us to understand better if specific metabolites can alter K7M2 cancer cells or T immune cell function. Additionally, studies could utilize short-chain fatty acid analysis (SCFAA) to determine what metabolites bacteria produce in non-Abx treated mice. These metabolites could be

administered to mice within their diets, potentially reversing microbiome depletion's impact on T immune cells.

Potential impacts of microbiome depletion can be completed on mice who only receive α-PD-L1 or α-CTLA-4 treatment alone to see if there is any impact on either antibody treatment alone, though this study was not the aim of the experiments described here, which was to determine if depletion of the microbiome had an impact on combination ICB. The microbiome represents a malleable factor in cancer therapy, as it can be altered through multiple means. Thus, the work described here is essential because if microbiome composition does impact T cell function, especially within the TME and further regarding therapies that rely on T cell function, understanding how it affects T cells and the immunotherapies that depend on them may allow us to alter microbiome composition for subjects that have decreased immune cell function due to antibiotic use or microbial dysbiosis.

CHAPTER 6

METHODS FOR VALIDATION OF A T CELL EPITOPE PREDICTION ALGORITHM ENSEMBLEMHC IN VIVO

Abstract

Bioinformatics-based approaches that calculate the ability of specific epitopes to engage T immune cells have made recent strides in the design of therapeutics against cancers. Epitope prediction models (EPMs) are software systems that analyze cellular data and determine which epitopes are essential for us to look at when designing treatments and therapeutics. One way EPMs can do this is by identifying neoepitopes in specific cancers. Our collaborating lab has recently shown that their EPM, "EnsembleMHC," has been able to predict epitopes and neoepitopes in diseases and cancers with higher accuracy when compared to other commonly used EPMs. One potential setback is that results associated with the efficacy of EnsembleMHC have only been completed outside of a living system. The calculated accuracy of its predictions is based on simulated environments and a comparison of predictions to real-world clinical trends. Though this shows promise as a means of determining what proteins might be necessary to target for therapies against certain diseases and cancer; it excludes variables present in a living system that computational biology cannot predict. To that end, we propose a study addressing the problem that past predicted accuracy of EPMs like EnsembleMHC was not based on results from a living system but instead on simulated predictions. Testing the effectiveness of EnsembleMHC to predict cancer neoepitopes that are important for therapeutics to target will allow us to gain further insight into its reliability and will first need to be completed in an established mouse model, such as the established K7M2 mouse model. We first

propose determining neoepitope proteins specific to K7M2 cell lines by using RNAseq and EnsembleMHC. This data gathered from RNAseq will allow us to carry out experiments in live mice that will test whether EnsembleMHC correctly predicted neoepitopes present in the K7M2 cells. We hypothesize EnsembleMHC will accurately predict neo-epitopes in K7M2 cells and that after mice are exposed to them, they will clear K7M2 cells. We believe these investigations on the accuracy of EPMs like EnsembleMHC must be completed if we are to advance bioinformatics applications to the creation of therapeutics for diseases like cancer.

Introduction

Prediction of epitopes that engage immune cells like T cells is important for the design of therapeutics against cancers. They allow researchers to understand better what protein therapies should be targeting based on their likelihood of recruiting immune cells to attack specific cells (Soria-Guerra et al. 2015). These predictions are currently used to determine the effectiveness of treatments like checkpoint blockade and have the potential to one day aid in developing vaccines against certain cancers (Sarkizova et al. 2019; Narang et al. 2019). Research in recent decades has made considerable strides in the ability of Epitope Prediction Models (EMPs) to accurately predict epitopes of interest for the design of cancer therapeutics. In addition to a better understanding of the human genome, the expansion of the size of computed protein datasets has made it possible for researchers to use EPMs to analyze large sets of data more accurately across the human population (Soria-Guerra et al. 2015; Tanjo et al. 2020). Our collaborators have recently developed an EPM that combines several other EPMs to improve epitope prediction accuracy (Tanjo et al.

2020; Wilson et al. 2021a). They have termed this EPM, EnsembleMHC, and have found that it can more accurately predict epitopes that are important to the immune system than all other EPMs commonly used (Wilson et al. 2021b). An issue with EPMs is that they are often meant to complement laboratory findings rather than serve to replace them (Brusic, Bajic, and Petrovsky 2004). A significant reason for this is that computational models may not predict factors present in a living system that could affect how important an epitope is to the immune response (Brusic, Bajic, and Petrovsky 2004; Paul et al. 2020). Such a circumstance could lead researchers to form incorrect conclusions on what proteins should be targeted for a particular therapy and may not be able to apply their findings to translational medicine applications.

Though EPMs show much promise in predicting epitopes that are important to focus on for cancer therapeutics, many EPMs, like EnsembleMHC, have never been tested in a living system. This prospect can be an issue, as we don't know how accurately EPMs can predict neoepitopes of importance until they are apparent in a live system. For this reason, we describe here methods to test EndembleMHC in a living system by comparing the protein makeup of two mouse bone cell lines. One cell line is a cancerous metastatic osteosarcoma (mOS) K7M2 cell line, and the other is a non-cancerous cell line made of primary osteoblasts. We hope to determine neoepitope proteins specific to K7M2 cell lines by using RNAseq and EnsembleMHC then compare the results of the mouse trial with the initial EnsembleMHC neoepitope predictions to determine the accuracy of EmsembleMHC in its prediciton of neoepitopes that are important for the immune system to target for osteosarcoma cancer in a mouse model. This study will directly compare healthy osteoblast cells to cancerous osteoblasts cells that are both of mouse origin. This will address the

problem that much past research using EPMs has not used direct comparisons of living cells to determine significant epitopes. This proposed research could also address the problem that past predicted accuracy of EPMs like EnsembleMHC was not based on results from a living system but instead on simulated predictions. Testing the effectiveness of EnsembleMHC to predict cancer neoepitopes that are important for therapeutics to target will allow us to gain further insight into EPMs reliability and possibly pave the way for the future design of cancer therapeutics.

We hypothesize that EnsembleMHC can predict the neoepitopes present in K7M2 cells accurately and that when these neoepitopes are given in the form of vaccination to mice and later inoculated with K7M2 cells, the mice will be able to clear the K7M2 cells, indicating that EnsembleMHC was able to predict neoepitopes of importance with high accuracy. Support for this hypothesis is drawn from recent studies that looked at the likelihood of certain people developing severe SARS-CoV-2 if exposed and infected using EnsembleMHC and compared those findings to real-world outcomes of patients with the same genetic factors (Wilson et al. 2020). The study found that they could predict with high accuracy what proteins were important for the immune system to recognize to effectively clear SARS-CoV-2 and that people with a predisposition not to recognize those identified proteins had a worse outcome when infected with SARS-CoV-2 (Wilson et al. 2020). Such a finding supports that the EnsembleMHC software could correctly predict which proteins were important for the immune system to recognize. Additionally, this study found that EnsembleMHC was more accurate than seven of the commonly used EPMs based on each EPM to predict epitopes that we already know are important for the immune system to recognize (Wilson et al. 2020). Thus, the research described here would

allow for the validation of the EnsembleMHC prediction algorithm and would pave the way for the design of potentially better design of immunotherapies that rely on T cells.

Proposed Methods

Murine Neonatal Calvaria and Long Bone Harvest

First, calvaria should be harvested from neonatal mice under 48 hours old by placing them on ice and under a guillotine. The surface of each mouse should be sprayed with 70% ETOH. Next, the skin of the neck should be peeled back, and the calvaria should be removed from mice and placed on ice. Further, the long bones of mice should be harvested and disassociated from mice, then skin and other tissue should be peeled away from murine femurs and long bones and placed on ice.

Disassociation and Culturing of Primary Osteoblasts

Murine calvaria should be cut into pieces ~1-3mm^3 and incubated in 0.25% trypsin containing 0.02% EDTA for 25 minutes at 37 °C to digest bone and fibrous tissues. Bone chips should be digested in DMEM containing 0.1% Collagenase II (Gibco) and 0.05% trypsin for 1 hour at 37 °C with a metal stir bar at 200. r.p.m. Released cells should be collected and centrifuged at 8 min at 1000 r.p.m. Cells should then be resuspended in 5mLs of DMEM/ 10% FBS and transferred to a 25 cm^2 plastic culture flask. Cells should be incubated for 20 mins before nonadherent cells are transferred to another flask. This step should be repeated twice to remove most fibroblasts, and the culture media was changed every 2-3 days. Cells should be verified mycoplasma free before tumor inoculation to ensure that these do not show up as neoepitopes upon the comparison of the cell lines.

Culturing of K7M2 and K7M2-Luc- Cells

K7M2 cells are needed for comparison to primary osteoblasts and should be grown in 10% fetal bovine serum (FBS), 1% of 100x penicillin, Streptavidin, and glutamine (PSG), in Dulbecco's Modified Eagle's medium (DMEM). Cells should be verified mycoplasma free before tumor inoculation to ensure that these do not show up as neoepitopes upon the comparison of the cell lines. K7M2-Luc cells will be cultured in the same way as K7M2 cells but will instead be used to inoculate mice.

RNA extraction

RNA extraction should be completed next on cells after they reach confluency at passages 3-4 using a Qiagen RNA extraction kit, following the manufacturer's instructions for both primary cells and K7M2 cells. It should be considered that mRNA expression likely varies among mice. For this reason, mRNA extraction will be completed in triplicate from three different mice for non-cancerous samples and three separate K7M2 samples.

Testing of RNA Quality and RNAseq Analysis.

RNA quality can be tested using a TapeStation and obtaining an RNA Integrity Number (RIN), assessing the quality of genomics samples. After RNA is tested and determined to be of usable quality, RNA should be run through RNAseq in triplicate for both K7M2 cells and primary cells. RNAseq data further will be compared between primary osteoblasts and K7M2 cells. The differences noted between cell lines will then be run through EnsembleMHC to determine which of the supposed neoepitopes are likely to bind in a high capacity to murine MHC, thus eliciting significant T-cell responses.

Peptides and Mouse Trial Design

After determination of which peptides are of high binding capacity, at least ten 5-wk-old mice will be vaccinated subcutaneously with the predicted T cell epitopes; after 4 wks when a complete T cell response is made, we will innoculate these mice and age-matched mice with $2*10^6$ luciferase-tagged K7M2 cells and monitor tumor progress for 100 days **(Fig. 30)** via *In Vivo* Imaging (IVIS) as described by Christie et al. (Christie et al. 2021). Luciferase is an enzyme that luminesces when exposed to luciferin. For this reason, K7M2 cells tagged with luciferase will allow us to track the tumor burden of mice in real time after they are injected with luciferin and viewed on IVIS. Luciferase activity can decay, so many days later, cancer may also be in other places that do not appear on IVIS. To account, statistical analysis will be performed on all tumor data.

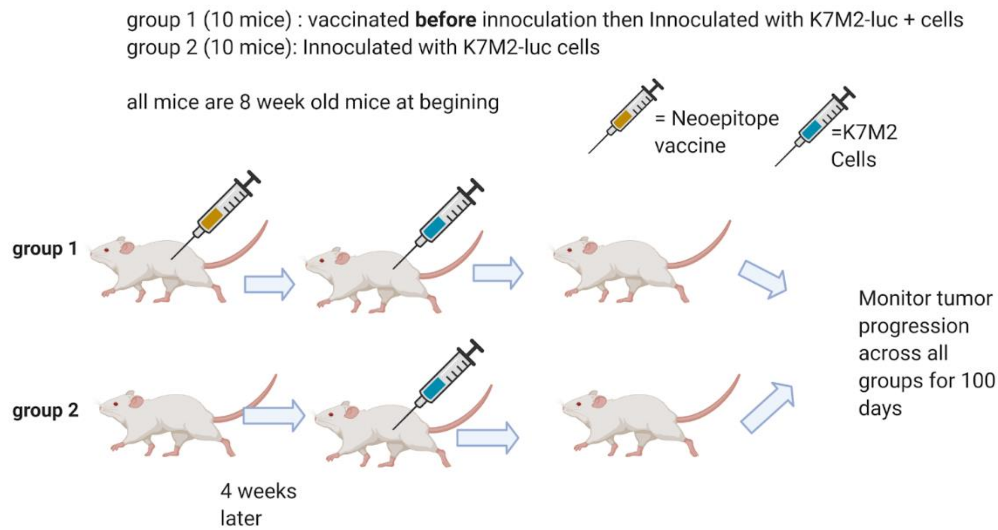


Figure 30: Design of *in vivo* Study to Determine the Efficacy of EnsembleMHC Prediction Algorithm. One group of mice would be vaccinated with neoepitopes that were proven to be significant for enhancing T cell responses to mOS. The second group would not receive the vaccination. Both groups would be injected with K7M2 cells which should contain neo-epitopes that were determined to be of significance for eliciting T cell responses by EnsembleMHC.

Representative Results

Culturing of Primary Osteoblasts

After the harvest of the calvaria and long bones from neonatal mice **(Fig. 31),** primary osteoblasts should be cultured from both calvaria and long bones, though long bones could yield a much higher quantity of cells **(Fig. 32)**. Both K7M2 and primary osteoblast cells will be adherent and will appear similar in size and morphology as shown in Figure 32.

Potential Outcome of Mouse Trial

We expect mice vaccinated with peptides predicted by EnsembleMHC to show more significant tumor regression than those not vaccinated, evident by reduced tumor burden via IVIS imaging. We also expect these mice will generate T-cells specific to peptides which can be tested in a future study.

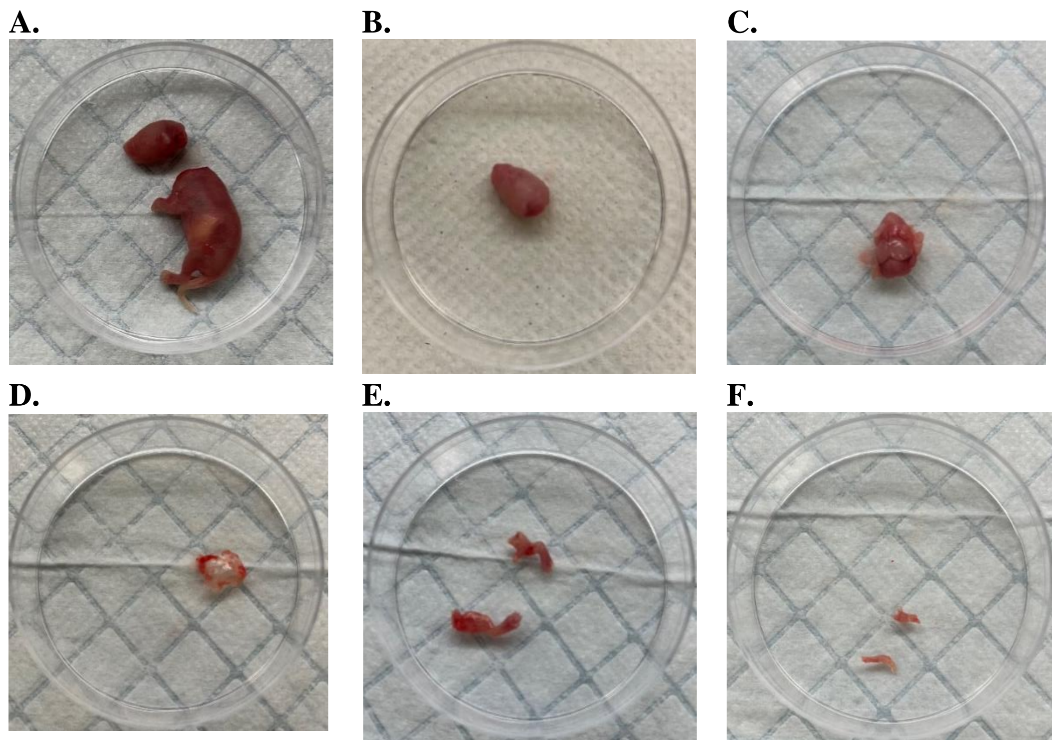


Figure 31: Harvest of Murine Calvaria and Long Bones. A) A Neo-natal mouse less than 48 hrs. after birth were euthanized, sprayed with 70% ethanol and used in these experiments. **B)** Mouse head was separated from the body. **C)** Skin along the top of the skull was peeled back so that calvaria could be viewed**. D)** Murine calvaria was removed. **E)** For harvest of long bones, legs from neonatal mice were removed**. F)** Skin, muscle and adipose tissues were removed from long bones.

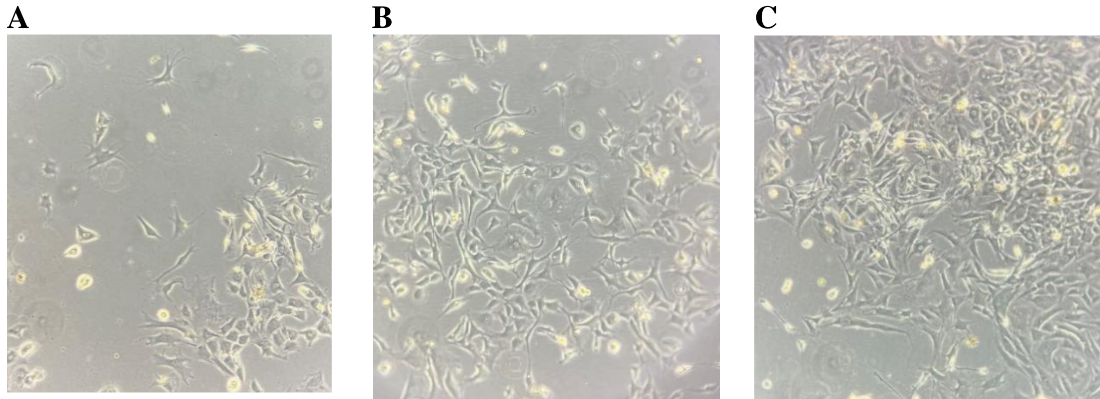


Figure 32: Primary Osteoblast Cultures Before First Passage. Cells were passaged when they reached 60-80% confluency. A) Long bone cells from mouse 1. B) Long bone cells from mouse 2. C) Long bone cells from mouse 3.

Discussion

The bioinformatics field has advanced dramatically in recent decades (Soria-Guerra et al. 2015), with applications ranging from risk assessment for certain diseases, such as determining one's risk for breast cancer, to developing therapeutic interventions for malignancies, such as cancer vaccines (Sarkizova et al. 2019; Narang et al. 2019). Current epitope prediction models (EPMs), like EnsembleMHC, can determine proteins of interest with far greater accuracy than previous models (Tanjo et al. 2020; Wilson et al. 2021a). In fact, EnsembleMHC demonstrated a remarkable ability to accurately predict epitopes that were most important for T cell recognition during SARS-CoV-2 infection, epitopes whose influence was validated when predictions were compared to real-world data, as persons who had the predisposition to present the predicted epitopes to their immune system due to having specific MHC haplotypes were less likely to develop severe SARS-CoV-2, underscoring the massive potential of the prediction algorithm. (Wilson et al. 2021a).

EPMs are meant to complement laboratory findings rather than replace them (Brusic, Bajic, and Petrovsky 2004). One obstacle computational models face is that they may not predict factors in a living system that could affect how significant an epitope is to the immune response, including innate and adaptive immune elements that may be present in a living system that remain unaccounted for in a prediction model (Brusic, Bajic, and Petrovsky 2004; Paul et al. 2020). This circumstance could mislead researchers into drawing inaccurate conclusions regarding what proteins should be targeted for potential therapeutic intervention. Though EPMs hold much promise in predicting epitopes important for cancer therapeutics, many have never been tested *in vivo*. Extensions from our work aim to address the problem that the past predicted accuracy of EnsembleMHC was not based on results from a living

system but instead on simulated predictions. We propose to test this using our lab's established K7M2 metastatic osteosarcoma (mOS) mouse model, made from cancerous osteoblast cells from Balb/c mice that form mOS, which serves as a convenient option to test this research as the outcomes of control mice have been tested and replicated multiple times in our lab (Lussier, O'Neill, et al. 2015; Lussier, Johnson, et al. 2015).

The first step of this research lies in comparing RNA from primary osteoblasts harvested from neonatal mice with RNA from K7M2 cancer cells. Osteoblasts harvested from mice are most likely to divide rapidly if taken from neonatal mice 48 hrs old or younger (Bakker and Klein-Nulend 2012). Further, the maturation of osteoblast cultures can be monitored by staining for the presence of alkaline phosphatase, a technique that we can apply to future cell cultures to validate that the correct cells have been cultured (K. S. Leung et al. 1993). Both cancerous and primary cells should be sent for RNAseq analysis and differences in RNA expression should be determined to understand neo-epitope presence. Further, we propose that this data is used to evaluate epitopes likely to engage immune cells via analysis with EnsembleMHC, then those epitopes determined to be able to elicit effective T cell responses be used to vaccinate mice. Next, mice should be inoculated with K7M2, and it should be determined if there is a difference in cancer progression among vaccinated vs. unvaccinated groups.

One important consideration when interpreting the results of this study is that the comparisons we hope to complete are in Balb/c mouse models. Balb/c mice are genetically similar, so, likely, the results found in one mouse will also apply to another mouse. However, these comparisons will not be true in other mouse models. Additionally, this comparison will be completed in murine K7M2 cells compared to primary osteoblast tissue

in mice and so is not necessarily translatable to human mOS since all cancers accumulate specific mutations. In the same breath, the purpose of this model is to validate the ability of EnsembleMHC to predict T cell epitopes *in vivo* rather than *in silico*, so although the specific epitopes determined within this model cannot be applied to humans, the prediction model itself can be. For the future clinical implementation of this model, individuals will likely need to be screened individually to predict which neo-epitopes within particular cancer accurately should be targeted for the patient can be made. Additionally, T-cell prediction algorithms are correct only a fraction of the time (Wilson et al. 2021a). Proper conclusions regarding the ability of a particular epitope to elicit T-cell responses would require an *in vivo* study.

Future progress in these studies could seek to determine first whether neo-epitopes can be predicted for other tumor models using the same methods. Additionally, studies that compare the effectiveness of T-cell prediction *in vivo* would be beneficial. Eventually, the methods described here may be able to contribute to the production of individualized cancer vaccines for various malignancies.

CHAPTER 7

DISCUSSION

Though the innate and adaptive immune systems have distinct functions and mechanisms, they work together to provide a coordinated defense against pathogens. Recognition of pathogens by the innate immune system is initiated by pathogen recognition receptors (PRRs), which recognize and are activated by conserved molecular structures on pathogens known as pathogen-associated molecular patterns (PAMPs). PRRs fall into several families, each recognizing a distinct pattern leading to specific consecutive immune activation steps, allowing the innate immune system to provide a tailored response to an infection based on the molecular pattern recognized (Akira, Uematsu, and Takeuchi 2006; Liao and Su 2021). The activation steps that follow pathogen recognition can further inform the adaptive immune system of the pathogen's presence by the release of cytokines, chemokines, and other molecules that impact cells of the immune system, resulting in the recruitment of cells to the site of infection, destruction of pathogens, promotion of inflammation or elicitation of other effector functions by immune cells (Koyama et al. 2008; Akira, Uematsu, and Takeuchi 2006). Moreover, PRRs can activate intracellular proteins that set into motion several changes within a cell. These changes, in turn, further activate intracellular anti-microbial processes to clear a pathogen from an infected cell, limiting its capacity to propagate.

The oligoadenylate synthetase (OAS) family of proteins plays a notable role in select intracellular anti-microbial processes. These proteins comprise a network of PRRs responsible for recognizing viral dsRNA within a cell, resulting in downstream changes that alter cellular function and result in virus clearance through the activation of RNase-L

and subsequent destruction of cytosolic RNA (Kristiansen et al. 2011). Briefly, dsRNA binds OAS proteins, resulting in a conformational change within the OAS protein that causes the protein to become enzymatically active (Silverman and Weiss 2014). The now-active OAS protein utilizes ATP within the cell as a substrate to synthesize 2-5A (2'-5' oligoadenylate). 2-5A binds to the latent RNase-L monomer, causing it to dimerize and embrace nuclease activity that allows for the cleavage of both viral and single-stranded RNA (Silverman and Weiss 2014). The three enzymatically active isoforms of OAS- OAS1, OAS2, and OAS3- all are believed to function through the same pathway of viral suppression via the activation of RNase-L and succeeding destruction of cytosolic RNA (Silverman and Weiss 2014). However, recent studies in an A549 human lung adenocarcinoma cell line have shown that eliminating OAS3 results in the loss of RNase-L activity, while eliminating OAS1 and OAS2 does not. These findings indicate that OAS1 and OAS2 isoforms may function through other mechanisms yet to be fully known (Li et al. 2016).

Additionally, how specific OAS proteins impact the clearance of viral infections across different families remains elusive. However, several accounts of polymorphisms, particularly OAS proteins in humans, show associations with increased susceptibility to or advancement of infection with certain viruses (Lim et al. 2009; Wickenhagen et al. 2021; Marques et al. 2008; Alagarasu et al. 2013; Barkhash et al. 2014). For instance, several studies in both humans and laboratory mice show that deficiencies in OAS1 proteins can increase susceptibility to west Nile virus (WNV) infection (Mashimo et al. 2002; Lim et al. 2009). Further, recent evidence suggests that the importance of specific OAS proteins may be associated with the cellular localization of viral replication within a cell

(Wickenhagen et al. 2021; Soveg et al. 2021). In fact, C-terminal prenylation of OAS1-46 traffics these proteins to endomembrane systems, which during viral infections as SARS-CoV-2, can be sites rich in viral dsRNA. Trafficking of OAS1-46 to these sites results in an increased likelihood that dsRNA structures in the SARS-CoV-2 genome will bind OAS1-46 and initiate potent anti-viral mechanisms through the activation of RNase-L. It is believed that this is the reason that persons with an inability to prenylated OAS1-46 showed increased susceptibility to SARS-CoV-2 (Wickenhagen et al. 2021; Soveg et al. 2021), supporting the notion that the impact of OAS proteins on viral replication is associated with the cellular localization of viral replication, though we still do not fully understand the mechanisms related to OAS virus-specific impacts on viral inhibition.

Research regarding the impact of OAS proteins on viral replication specific to particular viral families represents a gap in our understanding of anti-viral innate immune mechanisms. Understanding these mechanisms further could allow improvements in patient's with a viral illness, as we would be better able to predict which patients are at risk for severe or progressive disease based on genetic screenings. Additionally, a better understanding of innate immune pathways that impact viral replication could aid in developing new antiviral medications and therapies that target specific pathways. We wished to elucidate further the impact different OAS proteins had on viral replication across diverse RNA virus families. Additionally, we aimed to characterize whether OAS proteins' impact on viral suppression depends on RNase-L or if potential other mechanisms exist that allow for virus replication.

In Chapter 2, "Characterizing the Impact of Specific OAS Proteins on Viral Replication in a Human Lung, Adenocarcinoma Cell Line," we hypothesized that if the

impact of OAS proteins on viral replication depends on where in a cell a virus replicated and viral replication strategies are similar across viral families, then the ability of particular OAS proteins to impact viral replication would be similar across viral families. Evidence for this logic exists across several previous studies that indicate the location of viral replication is essential regarding which innate immune sensors are activated. Additionally, we predicted that if the antiviral function of OAS3 relies on RnsasL, then RnasLKO cell lines would also display ablated anti-viral properties towards viruses that OAS3 primarily impacts. We tested our hypotheses by creating TET-ON cells overexpressing OAS1-42, OAS1-46, OAS2, and OAS3 when induced with Doxycycline and infecting these cells with various viruses, both with and without induction with Doxycycline, to determine the impact of overexpression of certain proteins on virus replication.

Our findings suggest that OAS-mediated suppression of viruses varies even across viruses within the same family. *Alphaviruses* both showed that OAS3 overexpression resulted in decreases in virus replication, though only CHKV showed that overexpression of OAS1-42 resulted in reductions in virus replication. These results shed light on which proteins may be most important for combat against viral replication for SINV and CHKV. These results are also consistent with previous data that indicated both SINV and CHKV are impacted by OAS3 (Li et al. 2016; Bréhin et al. 2009). *Flavivurses*, Zika virus (ZIKV), and West Nile Virus (WNV) were both impacted differently by overexpression of OAS proteins. We show here that ZIKV replication is primarily reduced by overexpression of OAS1-42, which is mainly consistent with real-world clinical data that suggests mutations in OAS1 can cause people to be more susceptible to adverse ZIKV outcomes. However, WNV seemed to be most impacted by OAS2 and OAS3 overexpression. These results

indicate that other factors may impact the ability of OAS proteins to suppress viral replication besides the cellular localization of viruses during their replication cycle.

Additionally, we show here that in RNase-L-KO cells, overexpression of OAS3 for CHKV and SINV replication was ablated, indicating that RNase-L is a major component of OAS3-mediated viral suppression. However, there was reduced but not absent viral replication in cases of OAS1-42 and OAS2 overexpression for CHKV, ZIKV, and WNV, indicating that there may be other factors on which OAS1 and OAS2 rely on to suppress viral replication that yet to be determined.

Studies regarding elements of the innate immune system and their associations with the inhibition of viral replication allow for a better understanding of how the innate immune system reacts to both viral infections and disease. When extended, these studies can also allow for a better understanding of the adaptive immune system. In fact, recent studies demonstrate a direct interconnectedness of OAS proteins to the adaptive immune system, showing that both under and over-expression of OAS2 proteins can mediate T-cell receptor activity expression during some cancers via regulation of caspase-3, particularly in the context of oral cancers (Dar et al. 2016). Future research efforts can further analyze the interplay between innate immune elements like OAS proteins and RNase-L and their functional impact on both T immune cells and B cell responses. For example, if overexpression of OAS3 impedes the replication of a particular virus, the results of our study indicate that its ability to hinder viral replication also relies on RNase-L . Thus, future studies could determine if RNase-L KO mice (A. Zhou et al. 1997) also show a decreased ability to combat that particular virus but this *in vivo* model also would allow the analysis

of T cell responses and determine if those are impacted, providing insight into what elements are needed to create an effective T cell response against viral infection.

Beyond understanding the T-cell response to viral infections, copious efforts have been made for decades to understand and modulate the T-cell response to malignant cells across cancers (Pardoll and Topalian 1998; J. Han et al. 2020). Several excellent reviews have further analyzed the broad implications of T cell responsiveness to cancers and how knowledge gained from this research can be harnessed to improve cancer immunotherapies (van der Leun, Thommen, and Schumacher 2020; C. C. Smith et al. 2019). Immune checkpoint blockade (ICB) represents one form of cancer immunotherapy that has shown much success in recent decades across several cancers in both pre-clinical and clinical trials (Larkin et al. 2015). ICB that employs anti-CTLA-4, anti-PD-L1, and anti-PD-1 alone or in combination with one another have shown promise in improving survival rates amongst various malignancies via T cell-mediated control of cancer cells (Fairfax et al. 2020; Y. J. Park, Kuen, and Chung 2018; Brahmer et al. 2012; Topalian et al. 2012).

Our lab utilizes a murine K7M2 metastatic osteosarcoma (mOS) model to study T-cell responses to cancer. Osteosarcoma is the most common bone cancer in children making up ~3% of total child cancer cases, with limited treatment options (CK et al. 2016; Abarrategi et al. 2016). Osteosarcoma is believed to originate from mesenchymal cells and is subclassified according to its predominant stroma, often comprised of osteoblastic, chondroblastic, fibroblastic, or giant cell-rich tumors, among others (Abarrategi et al. 2016). However, the predominant stroma is not believed to have prognostic importance or implications for the effectiveness of current therapies, though it can be utilized for future immunotherapy development (Abarrategi et al. 2016). Most osteosarcoma cases initiate in

long bones, with common metastasis to the mucosa of the lungs in both human and murine cancers (Lindsey, Markel, and Kleinerman 2017; Abarrategi et al. 2016). Traditional treatment methods for metastatic osteosarcoma (mOS) involve surgery, radiation therapy, chemotherapy, or a combination of all three. Still, they have shown limited efficacy and high mortality rates even after the conclusion of treatment (Lindsey, Markel, and Kleinerman 2017). In fact, survival rates decline from ~70% to ~20% upon metastasis, even with modern treatment options (Czarnecka et al. 2020). A drive to elucidate and resolve these obstacles has led to discoveries of mOS resistance mechanisms to traditional treatment methods and has augmented the pioneering of alternative therapies to treat the disease (Chou and Gorlick 2006).

Immune checkpoint blockade represents one notable therapy pursued for the treatment of mOS. Additionally, multiple studies have indicated that cytotoxic T lymphocytes (CTLs) limit mOS progression and that the mechanisms of ICB rely on cytotoxic T cells to be fully efficient (Lussier, O'Neill, et al. 2015; Lussier, Johnson, et al. 2015). Bearing this in mind, we previously investigated the impact of ICB composed of anti-CTLA-4/anti-PD-L1 for metastatic osteosarcoma (mOS) in genetically identical mice and showed that treating mOS with this treatment regimen in a K7M2 Balb/cJ murine model results in complete control of tumors and immunity to later tumor inoculation with the same K7M2 cell line in 60% of mice (Lussier, Johnson, et al. 2015).

The discrepancy among mice regarding their response to the combination therapy is curious, considering the mice used in the study are inbred Balb/c mice with similar genetic makeup. The mice used in the study were also the same age and were housed under the same living conditions. One possibility of variance is differences in circulating

antibodies (Abs). Mice and humans produce natural Abs formed during development without antigen induction (Holodick, Rodríguez-Zhurbenko, and Hernández 2017). Additionally, B cells undergo the process of somatic recombination to generate a repertoire of Abs that can vastly expand following antigen stimulus; since this process occurs semi-randomly and independent of germline-encoded DNA, even subjects with the same DNA can have differences in circulating Abs (Tonegawa 1983; Jacob et al. 1991).

In addition to natural Abs presence and Abs generated through the process of somatic recombination, circulating Abs can often be indicators of risk for or development of certain diseases believed to have been produced in response to novel peptide stimulation instigated by the disease. Recent studies have characterized Ab presence in sera by using microarrays with >300,000 short peptides. The binding pattern of the Abs on the microarray is termed an "immunosignature." Analysis of immunosignatures has been shown to predict the identity of infectious diseases with >95% accuracy (Holodick, Rodríguez-Zhurbenko, and Hernández 2017). We initially characterized the Ab response between ICB responders and non-responders in mOS-bearing mice but found that immunosignatures from pre-bleeds, prior even to tumor inoculation, could distinguish responders from non-responders before ICB treatment on a microarray of 10,000 unique peptides. As mentioned in previous chapters, traditionally, immunosignatures have diagnostically predicted disease via the binding pattern of Abs in sera to the many peptides on these arrays, resulting in reproducible patterns of specific illnesses (Restrepo, Stafford, and Johnston 2013; Helmink et al. 2019). However, this technology has not been used to identify predicted treatment efficacy responders vs. non-responders.

In Chapter 3: Distinguishing Circulating Antibody Repertoires of Responders vs. Non-Responders to Immune Checkpoint Blockade (ICB) for Metastatic Osteosarcoma (mOS) in Balb/c mice, we sought first to determine if we could replicate our previous studies, which showed a ~60% survival rate in Balb/c mice that were inoculated with anti-CTLA-4/ anti-PD-L1 immunotherapy after inoculation with mOS. We then aimed to determine if pre-bleeds from these mice could show distinct antibody repertoires between responders and non-responders to ICB for mOS. Based on pre-bleed samples taken from mice inoculated with mOS and then treated with anti-CTLA-4/anti-PD-L1, we showed that mice likely to respond to immunotherapy typically had Abs bound to 74 specific peptides. We were further able to validate the ability to determine which mice would respond to immunotherapy and which would not by again running blinded samples on the identical peptide arrays. The findings from this research have several clinical implications.

First, this study shows that by constructing a training set of peptide arrays using responder vs. non-responder Antibody (Ab) profiles to immune checkpoint blockade (ICB, immunosignatures and similar peptide arrays can serve as a powerful diagnostic tool that can predict an individual's response to ICB for mOS with ~80% accuracy. This knowledge would enable clinicians to make informed decisions regarding patient care, as they could direct their patients to resources that maximize their treatment outcomes. Further, analysis of peptides to which Abs in responders bound with high capacity showed several consensus sequences. Notably, the peptides used on these arrays were randomly generated, implying that they could represent an actual linear peptide or be mimotopes that do not represent a linear peptide present in nature (Meloen, Puijk, and Slootstra n.d.).

Future studies could move in several directions, first to determine if these sequences are indicative of Abs that are instigating an increased immune response to mOS after ICB; one potential experiment could be to inject mice with epitopes that correlated in high Ab binding capacity in responders, into mice before mOS inoculation or ICB treatment. This would allow us to determine whether the Ab presence is causative of the response to ICB for mOS. If the generation of an Ab response to epitopes determined to be of the high binding capacity of Abs from responders shows increased responsiveness to ICB for mOS, these results could be applied clinically by seeing if vaccination before ICB treatment can impact and maximize results of ICB. Additionally, certain factors have already been correlated with changes in circulating Ab composition.

For instance, alterations in microbiome composition typically result in subsequent changes to circulating Ab composition, believed to be in response to antigens presented by microbes (Vivarelli et al. 2019; Davar et al. 2021a). Additionally, recent studies have discovered that ICB's effectiveness can be associated with or even reliant on microbiome composition (Davar et al. 2021b). For this reason, we speculated if differences in Ab composition between responders and non-responders could be related to pre-existing microbiome composition. We further tested this theory in subsequent chapters.

In Chapter 4: The Impact of Microbiome Dysbiosis on T Cell Function Within the Tumor Microenvironment (TME), I explain first how T cells canonically react to cancerous cells; I then explain how the presence of certain microbes can impact T cell function within the TME. Certain bacterial presence has been shown to influence both innate and adaptive immune cell function. This influence is primarily a result of metabolites secreted as fermentation products by these bacteria. Short-chain fatty acids (SCFAs) have been shown

to play direct roles in immune cell function (M. Sun et al. 2018; J. Zhang et al. 2019; Luu et al. 2021; Schiweck et al. 2022). Additionally, variations in microbial populations can alter subsequent metabolites produced by microbes, which can further impact immune cell function (W. Yang and Cong 2021).

Within the TME, this is particularly important, as there are not only intratumoral microbial populations for which metabolite presence can affect immune cell function, but the presence of specific microbes in mucosal tissue can also impact the function of immune cells. These findings hold significance in the context of cancer treatments that rely on the direct engagement of T immune cells, especially immunotherapy treatments. This reinforces the idea that the microbiome influences immunotherapy treatments reliant on immune cells, such as T cells. Recognizing and understanding these insights better is imperative for clinicians and researchers who hope to optimize patient outcomes. Additionally, they inspire research efforts to understand the microbiome's impact on immunotherapy treatments for specific cancers.

In Chapter 5: The Impact of Microbiome Modulations on T Cell-Mediated Immunotherapy Efficacy for Metastatic Osteosarcoma (mOS), we sought to determine the impact of the microbiome on immunotherapy composed of anti-CTLA-4/anti-PDL1 for mOS. First, we harvested fecal samples of mice before they were given ICB or mOS. After knowing whether the mice in the study were responders or nonresponders to ICB, we sent fecal samples for 16S ribosomal unit sequencing. Genus level resolution and characterization of microbial populations in these samples showed no specific difference in microbial populations before tumor inoculation and ICB. This indicates that genus-level microbial populations likely do not cause responders or non-responders to ICB for mOS.

To determine if the microbiome impacted ICB for mOS in any capacity, we decided to apply an antibiotic regimen known to deplete, to a significant level, the microbiome in specific pathogen-free mice. This antibiotic regimen composed of ampicillin, vancomycin, neomycin, and metronidazole, termed AVNM, was previously shown to eliminate most of the microbiome while still not killing mice that were ten weeks old. We first found that variation in weight among mice is dependent on the age of mice at the start of antibiotic treatment. Additionally, when mice were inoculated at 10 weeks of age with K7M2 cells, mice treated with Abx did not show reduced responsiveness to ICB or decreased ability to combat mOS. After inoculation with the cells, it was found that there was a mycoplasma presence in the cell line. It is possible that the Abx that was administered during treatment to the mice eliminated the mycoplasma in the injected cells, causing additional elimination of the tumor cells from the mice. This concept is similar to that which was introduced in Chapter 1, describing Coley's toxin. In this situation, intratumoral bacteria activated and bound to PRRs, which triggered the innate immune system further and activated the adaptive immune system in response.

We completed an additional trial with K7M2 cells whose stocks were tested to be mycoplasma negative, and this trial will continue to be monitored to determine if the results found here are replicative or only present during mycoplasma presence. In this study, we noted splenomegaly is present in mice who succumb to tumors and are on antibiotic regimens. In contrast, mice not on antibiotic regimens and who developed endpoint metastasis did not display splenomegaly. Further, mice that were typically on the same antibiotic regimen but did not have active tumor burden did not show the same splenomegaly. This result is significant because it implies that there is still an immune

response occurring in the mice that reach the endpoint but implies that since the mice are still succumbing to tumors, the immune response may not be effective. It is possible that immune cells cannot traffic to the areas they are needed in due to microbiome depletion. Future studies can look into markers expressed in these splenic cells to determine whether or not immune cells are "stuck" in the spleen when microbiome depletion has occurred. If ways in which the microbiome impacts immunotherapy and immune cell function during cancer progression are better understood, researchers may be able to improve T-cell-mediated immunotherapy regimens in the future.

Another way to improve T cell-medicated immunotherapy regimens is to understand better how to effectively engage T cells to eliminate cancer. In Chapter 6, Methods for Validation of a T cell Epitope Prediction Algorithm EnsembleMHC *in vivo,* we describe methods that would allow for the testing of the accuracy of a T cell prediction algorithm. We describe utilizing the K7M2 Balb/c model described in these studies to test how well EnsembleMHC can predict T cell epitopes. These studies can be extended in several capacities, as we could better predict how to engage T-cell responses and extend this knowledge to therapeutic cancer vaccines and CART cell therapies.

Taken together, results from this research have implications for both antiviral therapeutic approaches and anti-cancer therapeutic approaches, as they indicate knowledge related to how the innate immune system responds to viral infection as well as how certain factors can impact the adaptive immune system during immunotherapy.

REFERENCES

Abarrategi, Ander, Juan Tornin, Martinez Cruzado Lucia, Ashley Hamilton, Martinez Campos Enrique, Juan P. Rodrigo, M. Victoria González, Nicola Baldini, Garcia Castro Javier, and Rene Rodriguez. 2016. "Osteosarcoma: Cells-of-Origin, Cancer Stem Cells, and Targeted Therapies." *Stem Cells International*. Hindawi Limited. https://doi.org/10.1155/2016/3631764.

Abiko, Kaoru, Masaki Mandai, Junzo Hamanishi, Yumiko Yoshioka, Noriomi Matsumura, Tsukasa Baba, Ken Yamaguchi, et al. 2013. "PD-L1 on Tumor Cells Is Induced in Ascites and Promotes Peritoneal Dissemination of Ovarian Cancer through CTL Dysfunction." *Clinical Cancer Research* 19 (6): 1363–74. https://doi.org/10.1158/1078-0432.CCR-12-2199/85155/AM/PD-L1-ON-TUMOR-CELLS-IS-INDUCED-IN-ASCITES-AND.

Aggarwal, Bharat B. 2003. "Signalling Pathways of the TNF Superfamily: A Double-Edged Sword." *Nature Reviews Immunology 2003 3:9* 3 (9): 745–56. https://doi.org/10.1038/nri1184.

Ahmadzadeh, Mojgan, Laura A. Johnson, Bianca Heemskerk, John R. Wunderlich, Mark E. Dudley, Donald E. White, and Steven A. Rosenberg. 2009. "Tumor Antigen–Specific CD8 T Cells Infiltrating the Tumor Express High Levels of PD-1 and Are Functionally Impaired." *Blood* 114 (8): 1537–44. https://doi.org/10.1182/BLOOD-2008-12-195792.

Akira, Shizuo, Satoshi Uematsu, and Osamu Takeuchi. 2006. "Pathogen Recognition and Innate Immunity." *Cell* 124 (4): 783–801. https://doi.org/10.1016/J.CELL.2006.02.015.

Al-Shibli, Khalid I., Tom Donnem, Samer Al-Saad, Magnus Persson, Roy M. Bremnes, and Lill Tove Busund. 2008. "Prognostic Effect of Epithelial and Stromal Lymphocyte Infiltration in Non-Small Cell Lung Cancer." *Clinical Cancer Research* 14 (16): 5220–27. https://doi.org/10.1158/1078-0432.CCR-08-0133.

Alagarasu, K., T. Honap, I. M. Damle, A. P. Mulay, P. S. Shah, and D. Cecilia. 2013. "Polymorphisms in the Oligoadenylate Synthetase Gene Cluster and Its Association with Clinical Outcomes of Dengue Virus Infection." *Infection, Genetics and Evolution* 14 (1): 390–95. https://doi.org/10.1016/J.MEEGID.2012.12.021.

Alcazer, Vincent, Paola Bonaventura, Laurie Tonon, Sandrine Wittmann, Christophe Caux, and Stéphane Depil. 2019. "Neoepitopes-Based Vaccines: Challenges and Perspectives." *European Journal of Cancer* 108 (February): 55–60. https://doi.org/10.1016/J.EJCA.2018.12.011.

Almonte, Andrew A., Hareesha Rangarajan, Desmond Yip, and Aude M. Fahrer. 2021.

"How Does the Gut Microbiome Influence Immune Checkpoint Blockade Therapy?" *Immunology and Cell Biology* 99 (4): 361–72. https://doi.org/10.1111/IMCB.12423.

Anderson, Michael J., Kimberly Shafer-Weaver, Norman M. Greenberg, and Arthur A. Hurwitz. 2007. "Tolerization of Tumor-Specific T Cells Despite Efficient Initial Priming in a Primary Murine Model of Prostate Cancer." *The Journal of Immunology* 178 (3): 1268–76. https://doi.org/10.4049/JIMMUNOL.178.3.1268.

Andreeva, Liudmila, Björn Hiller, Dirk Kostrewa, Charlotte Lässig, Carina C. De Oliveira Mann, David Jan Drexler, Andreas Maiser, et al. 2017. "CGAS Senses Long and HMGB/TFAM-Bound U-Turn DNA by Forming Protein–DNA Ladders." *Nature 2017 549:7672* 549 (7672): 394–98. https://doi.org/10.1038/nature23890.

Andresen, Lars, Karen Aagaard Hansen, Helle Jensen, Stine Falsig Pedersen, Peter Stougaard, Helle Rüsz Hansen, Jesper Jurlander, and Søren Skov. 2009. "Propionic Acid Secreted from Propionibacteria Induces NKG2D Ligand Expression on Human-Activated T Lymphocytes and Cancer Cells." *The Journal of Immunology* 183 (2): 897–906. https://doi.org/10.4049/JIMMUNOL.0803014.

Apostolou, Panagiotis, Aggeliki Tsantsaridou, Ioannis Papasotiriou, Maria Toloudi, Marina Chatziioannou, and Gregory Giamouzis. 2011. "Bacterial and Fungal Microflora in Surgically Removed Lung Cancer Samples." *Journal of Cardiothoracic Surgery* 6 (1): 1–5. https://doi.org/10.1186/1749-8090-6-137/FIGURES/1.

Armitage, Richard J., William C. Fanslow, Laura Strockbine, Timothy A. Sato, Ky N. Clifford, Brian M. Macduff, Dirk M. Anderson, et al. 1992. "Molecular and Biological Characterization of a Murine Ligand for CD40." *Nature 1992 357:6373* 357 (6373): 80–82. https://doi.org/10.1038/357080a0.

Arpaia, Nicholas, Clarissa Campbell, Xiying Fan, Stanislav Dikiy, Joris Van Der Veeken, Paul Deroos, Hui Liu, et al. 2013. "Metabolites Produced by Commensal Bacteria Promote Peripheral Regulatory T-Cell Generation." *Nature 2013 504:7480* 504 (7480): 451–55. https://doi.org/10.1038/nature12726.

Arya, Dev P. 2005. "Aminoglycoside-Nucleic Acid Interactions: The Case for Neomycin." *Topics in Current Chemistry* 253 (January): 149–78. https://doi.org/10.1007/B100446/COVER.

Baglioni, Corrado, Michael A. Minks, and Patricia A. Maroney. 1978. "Interferon Action May Be Mediated by Activation of a Nuclease by PppA2′p5′A2′p5′A." *Nature 1978 273:5664* 273 (5664): 684–87. https://doi.org/10.1038/273684a0.

Bakker, Astrid D., and Jenneke Klein-Nulend. 2012. "Osteoblast Isolation from Murine

Calvaria and Long Bones." *Methods in Molecular Biology* 816: 19–29. https://doi.org/10.1007/978-1-61779-415-5_2.

Banerjee, Sagarika, Tian Tian, Zhi Wei, Natalie Shih, Michael D. Feldman, Kristen N. Peck, Angela M. DeMichele, James C. Alwine, and Erle S. Robertson. 2018. "Distinct Microbial Signatures Associated with Different Breast Cancer Types." *Frontiers in Microbiology* 9 (MAY): 951. https://doi.org/10.3389/FMICB.2018.00951/BIBTEX.

Banerjee, Sagarika, Zhi Wei, Tian Tian, Dipayan Bose, Natalie N.C. Shih, Michael D. Feldman, Thaer Khoury, Angela De Michele, and Erle S. Robertson. 2021. "Prognostic Correlations with the Microbiome of Breast Cancer Subtypes." *Cell Death & Disease 2021 12:9* 12 (9): 1–14. https://doi.org/10.1038/s41419-021-04092-x.

Banerjee, Shuvojit, Arindam Chakrabarti, Babal Kant Jha, Susan R. Weiss, and Robert H. Silverman. 2014. "Cell-Type-Specific Effects of RNase L on Viral Induction of Beta Interferon." *MBio* 5 (2): 856–70. https://doi.org/10.1128/MBIO.00856-14/ASSET/618087CA-7A0C-4EED-A784-083537CBE1A3/ASSETS/GRAPHIC/MBO0011417530002.JPEG.

Barber, Glen N. 2015. "STING: Infection, Inflammation and Cancer." *Nature Reviews Immunology 2015 15:12* 15 (12): 760–70. https://doi.org/10.1038/nri3921.

Barkhash, Andrey V., Galina V. Kochneva, Elena V. Chub, Svetlana V. Mikhailova, and Aida G. Romaschenko. 2014. "Association between Polymorphisms in OAS2 and CD209 Genes and Predisposition to Chronic Hepatitis C in Russian Population." *Microbes and Infection* 16 (5): 445–49. https://doi.org/10.1016/J.MICINF.2014.02.004.

Behr, Felix M., Ammarina Chuwonpad, Regina Stark, and Klaas P.J.M. van Gisbergen. 2018. "Armed and Ready: Transcriptional Regulation of Tissue-Resident Memory CD8 T Cells." *Frontiers in Immunology* 9 (July): 1770. https://doi.org/10.3389/FIMMU.2018.01770/BIBTEX.

Behring, E. Von and Kitasato S. 1890. "Ueber Das Zustandekommen Der Diphtherie-Immunität Und Der Tetanus-Immunität Bei Thieren." *Zeitschrift Für Hygiene Und Infektionskrankheiten,* 7: 441–48. https://doi.org/10.1016/0161-5890(91)90033-G.

Belladonna, Maria L., Claudia Volpi, Roberta Bianchi, Carmine Vacca, Ciriana Orabona, Maria T. Pallotta, Louis Boon, et al. 2008. "Cutting Edge: Autocrine TGF-Beta Sustains Default Tolerogenesis by IDO-Competent Dendritic Cells." *Journal of Immunology (Baltimore, Md. : 1950)* 181 (8): 5194–98. https://doi.org/10.4049/JIMMUNOL.181.8.5194.

Bender, Mackenzie J, Alex C McPherson, Catherine M Phelps, Surya P Pandey, Colin R Laughlin, Jake H Shapira, Luzmariel Medina Sanchez, et al. 2023. "Dietary Tryptophan Metabolite Released by Intratumoral Lactobacillus Reuteri Facilitates Immune Checkpoint Inhibitor Treatment." *Cell* 186 (9): 1846-1862.e26. https://doi.org/10.1016/j.cell.2023.03.011.

Beura, Lalit K., Sara E. Hamilton, Kevin Bi, Jason M. Schenkel, Oludare A. Odumade, Kerry A. Casey, Emily A. Thompson, et al. 2016. "Normalizing the Environment Recapitulates Adult Human Immune Traits in Laboratory Mice." *Nature 2016 532:7600* 532 (7600): 512–16. https://doi.org/10.1038/nature17655.

Bordet, J. 1895. "Studien Über Die Hämolysine: Ihre Physiologische Bedeutung Und Anwendung in Der Pathologie." *Zeitschrift Für Hygiene Und Infektionskrankheiten* 19: 423–95. https://doi.org/10.1258/JMB.2009.009061.

Bordet, Jules. 1895. "Études Sur La Sérum Thérapeutique." *Annales de l'Institut Pasteur* 9: 462–534.

Brahmer, Julie R., Scott S. Tykodi, Laura Q.M. Chow, Wen-Jen Hwu, Suzanne L. Topalian, Patrick Hwu, Charles G. Drake, et al. 2012. "Safety and Activity of Anti-PD-L1 Antibody in Patients with Advanced Cancer." *The New England Journal of Medicine* 366 (26): 2455–65. https://doi.org/10.1056/NEJMOA1200694.

Bréhin, Anne Claire, Isabelle Casadémont, Marie Pascale Frenkiel, Cécile Julier, Anavaj Sakuntabhai, and Philippe Desprès. 2009. "The Large Form of Human 2′,5′-Oligoadenylate Synthetase (OAS3) Exerts Antiviral Effect against Chikungunya Virus." *Virology* 384 (1): 216–22. https://doi.org/10.1016/J.VIROL.2008.10.021.

Brinton, Margo A. 2013. "Replication Cycle and Molecular Biology of the West Nile Virus." *Viruses 2014, Vol. 6, Pages 13-53* 6 (1): 13–53. https://doi.org/10.3390/V6010013.

Brownlie, Rebecca J., and Rose Zamoyska. 2013. "T Cell Receptor Signalling Networks: Branched, Diversified and Bounded." *Nature Reviews Immunology 2013 13:4* 13 (4): 257–69. https://doi.org/10.1038/nri3403.

Brusic, Vladimir, Vladimir B. Bajic, and Nikolai Petrovsky. 2004. "Computational Methods for Prediction of T-Cell Epitopes—a Framework for Modelling, Testing, and Applications." *Methods* 34 (4): 436–43. https://doi.org/10.1016/J.YMETH.2004.06.006.

Butel, Janet S. 2000. "Viral Carcinogenesis: Revelation of Molecular Mechanisms and Etiology of Human Disease." *Carcinogenesis* 21 (3): 405–26. https://doi.org/10.1093/CARCIN/21.3.405.

Butz, Eric A., and Michael J. Bevan. 1998. “Massive Expansion of Antigen-Specific CD8+ T Cells during an Acute Virus Infection.” *Immunity* 8 (2): 167. /pmc/articles/PMC2776648/.

Castellarin, Mauro, René L. Warren, J. Douglas Freeman, Lisa Dreolini, Martin Krzywinski, Jaclyn Strauss, Rebecca Barnes, et al. 2012. “Fusobacterium Nucleatum Infection Is Prevalent in Human Colorectal Carcinoma.” *Genome Research* 22 (2): 299–306. https://doi.org/10.1101/GR.126516.111.

Catherine Hagan, D.V.M. Ph.D. 2017. “When Are Mice Considered Old?” The Jackson Laboratory. November 7, 2017. https://www.jax.org/news-and-insights/jax-blog/2017/november/when-are-mice-considered-old.

Chávez-Galán, L., M. C. Arenas-Del Angel, E. Zenteno, R. Chávez, and Ricardo Lascurain. 2009. “Cell Death Mechanisms Induced by Cytotoxic Lymphocytes.” *Cellular & Molecular Immunology 2009 6:1* 6 (1): 15–25. https://doi.org/10.1038/cmi.2009.3.

Chen, Huihui, Hui Sun, Fuping You, Wenxiang Sun, Xiang Zhou, Lu Chen, Jing Yang, et al. 2011. “Activation of STAT6 by STING Is Critical for Antiviral Innate Immunity.” *Cell* 147 (2): 436–46. https://doi.org/10.1016/J.CELL.2011.09.022.

Chen, Jane Jane, and Irving M. London. 1995. “Regulation of Protein Synthesis by Heme-Regulated EIF-2α Kinase.” *Trends in Biochemical Sciences* 20 (3): 105–8. https://doi.org/10.1016/S0968-0004(00)88975-6.

Cheng, Ru Yue, Ming Li, Shan Shan Li, Miao He, Xiao Hong Yu, Lei Shi, and Fang He. 2017. “Vancomycin and Ceftriaxone Can Damage Intestinal Microbiota and Affect the Development of the Intestinal Tract and Immune System to Different Degrees in Neonatal Mice.” *Pathogens and Disease* 75 (8): 104. https://doi.org/10.1093/FEMSPD/FTX104.

Choi, Un Yung, Ji Seon Kang, Yune Sahng Hwang, and Young Joon Kim. 2015. “Oligoadenylate Synthase-like (OASL) Proteins: Dual Functions and Associations with Diseases.” *Experimental & Molecular Medicine 2015 47:3* 47 (3): e144–e144. https://doi.org/10.1038/emm.2014.110.

Chou, Alexander J., and Richard Gorlick. 2006. “Chemotherapy Resistance in Osteosarcoma: Current Challenges and Future Directions.” *Expert Review of Anticancer Therapy*. Taylor & Francis. https://doi.org/10.1586/14737140.6.7.1075.

Christie, John D., Nicole Appel, Hannah Canter, Jazmin Galvan Achi, Natalie M. Elliott, Ana Lemos de Matos, Lina Franco, et al. 2021. “Systemic Delivery of TNF-Armed Myxoma Virus plus Immune Checkpoint Inhibitor Eliminates Lung Metastatic Mouse Osteosarcoma.” *Molecular Therapy - Oncolytics* 22 (September): 539–54.

https://doi.org/10.1016/J.OMTO.2021.07.014.

CK, Lettieri, Appel N, Labban N, Lussier DM, Blattman JN, and Hingorani P. 2016. “Progress and Opportunities for Immune Therapeutics in Osteosarcoma.” *Immunotherapy* 8 (10): 1233–44. https://doi.org/10.2217/IMT-2016-0048.

Claesson, Marcus J., Ian B. Jeffery, Susana Conde, Susan E. Power, Eibhlís M. O’connor, Siobhán Cusack, Hugh M.B. Harris, et al. 2012. “Gut Microbiota Composition Correlates with Diet and Health in the Elderly.” *Nature 2012 488:7410* 488 (7410): 178–84. https://doi.org/10.1038/nature11319.

Cooper, Max D., Raymond D.A. Peterson, and Robert A. Good. 1965. “Delineation of the Thymic and Bursal Lymphoid Systems in the Chicken.” *Nature 1965 205:4967* 205 (4967): 143–46. https://doi.org/10.1038/205143A0.

Cornel, Annelisa M., Iris L. Mimpen, and Stefan Nierkens. 2020. “MHC Class I Downregulation in Cancer: Underlying Mechanisms and Potential Targets for Cancer Immunotherapy.” *Cancers 2020, Vol. 12, Page 1760* 12 (7): 1760. https://doi.org/10.3390/CANCERS12071760.

Costantini, Lara, Stefano Magno, Davide Albanese, Claudio Donati, Romina Molinari, Alessio Filippone, Riccardo Masetti, and Nicolò Merendino. 2018. “Characterization of Human Breast Tissue Microbiota from Core Needle Biopsies through the Analysis of Multi Hypervariable 16S-RRNA Gene Regions.” *Scientific Reports 2018 8:1* 8 (1): 1–9. https://doi.org/10.1038/s41598-018-35329-z.

Coutzac, Clélia, Jean Mehdi Jouniaux, Angelo Paci, Julien Schmidt, Domenico Mallardo, Atmane Seck, Vahe Asvatourian, et al. 2020. “Systemic Short Chain Fatty Acids Limit Antitumor Effect of CTLA-4 Blockade in Hosts with Cancer.” *Nature Communications 2020 11:1* 11 (1): 1–13. https://doi.org/10.1038/s41467-020-16079-x.

Creagh, Emma M., and Luke A.J. O’Neill. 2006. “TLRs, NLRs and RLRs: A Trinity of Pathogen Sensors That Co-Operate in Innate Immunity.” *Trends in Immunology* 27 (8): 352–57. https://doi.org/10.1016/J.IT.2006.06.003.

Crotty, Shane. 2019. “T Follicular Helper Cell Biology: A Decade of Discovery and Diseases.” *Immunity* 50 (5): 1132–48. https://doi.org/10.1016/J.IMMUNI.2019.04.011.

Cummings, J. H., E. W. Pomare, H. W.J. Branch, C. P.E. Naylor, and G. T. MacFarlane. 1987. “Short Chain Fatty Acids in Human Large Intestine, Portal, Hepatic and Venous Blood.” *Gut* 28 (10): 1221–27. https://doi.org/10.1136/GUT.28.10.1221.

Czarnecka, Anna M., Kamil Synoradzki, Wiktoria Firlej, Ewa Bartnik, Pawel Sobczuk,

Michal Fiedorowicz, Pawel Grieb, and Piotr Rutkowski. 2020. "Molecular Biology of Osteosarcoma." *Cancers* 12 (8): 2130. https://doi.org/10.3390/cancers12082130.

Czesnikiewicz-Guzik, Marta, and Dominik N. Müller. 2018. "Scientists on the Spot: Salt, the Microbiome, and Cardiovascular Diseases." *Cardiovascular Research*. Oxford University Press. https://doi.org/10.1093/cvr/cvy171.

D'Angelo, Sandra P., Michelle R. Mahoney, Brian A. Van Tine, James Atkins, Mohammed M. Milhem, Balkrishna N. Jahagirdar, Cristina R. Antonescu, et al. 2018. "Nivolumab with or without Ipilimumab Treatment for Metastatic Sarcoma (Alliance A091401): Two Open-Label, Non-Comparative, Randomised, Phase 2 Trials." *The Lancet Oncology* 19 (3): 416–26. https://doi.org/10.1016/S1470-2045(18)30006-8.

Daniels-Wells, Tracy R., Gustavo Helguera, Richard K. Leuchter, Rafaela Quintero, Maggie Kozman, José A. Rodríguez, Elizabeth Ortiz-Sánchez, et al. 2013. "A Novel IgE Antibody Targeting the Prostate-Specific Antigen as a Potential Prostate Cancer Therapy." *BMC Cancer* 13 (1): 1–13. https://doi.org/10.1186/1471-2407-13-195/TABLES/3.

Dar, Asif A., Trupti N. Pradhan, Dakshayni P. Kulkarni, Sagar U. Shah, Kanury V. Rao, Devendra A. Chaukar, Anil K. D'Cruz, and Shubhada V. Chiplunkar. 2016. "Extracellular 2′5′-Oligoadenylate Synthetase 2 Mediates T-Cell Receptor CD3-ζ Chain down-Regulation via Caspase-3 Activation in Oral Cancer." *Immunology* 147 (2): 251–64. https://doi.org/10.1111/IMM.12560.

Davar, Diwakar, Amiran K. Dzutsev, John A. McCulloch, Richard R. Rodrigues, Joe-Marc Chauvin, Robert M. Morrison, Richelle N. Deblasio, et al. 2021a. "Fecal Microbiota Transplant Overcomes Resistance to Anti–PD-1 Therapy in Melanoma Patients." *Science* 371 (6529): 595–602. https://doi.org/10.1126/SCIENCE.ABF3363.

Davar, Diwakar, Amiran K. Dzutsev, John A. McCulloch, Richard R. Rodrigues, Joe Marc Chauvin, Robert M. Morrison, Richelle N. Deblasio, et al. 2021b. "Fecal Microbiota Transplant Overcomes Resistance to Anti-PD-1 Therapy in Melanoma Patients." *Science* 371 (6529): 595–602. https://doi.org/10.1126/SCIENCE.ABF3363/SUPPL_FILE/ABF3363_MDAR_REPRODUCIBILITY_CHECKLIST.PDF.

David, Lawrence A., Corinne F. Maurice, Rachel N. Carmody, David B. Gootenberg, Julie E. Button, Benjamin E. Wolfe, Alisha V. Ling, et al. 2013. "Diet Rapidly and Reproducibly Alters the Human Gut Microbiome." *Nature 2013 505:7484* 505 (7484): 559–63. https://doi.org/10.1038/nature12820.

Davis, Zachary B., Daniel A. Vallera, Jeffrey S. Miller, and Martin Felices. 2017.

"Natural Killer Cells Unleashed: Checkpoint Receptor Blockade and BiKE/TriKE Utilization in NK-Mediated Anti-Tumor Immunotherapy." *Seminars in Immunology* 31 (June): 64–75. https://doi.org/10.1016/J.SMIM.2017.07.011.

Dhodapkar, Madhav V., Matthew D. Geller, David H. Chang, Kanako Shimizu, Shin Ichiro Fujii, Kavita M. Dhodapkar, and Joseph Krasovsky. 2003. "A Reversible Defect in Natural Killer T Cell Function Characterizes the Progression of Premalignant to Malignant Multiple Myeloma." *The Journal of Experimental Medicine* 197 (12): 1667–76. https://doi.org/10.1084/JEM.20021650.

Dijkgraaf, Feline E., Lianne Kok, and Ton N.M. Schumacher. 2021. "Formation of Tissue-Resident CD8+ T-Cell Memory." *Cold Spring Harbor Perspectives in Biology* 13 (8): a038117. https://doi.org/10.1101/CSHPERSPECT.A038117.

DiPalma, Michelle P., and Joseph N. Blattman. 2023. "The Impact of Microbiome Dysbiosis on T Cell Function within the Tumor Microenvironment (TME)." *Frontiers in Cell and Developmental Biology* 11 (March): 277. https://doi.org/10.3389/FCELL.2023.1141215/BIBTEX.

Du, Xinhui, Hua Wei, Boya Zhang, Bangmin Wang, Zhehuang Li, Lon Kai Pang, Ruiying Zhao, and Weitao Yao. 2023. "Molecular Mechanisms of Osteosarcoma Metastasis and Possible Treatment Opportunities." *Frontiers in Oncology* 13 (May): 1833. https://doi.org/10.3389/FONC.2023.1117867.

Duan, Jinyou, Hachung Chung, Erin Troy, and Dennis L. Kasper. 2010. "Microbial Colonization Drives Expansion of IL-1 Receptor 1-Expressing and IL-17-Producing γ/δ T Cells." *Cell Host and Microbe* 7 (2): 140–50. https://doi.org/10.1016/j.chom.2010.01.005.

Ehrlich, P. 1891. "Experimentelle Untersuchungen Über Immunität: I. Über Ricin Und Abrin." *Zeitschrift Für Hygiene Und Infektionskrankheiten* 10 (4–5): 424–47.

Eskildsen, Signe, Just Justesen, Mikkel Heide Schierup, and Rune Hartmann. 2003. "Characterization of the 2'-5'-Oligoadenylate Synthetase Ubiquitin-like Family." *Nucleic Acids Research* 31 (12): 3166–73. https://doi.org/10.1093/NAR/GKG427.

Eyre, Rachel, Richard G. Feltbower, Peter W. James, Karen Blakey, Emmanuel Mubwandarikwa, David Forman, Patricia A. McKinney, Mark S. Pearce, and Richard J.Q. McNally. 2010. "The Epidemiology of Bone Cancer in 0 - 39 Year Olds in Northern England, 1981 - 2002." *BMC Cancer* 10 (1): 1–7. https://doi.org/10.1186/1471-2407-10-357/FIGURES/2.

Fairfax, Benjamin P., Chelsea A. Taylor, Robert A. Watson, Isar Nassiri, Sara Danielli, Hai Fang, Elise A. Mahé, et al. 2020. "Peripheral CD8+ T Cell Characteristics Associated with Durable Responses to Immune Checkpoint Blockade in Patients

with Metastatic Melanoma." *Nature Medicine 2020 26:2* 26 (2): 193–99. https://doi.org/10.1038/s41591-019-0734-6.

Fearon, D. T., and R. H. Carter. 1999. "The CD19/CR2/TAPA-1 Complex of B Lymphocytes: Linking Natural to Acquired Immunity." *Annual Review of Immunology* 13: 127–49.

Felices, Martin, Todd R. Lenvik, Zachary B. Davis, Jeffrey S. Miller, and Daniel A. Vallera. 2016. "Generation of BiKEs and TriKEs to Improve NK Cell-Mediated Targeting of Tumor Cells." *Methods in Molecular Biology (Clifton, N.J.)* 1441: 333–46. https://doi.org/10.1007/978-1-4939-3684-7_28.

Ferrantini, Maria, Imerio Capone, and Filippo Belardelli. 2008. "Dendritic Cells and Cytokines in Immune Rejection of Cancer." *Cytokine & Growth Factor Reviews* 19 (1): 93–107. https://doi.org/10.1016/J.CYTOGFR.2007.10.003.

Ferrere, Gladys, Maryam Tidjani Alou, Peng Liu, Anne Gaëlle Goubet, Marine Fidelle, Oliver Kepp, Sylvère Durand, et al. 2021. "Ketogenic Diet and Ketone Bodies Enhance the Anticancer Effects of PD-1 Blockade." *JCI Insight* 6 (2). https://doi.org/10.1172/JCI.INSIGHT.145207.

Ferreri, Andrés J.M., Caterina Cecchetti, Barbara Kiesewetter, Marianna Sassone, Teresa Calimeri, Salvatore Perrone, Maurilio Ponzoni, and Markus Raderer. 2018. "Clarithromycin as a 'Repurposing Drug' against MALT Lymphoma." *British Journal of Haematology* 182 (6): 913–15. https://doi.org/10.1111/BJH.14878.

Figueroa-González, Gabriela, and Carlos Pérez-Plasencia. 2017. "Strategies for the Evaluation of DNA Damage and Repair Mechanisms in Cancer." *Oncology Letters* 13 (6): 3982–88. https://doi.org/10.3892/OL.2017.6002/HTML.

Finlay, David K., Ella Rosenzweig, Linda V. Sinclair, Feijoo Carnero Carmen, Jens L. Hukelmann, Julia Rolf, Andrey A. Panteleyev, Klaus Okkenhaug, and Doreen A. Cantrell. 2012. "PDK1 Regulation of MTOR and Hypoxia-Inducible Factor 1 Integrate Metabolism and Migration of CD8+ T Cells." *Journal of Experimental Medicine* 209 (13): 2441–53. https://doi.org/10.1084/JEM.20112607.

Freilich, Shiri, Anat Kreimer, Elhanan Borenstein, Nir Yosef, Roded Sharan, Uri Gophna, and Eytan Ruppin. 2009. "Metabolic-Network-Driven Analysis of Bacterial Ecological Strategies." *Genome Biology* 10 (6): 1–8. https://doi.org/10.1186/GB-2009-10-6-R61/FIGURES/2.

Friswell, Melissa K., Helen Gika, Ian J. Stratford, Georgios Theodoridis, Brian Telfer, Ian D. Wilson, and Andrew J. McBain. 2010. "Site and Strain-Specific Variation in Gut Microbiota Profiles and Metabolism in Experimental Mice." *PLoS ONE* 5 (1). https://doi.org/10.1371/journal.pone.0008584.

Frith, Martin C., Neil F.W. Saunders, Bostjan Kobe, and Timothy L. Bailey. 2008. "Discovering Sequence Motifs with Arbitrary Insertions and Deletions." *PLOS Computational Biology* 4 (5): e1000071. https://doi.org/10.1371/JOURNAL.PCBI.1000071.

Furth, R. van, Z. A. Cohn, J. G. Hirsch, J. H. Humphrey, W. G. Spector, and H. L. Langevoort. 1972. "The Mononuclear Phagocyte System: A New Classification of Macrophages, Monocytes, and Their Precursor Cells." *Bulletin of the World Health Organization* 46 (6): 845. /pmc/articles/PMC2480884/?report=abstract.

Furusawa, Yukihiro, Yuuki Obata, Shinji Fukuda, Takaho A. Endo, Gaku Nakato, Daisuke Takahashi, Yumiko Nakanishi, et al. 2013. "Commensal Microbe-Derived Butyrate Induces the Differentiation of Colonic Regulatory T Cells." *Nature 2013 504:7480* 504 (7480): 446–50. https://doi.org/10.1038/nature12721.

Gal-Ben-Ari, Shunit, Iliana Barrera, Marcelo Ehrlich, and Kobi Rosenblum. 2019. "PKR: A Kinase to Remember." *Frontiers in Molecular Neuroscience* 11 (January): 480. https://doi.org/10.3389/FNMOL.2018.00480/BIBTEX.

Gao, Qiang, Xiao Ying Wang, Shuang Jian Qiu, Ichiro Yamato, Masayuki Sho, Yoshiyuki Nakajima, Jian Zhou, et al. 2009. "Overexpression of PD-L1 Significantly Associates with Tumor Aggressiveness and Postoperative Recurrence in Human Hepatocellular Carcinoma." *Clinical Cancer Research* 15 (3): 971–79. https://doi.org/10.1158/1078-0432.CCR-08-1608.

Gao, Yunfei, Wancai Yang, Meng Pan, Eileen Scully, Michael Girardi, Leonard H. Augenlicht, Joe Craft, and Zhinan Yin. 2003. "Γδ T Cells Provide an Early Source of Interferon γ in Tumor Immunity." *Journal of Experimental Medicine* 198 (3): 433–42. https://doi.org/10.1084/JEM.20030584.

García, M. A., E. F. Meurs, and M. Esteban. 2007. "The DsRNA Protein Kinase PKR: Virus and Cell Control." *Biochimie* 89 (6–7): 799–811. https://doi.org/10.1016/J.BIOCHI.2007.03.001.

Gerull, William D., Varun Puri, and Benjamin D. Kozower. 2021. "The Epidemiology and Biology of Pulmonary Metastases." *Journal of Thoracic Disease* 13 (4): 2585. https://doi.org/10.21037/JTD.2020.04.28.

Ginhoux, Florent, and Steffen Jung. 2014. "Monocytes and Macrophages: Developmental Pathways and Tissue Homeostasis." *Nature Reviews Immunology 2014 14:6* 14 (6): 392–404. https://doi.org/10.1038/nri3671.

Gong, Jun, Alexander Chehrazi-Raffle, Veronica Placencio-Hickok, Michelle Guan, Andrew Hendifar, and Ravi Salgia. 2019. "The Gut Microbiome and Response to Immune Checkpoint Inhibitors: Preclinical and Clinical Strategies." *Clinical and*

Translational Medicine 2019 8:1 8 (1): 1–14. https://doi.org/10.1186/S40169-019-0225-X.

Gonzalez, Santiago F., Søren E. Degn, Lisa A. Pitcher, Matthew Woodruff, Balthasar A. Heesters, and Michael C. Carroll. 2011. "Trafficking of B Cell Antigen in Lymph Nodes." *Annual Review of Immunology* 29 (1): 215–33. https://doi.org/10.1146/annurev-immunol-031210-101255.

Gopalakrishnan, V., C. N. Spencer, L. Nezi, A. Reuben, M. C. Andrews, T. V. Karpinets, P. A. Prieto, et al. 2018. "Gut Microbiome Modulates Response to Anti-PD-1 Immunotherapy in Melanoma Patients." *Science* 359 (6371): 97–103. https://doi.org/10.1126/science.aan4236.

Greathouse, K. Leigh, James R. White, Ashely J. Vargas, Valery V. Bliskovsky, Jessica A. Beck, Natalia von Muhlinen, Eric C. Polley, et al. 2018. "Interaction between the Microbiome and TP53 in Human Lung Cancer." *Genome Biology* 19 (1): 1–16. https://doi.org/10.1186/S13059-018-1501-6/FIGURES/4.

Groh, Veronika, Anja Brühl, Hani El-Gabalawy, J. Lee Nelson, and Thomas Spies. 2003. "Stimulation of T Cell Autoreactivity by Anomalous Expression of NKG2D and Its MIC Ligands in Rheumatoid Arthritis." *Proceedings of the National Academy of Sciences* 100 (16): 9452–57. https://doi.org/10.1073/PNAS.1632807100.

Grundy, Isobel. 2000. "Montagu's Variolation." *Endeavour* 24 (1): 4–7. https://doi.org/10.1016/S0160-9327(99)01244-2.

Gubin, Matthew M., Maxim N. Artyomov, Elaine R. Mardis, and Robert D. Schreiber. 2015. "Tumor Neoantigens: Building a Framework for Personalized Cancer Immunotherapy." *The Journal of Clinical Investigation* 125 (9): 3413–21. https://doi.org/10.1172/JCI80008.

Gutzmer, Ralf, Margarete Lisewski, Jörg Zwirner, Susanne Mommert, Carola Diesel, Miriam Wittmann, Alexander Kapp, and Thomas Werfel. 2004. "Human Monocyte-Derived Dendritic Cells Are Chemoattracted to C3a after up-Regulation of the C3a Receptor with Interferons." *Immunology* 111 (4): 435–43. https://doi.org/10.1111/J.1365-2567.2004.01829.X.

Haabeth, Ole Audun Werner, Anders Aune Tveita, Marte Fauskanger, Fredrik Schjesvold, Kristina Berg Lorvik, Peter O. Hofgaard, Hilde Omholt, et al. 2014. "How Do CD4+ T Cells Detect and Eliminate Tumor Cells That Either Lack or Express MHC Class II Molecules?" *Frontiers in Immunology* 5 (APR): 174. https://doi.org/10.3389/FIMMU.2014.00174/BIBTEX.

Haan, Joke M.M. den, Ramon Arens, and Menno C. van Zelm. 2014. "The Activation of the Adaptive Immune System: Cross-Talk between Antigen-Presenting Cells, T

Cells and B Cells." *Immunology Letters* 162 (2): 103–12. https://doi.org/10.1016/J.IMLET.2014.10.011.

Hage, Racha El, Emma Hernandez-Sanabria, Marta Calatayud Arroyo, Ruben Props, and Tom Van De Wiele. 2019. "Propionate-Producing Consortium Restores Antibiotic-Induced Dysbiosis in a Dynamic in Vitro Model of the Human Intestinal Microbial Ecosystem." *Frontiers in Microbiology* 10 (MAY): 1206. https://doi.org/10.3389/FMICB.2019.01206/BIBTEX.

Hall, Roy A., Jacqueline H. Scherret, and John S. Mackenzie. 2001. "Kunjin Virus." *Annals of the New York Academy of Sciences* 951 (1): 153–60. https://doi.org/10.1111/J.1749-6632.2001.TB02693.X.

Halstead, S. B. n.d. "The Flaviviruses: Pathogenesis and Immunity - Google Books." Advances In Virus Research. Accessed June 22, 2018. https://books.google.com/books?hl=en&lr=&id=TPB2zVh6MzUC&oi=fnd&pg=PA421&dq=macrophage+and+antibody+dependenet+enhancement&ots=67SbDVAUyS&sig=g_6jV0I9QtdKCCn37dsCj6acr_s#v=onepage&q=macrophage and antibody dependenet enhancement&f=false.

Hammes, W. P., and F. C. Neuhaus. 1974. "On the Mechanism of Action of Vancomycin: Inhibition of Peptidoglycan Synthesis in Gaffkya Homari." *Antimicrobial Agents and Chemotherapy* 6 (6): 722–28. https://doi.org/10.1128/AAC.6.6.722.

Han, Hyojeong, Hannah Yan, and Katherine Y. King. 2021. "Broad-Spectrum Antibiotics Deplete Bone Marrow Regulatory T Cells." *Cells* 10 (2): 1–9. https://doi.org/10.3390/CELLS10020277.

Han, Jichang, Nikhil Khatwani, Tyler G. Searles, Mary Jo Turk, and Christina V. Angeles. 2020. "Memory CD8+ T Cell Responses to Cancer." *Seminars in Immunology* 49 (June): 101435. https://doi.org/10.1016/J.SMIM.2020.101435.

Hansen, Ulla Kring, Sofie Ramskov, Anne Mette Bjerregaard, Annie Borch, Rikke Andersen, Arianna Draghi, Marco Donia, et al. 2020. "Tumor-Infiltrating T Cells From Clear Cell Renal Cell Carcinoma Patients Recognize Neoepitopes Derived From Point and Frameshift Mutations." *Frontiers in Immunology* 11 (March): 373. https://doi.org/10.3389/FIMMU.2020.00373/BIBTEX.

Hao, Ning Bo, Mu Han Lü, Ya Han Fan, Ya Ling Cao, Zhi Ren Zhang, and Shi Ming Yang. 2012. "Macrophages in Tumor Microenvironments and the Progression of Tumors." *Clinical and Developmental Immunology* 2012. https://doi.org/10.1155/2012/948098.

Hayday, Adrian C., Domingo F. Barber, Nataki Douglas, and Eric S. Hoffman. 1999.

"Signals Involved in Gamma/Delta T Cell versus Alpha/Beta T Cell Lineage Commitment." *Seminars in Immunology* 11 (4): 239–49. https://doi.org/10.1006/SMIM.1999.0180.

Helmink, Beth A., M. A.Wadud Khan, Amanda Hermann, Vancheswaran Gopalakrishnan, and Jennifer A. Wargo. 2019. "The Microbiome, Cancer, and Cancer Therapy." *Nature Medicine*. Nature Publishing Group. https://doi.org/10.1038/s41591-019-0377-7.

Holechek, Susan A., Megan S. McAfee, Lizbeth M. Nieves, Vanessa P. Guzman, Kavita Manhas, Timothy Fouts, Kenneth Bagley, and Joseph N. Blattman. 2016. "Retinaldehyde Dehydrogenase 2 as a Molecular Adjuvant for Enhancement of Mucosal Immunity during DNA Vaccination." *Vaccine* 34 (46): 5629–35. https://doi.org/10.1016/j.vaccine.2016.09.013.

Holm, Christian K., Stine H. Rahbek, Hans Henrik Gad, Rasmus O. Bak, Martin R. Jakobsen, Zhaozaho Jiang, Anne Louise Hansen, et al. 2016. "Influenza A Virus Targets a CGAS-Independent STING Pathway That Controls Enveloped RNA Viruses." *Nature Communications 2016 7:1* 7 (1): 1–9. https://doi.org/10.1038/ncomms10680.

Holmes, Autumn C., Katherine Basore, Daved H. Fremont, and Michael S. Diamond. 2020. "A Molecular Understanding of Alphavirus Entry." *PLOS Pathogens* 16 (10): e1008876. https://doi.org/10.1371/JOURNAL.PPAT.1008876.

Holodick, Nichol E., Nely Rodríguez-Zhurbenko, and Ana María Hernández. 2017. "Defining Natural Antibodies." *Frontiers in Immunology* 8 (JUL): 872. https://doi.org/10.3389/fimmu.2017.00872.

"Home | American Cancer Society - Cancer Facts & Statistics." n.d. Accessed November 30, 2021. https://cancerstatisticscenter.cancer.org/?_ga=2.265714191.1238880037.1638292522-28619497.1638292520#!/.

Hong, Minsun, Sung il Yoon, and Ian A. Wilson. 2012. "Structure and Functional Characterization of the RNA-Binding Element of the NLRX1 Innate Immune Modulator." *Immunity* 36 (3): 337–47. https://doi.org/10.1016/J.IMMUNI.2011.12.018.

Hovanessian, Ara G. 2007. "On the Discovery of Interferon-Inducible, Double-Stranded RNA Activated Enzymes: The 2′–5′oligoadenylate Synthetases and the Protein Kinase PKR." *Cytokine & Growth Factor Reviews* 18 (5–6): 351–61. https://doi.org/10.1016/J.CYTOGFR.2007.06.003.

Hovanessian, Ara G., Ronald E. Brown, and Ian M. Kerr. 1977. "Synthesis of Low

Molecular Weight Inhibitor of Protein Synthesis with Enzyme from Interferon-Treated Cells." *Nature* 268 (5620): 537–40. https://doi.org/10.1038/268537A0.

Hu, Yongxian, Jingjing Li, Fang Ni, Zhongli Yang, Xiaohua Gui, Zhiwei Bao, Houli Zhao, et al. 2022. "CAR-T Cell Therapy-Related Cytokine Release Syndrome and Therapeutic Response Is Modulated by the Gut Microbiome in Hematologic Malignancies." *Nature Communications 2022 13:1* 13 (1): 1–14. https://doi.org/10.1038/s41467-022-32960-3.

Huang, Xuan Zhang, Peng Gao, Yong Xi Song, Yan Xu, Jing Xu Sun, Xiao Wan Chen, Jun Hua Zhao, and Zhen Ning Wang. 2019. "Antibiotic Use and the Efficacy of Immune Checkpoint Inhibitors in Cancer Patients: A Pooled Analysis of 2740 Cancer Patients." *OncoImmunology* 8 (12). https://doi.org/10.1080/2162402X.2019.1665973.

Hurwitz, Arthur A., and Stephanie K. Watkins. 2012. "Immune Suppression in the Tumor Microenvironment: A Role for Dendritic Cell-Mediated Tolerization of T Cells." *Cancer Immunology, Immunotherapy : CII* 61 (2): 289–93. https://doi.org/10.1007/S00262-011-1181-5.

Iida, Noriho, Amiran Dzutsev, C. Andrew Stewart, Loretta Smith, Nicolas Bouladoux, Rebecca A. Weingarten, Daniel A. Molina, et al. 2013. "Commensal Bacteria Control Cancer Response to Therapy by Modulating the Tumor Microenvironment." *Science* 342 (6161): 967–70. https://doi.org/10.1126/science.1240527.

Iida, Noriho, Amiran Dzutsev, C Andrew Stewart, Loretta Smith, Nicolas Bouladoux, Rebecca A Weingarten, Daniel A Molina, et al. n.d. "Commensal Bacteria Control Cancer Response to Therapy by Modulating the Tumor Microenvironment HHS Public Access." Accessed January 24, 2021. https://doi.org/10.1126/science.1240527.

Iwasaki, Akiko, and Ruslan Medzhitov. 2010. "Regulation of Adaptive Immunity by the Innate Immune System." *Science* 327 (5963): 291–95. https://doi.org/10.1126/SCIENCE.1183021/ASSET/3BBC1CA0-DF74-408C-A69B-B48257FD931F/ASSETS/GRAPHIC/327_291_F4.JPEG.

Jacob, Joshy, Garnett Kelsoe, Klaus Rajewsky, and Ursula Weiss. 1991. "Intraclonal Generation of Antibody Mutants in Germinal Centres." *Nature 1991 354:6352* 354 (6352): 389–92. https://doi.org/10.1038/354389a0.

Jacobs, Sarah R, and Blossom Damania. 2012. "NLRs, Inflammasomes, and Viral Infection." *Journal of Leukocyte Biology* 92 (3): 469–77. https://doi.org/10.1189/JLB.0312132.

Janeway, Charles A., and Ruslan Medzhitov. 2003. "Innate Immune Recognition."

Https://Doi.Org/10.1146/Annurev.Immunol.20.083001.084359 20 (November): 197–216. https://doi.org/10.1146/ANNUREV.IMMUNOL.20.083001.084359.

Janssen, Edith M., Edward E. Lemmens, Tom Wolfe, Urs Christen, Matthias G. Von Herrath, and Stephen P. Schoenberger. 2003. "CD4+ T Cells Are Required for Secondary Expansion and Memory in CD8+ T Lymphocytes." *Nature 2003 421:6925* 421 (6925): 852–56. https://doi.org/10.1038/nature01441.

Jenner, Edward. 1798. "An Inquiry into the Causes and Effects of the Variolae Vaccinae." *London: Sampson Low*. https://www.gutenberg.org/ebooks/29414.

Jiafeng Zhang. 2002. "Disease and Its Impact on Politics, Diplomacy, and the Military: The Case of Smallpox and the Manchus (1613-1795)." *Journal of the History of Medicine and Allied Sciences* 57 (2): 177–97.

Jorgovanovic, Dragica, Mengjia Song, Liping Wang, and Yi Zhang. 2020. "Roles of IFN-γ in Tumor Progression and Regression: A Review." *Biomarker Research 2020 8:1* 8 (1): 1–16. https://doi.org/10.1186/S40364-020-00228-X.

José, P J, M J Forrest, and T J Williams. 1981. "Human C5a Des Arg Increases Vascular Permeability." *The Journal of Immunology* 127 (6): 2376–80. https://doi.org/10.4049/JIMMUNOL.127.6.2376.

Kamp, Frits, and James A. Hamilton. 2006. "How Fatty Acids of Different Chain Length Enter and Leave Cells by Free Diffusion." *Prostaglandins, Leukotrienes and Essential Fatty Acids* 75 (3): 149–59. https://doi.org/10.1016/J.PLEFA.2006.05.003.

Katz, Jessica B., Alexander J. Muller, and George C. Prendergast. 2008. "Indoleamine 2,3-Dioxygenase in T-Cell Tolerance and Tumoral Immune Escape." *Immunological Reviews* 222 (1): 206–21. https://doi.org/10.1111/J.1600-065X.2008.00610.X.

Kespohl, Meike, Niyati Vachharajani, Maik Luu, Hani Harb, Sabine Pautz, Svenja Wolff, Nina Sillner, et al. 2017. "The Microbial Metabolite Butyrate Induces Expression of Th1- Associated Factors in CD4+ T Cells." *Frontiers in Immunology* 8 (AUG): 1036. https://doi.org/10.3389/FIMMU.2017.01036/BIBTEX.

Khan, Uqba, Kaylee Ho, Eun Kyeong Hwang, Cristian Peña, Julianna Brouwer, Katherine Hoffman, Doron Betel, et al. 2021. "Impact of Use of Antibiotics on Response to Immune Checkpoint Inhibitors and Tumor Microenvironment." *American Journal of Clinical Oncology: Cancer Clinical Trials* 44 (6): 247–53. https://doi.org/10.1097/COC.0000000000000813.

Khandelia, Piyush, Karen Yap, and Eugene V. Makeyev. 2011. "Streamlined Platform for Short Hairpin RNA Interference and Transgenesis in Cultured Mammalian Cells." *Proceedings of the National Academy of Sciences of the United States of*

America 108 (31): 12799–804. https://doi.org/10.1073/PNAS.1103532108/SUPPL_FILE/PNAS.201103532SI.PDF.

Kim, Chang H. 2021. "Control of Lymphocyte Functions by Gut Microbiota-Derived Short-Chain Fatty Acids." *Cellular & Molecular Immunology 2021 18:5* 18 (5): 1161–71. https://doi.org/10.1038/s41423-020-00625-0.

Kim, Chang H, and Judy Betz. 2018. "Immune Regulation by Microbiome Metabolites." *Immunology* 154 (2): 220–29. https://doi.org/10.1111/IMM.12930.

Kim, Donghyun, Melody Y. Zeng, and Gabriel Núñez. 2017. "The Interplay between Host Immune Cells and Gut Microbiota in Chronic Inflammatory Diseases." *Experimental and Molecular Medicine*. Nature Publishing Group. https://doi.org/10.1038/emm.2017.24.

Kim, Esther, Hyeok Ahn, and Hansoo Park. 2021. "A Review on the Role of Gut Microbiota in Immune Checkpoint Blockade Therapy for Cancer." *Mammalian Genome* 32 (4): 223–31. https://doi.org/10.1007/S00335-021-09867-3/TABLES/1.

Kim, Young Keun, Jeon Soo Shin, and Moon H. Nahm. 2016. "NOD-Like Receptors in Infection, Immunity, and Diseases." *Yonsei Medical Journal* 57 (1): 5. https://doi.org/10.3349/YMJ.2016.57.1.5.

Kobayashi, Koichi, Naohiro Inohara, Lorraine D. Hernandez, Jorge E. Galán, Gabriel Nüñez, Charles A. Janeway, Ruslan Medzhitov, and Richard A. Flavell. 2002. "RICK/Rip2/CARDIAK Mediates Signalling for Receptors of the Innate and Adaptive Immune Systems." *Nature 2002 416:6877* 416 (6877): 194–99. https://doi.org/10.1038/416194a.

Kobayashi, Koichi S., and Peter J. Van Den Elsen. 2012. "NLRC5: A Key Regulator of MHC Class I-Dependent Immune Responses." *Nature Reviews Immunology 2012 12:12* 12 (12): 813–20. https://doi.org/10.1038/nri3339.

Koch, Robert,. 1876. "Die Ätiologie Der Milzbrand-Krankheit,Begründet Auf Die Entwicklungsgeschichte Des Bacillus Anthracis." *Cohns Beiträge Zur Biologie Der Pflanzen* 2: 277–310. http://hdl.handle.net/2027/coo.31924011948639.

Kopenhaver, Jessica, Robert D. Carlson, and Adam E. Snook. 2020. "Mobilizing Toxins for Cancer Treatment: Historical Perspectives and Current Strategies." *Toxins 2020, Vol. 12, Page 416* 12 (6): 416. https://doi.org/10.3390/TOXINS12060416.

Korn, Thomas, Estelle Bettelli, Mohamed Oukka, and Vijay K. Kuchroo. 2009. "IL-17 and Th17 Cells." *Https://Doi.Org/10.1146/Annurev.Immunol.021908.132710* 27 (March): 485–517. https://doi.org/10.1146/ANNUREV.IMMUNOL.021908.132710.

Koyama, Shohei, Ken J. Ishii, Cevayir Coban, and Shizuo Akira. 2008. "Innate Immune Response to Viral Infection." *Cytokine* 43 (3): 336–41. https://doi.org/10.1016/J.CYTO.2008.07.009.

Kristiansen, Helle, Hans Henrik Gad, Signe Eskildsen-Larsen, Philippe Despres, and Rune Hartmann. 2011. "The Oligoadenylate Synthetase Family: An Ancient Protein Family with Multiple Antiviral Activities." *Journal of Interferon and Cytokine Research* 31 (1): 41–47.
https://doi.org/10.1089/JIR.2010.0107/ASSET/IMAGES/LARGE/FIGURE3.JPEG.

Lacy, Paige. 2006. "Mechanisms of Degranulation in Neutrophils." *Allergy, Asthma & Clinical Immunology* 2 (3): 1–11. https://doi.org/10.1186/1710-1492-2-3-98/FIGURES/2.

Larkin, James, Vanna Chiarion-Sileni, Rene Gonzalez, Jean Jacques Grob, C. Lance Cowey, Christopher D. Lao, Dirk Schadendorf, et al. 2015. "Combined Nivolumab and Ipilimumab or Monotherapy in Untreated Melanoma." *New England Journal of Medicine* 373 (1): 23–34. https://doi.org/10.1056/nejmoa1504030.

Laukens, Debby, Brigitta M Brinkman, Jeroen Raes, Martine De Vos, and Peter Vandenabeele. 2016. "Heterogeneity of the Gut Microbiome in Mice: Guidelines for Optimizing Experimental Design." *FEMS Microbiology Reviews* 036 (1): 117–32. https://doi.org/10.1093/femsre/fuv036.

Law, S. K., N. A. Lichtenberg, and R. P. Levine. 1980. "Covalent Binding and Hemolytic Activity of Complement Proteins." *Proceedings of the National Academy of Sciences* 77 (12): 7194–98. https://doi.org/10.1073/PNAS.77.12.7194.

Leoh, Lai Sum, Tracy R. Daniels-Wells, and Manuel L. Penichet. 2015. "Ige Immunotherapy against Cancer." *Current Topics in Microbiology and Immunology* 388: 109–49. https://doi.org/10.1007/978-3-319-13725-4_6/COVER.

Leun, Anne M. van der, Daniela S. Thommen, and Ton N. Schumacher. 2020. "CD8+ T Cell States in Human Cancer: Insights from Single-Cell Analysis." *Nature Reviews Cancer 2020 20:4* 20 (4): 218–32. https://doi.org/10.1038/s41568-019-0235-4.

Leung, Angela Ki Che. 2011. "'Variolation' and Vaccination in Late Imperial China, Ca 1570–1911." *History of Vaccine Development*, 5–12. https://doi.org/10.1007/978-1-4419-1339-5_2.

Leung, Jason Yat Sing, Mary Mah Lee Ng, and Justin Jang Hann Chu. 2011. "Replication of Alphaviruses: A Review on the Entry Process of Alphaviruses into Cells." *Advances in Virology* 2011. https://doi.org/10.1155/2011/249640.

Leung, K. S., K. P. Fung, A. H.L. Sher, C. K. Li, and K. M. Lee. 1993. "Plasma Bone-

Specific Alkaline Phosphatase as an Indicator of Osteoblastic Activity." *Journal of Bone and Joint Surgery - Series B* 75 (2): 288–92. https://doi.org/10.1302/0301-620X.75B2.8444951/LETTERTOEDITOR.

Li, Yize, Shuvojit Banerjee, Yuyan Wang, Stephen A. Goldstein, Beihua Dong, Christina Gaughan, Robert H. Silverman, and Susan R. Weiss. 2016. "Activation of RNase L Is Dependent on OAS3 Expression during Infection with Diverse Human Viruses." *Proceedings of the National Academy of Sciences of the United States of America* 113 (8): 2241–46. https://doi.org/10.1073/PNAS.1519657113/SUPPL_FILE/PNAS.201519657SI.PDF.

Liao, Zhiwei, and Jianguo Su. 2021. "Progresses on Three Pattern Recognition Receptor Families (TLRs, RLRs and NLRs) in Teleost." *Developmental & Comparative Immunology* 122 (September): 104131. https://doi.org/10.1016/J.DCI.2021.104131.

Lim, Jean K., Andrea Lisco, David H. McDermott, Linda Huynh, Jerrold M. Ward, Bernard Johnson, Hope Johnson, et al. 2009. "Genetic Variation in OAS1 Is a Risk Factor for Initial Infection with West Nile Virus in Man." *PLOS Pathogens* 5 (2): e1000321. https://doi.org/10.1371/JOURNAL.PPAT.1000321.

Lindahl, Lise M., Andreas Willerslev-Olsen, Lise M.R. Gjerdrum, Pia R. Nielsen, Edda Blümel, Anne H. Rittig, Pamela Celis, et al. 2019. "Antibiotics Inhibit Tumor and Disease Activity in Cutaneous T-Cell Lymphoma." *Blood* 134 (13): 1072–83. https://doi.org/10.1182/BLOOD.2018888107.

Lindsey, Brock A., Justin E. Markel, and Eugenie S. Kleinerman. 2017. "Osteosarcoma Overview." *Rheumatology and Therapy* 4 (1): 25–43. https://doi.org/10.1007/s40744-016-0050-2.

Liu, Chaohong, Nicole M. Chapman, Peer W. F. Karmaus, Hu Zeng, and Hongbo Chi. 2015. "MTOR and Metabolic Regulation of Conventional and Regulatory T Cells." *Journal of Leukocyte Biology* 97 (5): 837–47. https://doi.org/10.1189/JLB.2RI0814-408R.

Liu, Dongbo, Alessandra K. Cardozo, Martine I. Darville, and Décio L. Eizirik. 2002. "Double-Stranded RNA Cooperates with Interferon-γ and IL-1β to Induce Both Chemokine Expression and Nuclear Factor-KB-Dependent Apoptosis in Pancreatic β-Cells: Potential Mechanisms for Viral-Induced Insulitis and β-Cell Death in Type 1 Diabetes Mellitus." *Endocrinology* 143 (4): 1225–34. https://doi.org/10.1210/endo.143.4.8737.

Liu, Juan, Cheng Qian, and Xuetao Cao. 2016. "Post-Translational Modification Control of Innate Immunity." *Immunity* 45 (1): 15–30. https://doi.org/10.1016/J.IMMUNI.2016.06.020.

Liu, Shuzhen, Jonathan Lachapelle, Samuel Leung, Dongxia Gao, William D. Foulkes, and Torsten O. Nielsen. 2012. "CD8 +lymphocyte Infiltration Is an Independent Favorable Prognostic Indicator in Basal-like Breast Cancer." *Breast Cancer Research* 14 (2): R48. https://doi.org/10.1186/bcr3148.

Liu, Yuan, Beth Gordesky-Gold, Michael Leney-Greene, Nathan L. Weinbren, Matthew Tudor, and Sara Cherry. 2018. "Inflammation-Induced STING-Dependent Autophagy Restricts Zika Virus Infection in the Drosophila Brain." *Cell Host & Microbe* 24 (1): 57. https://doi.org/10.1016/J.CHOM.2018.05.022.

Loughlin, Kevin R. 2020. "William B. Coley: His Hypothesis, His Toxin, and the Birth of Immunotherapy." *Urologic Clinics of North America* 47 (4): 413–17. https://doi.org/10.1016/j.ucl.2020.07.001.

Louis, Petra, Georgina L. Hold, and Harry J. Flint. 2014. "The Gut Microbiota, Bacterial Metabolites and Colorectal Cancer." *Nature Reviews Microbiology 2014 12:10* 12 (10): 661–72. https://doi.org/10.1038/nrmicro3344.

Lundin, Annelie, Chek Mei Bok, Linda Aronsson, Britta Björkholm, Jan Åke Gustafsson, Sebastian Pott, Velmurugesan Arulampalam, Martin Hibberd, Joseph Rafter, and Sven Pettersson. 2008. "Gut Flora, Toll-like Receptors and Nuclear Receptors: A Tripartite Communication That Tunes Innate Immunity in Large Intestine." *Cellular Microbiology* 10 (5): 1093–1103. https://doi.org/10.1111/J.1462-5822.2007.01108.X.

Lundin, Katrin U., Valentina Screpanti, Hilde Omholt, Peter O. Hofgaard, Hideo Yagita, Alf Grandien, and Bjarne Bogen. 2004. "CD4+ T Cells Kill Id+ B-Lymphoma Cells: FasLigand-Fas Interaction Is Dominant in Vitro but Is Redundant in Vivo." *Cancer Immunology, Immunotherapy* 53 (12): 1135–45. https://doi.org/10.1007/S00262-004-0538-4/FIGURES/6.

Lussier, Danielle M., John L. Johnson, Pooja Hingorani, and Joseph N. Blattman. 2015. "Combination Immunotherapy with α-CTLA-4 and α-PD-L1 Antibody Blockade Prevents Immune Escape and Leads to Complete Control of Metastatic Osteosarcoma." *Journal for ImmunoTherapy of Cancer* 3 (1): 21. https://doi.org/10.1186/s40425-015-0067-z.

Lussier, Danielle M., Lauren O'Neill, Lizbeth M. Nieves, Megan S. McAfee, Susan A. Holechek, Andrea W. Collins, Paul Dickman, Jeffrey Jacobsen, Pooja Hingorani, and Joseph N. Blattman. 2015. "Enhanced T-Cell Immunity to Osteosarcoma through Antibody Blockade of PD-1/PD-L1 Interactions." *Journal of Immunotherapy* 38 (3): 96–106. https://doi.org/10.1097/CJI.0000000000000065.

Lussier, Danielle M., Eric C. Woolf, John L. Johnson, Kenneth S. Brooks, Joseph N. Blattman, and Adrienne C. Scheck. 2016. "Enhanced Immunity in a Mouse Model

of Malignant Glioma Is Mediated by a Therapeutic Ketogenic Diet.” *BMC Cancer* 16 (1): 1–10. https://doi.org/10.1186/s12885-016-2337-7.

Lussier, Danielle M, Lizbeth M Nieves, Megan S McAfee, Susan A Holechek, Andrea W Collins, Paul Dickman, Jeffrey Jacobsen, Pooja Hingorani, and Joseph N Blattman. 2015. “Enhanced T-Cell Immunity to Osteosarcoma Through Antibody Blockade of PD-1/PD-L1 Interactions HHS Public Access.” *J Immunother* 38 (3): 96–106. https://doi.org/10.1097/CJI.0000000000000065.

Luu, Maik, Sabine Pautz, Vanessa Kohl, Rajeev Singh, Rossana Romero, Sébastien Lucas, Jörg Hofmann, et al. 2019. “The Short-Chain Fatty Acid Pentanoate Suppresses Autoimmunity by Modulating the Metabolic-Epigenetic Crosstalk in Lymphocytes.” *Nature Communications 2019 10:1* 10 (1): 1–12. https://doi.org/10.1038/s41467-019-08711-2.

Luu, Maik, Zeno Riester, Adrian Baldrich, Nicole Reichardt, Samantha Yuille, Alessandro Busetti, Matthias Klein, et al. 2021. “Microbial Short-Chain Fatty Acids Modulate CD8+ T Cell Responses and Improve Adoptive Immunotherapy for Cancer.” *Nature Communications 2021 12:1* 12 (1): 1–12. https://doi.org/10.1038/s41467-021-24331-1.

M B Fischer, S Goerg M Ma, X Zhou, J Xia, O Finco, S Han, G Kelsoe, et al. 1996. “Regulation of the B Cell Response to T-Dependent Antigens by Classical Pathway Complement - PubMed.” *The Journal of Immunology* 157 (2): 549–56. https://pubmed.ncbi.nlm.nih.gov/8752901/.

Ma, Zhe, and Blossom Damania. 2016. “The CGAS-STING Defense Pathway and Its Counteraction by Viruses.” *Cell Host & Microbe* 19 (2): 150–58. https://doi.org/10.1016/J.CHOM.2016.01.010.

Macatonia, S E, N A Hosken, M Litton, P Vieira, C S Hsieh, J A Culpepper, M Wysocka, G Trinchieri, K M Murphy, and A O’Garra. 1995. “Dendritic Cells Produce IL-12 and Direct the Development of Th1 Cells from Naive CD4+ T Cells.” *The Journal of Immunology* 154 (10): 5071–79. https://doi.org/10.4049/JIMMUNOL.154.10.5071.

Mager, Lukas F., Regula Burkhard, Nicola Pett, Noah C.A. Cooke, Kirsty Brown, Hena Ramay, Seungil Paik, et al. 2020. “Microbiome-Derived Inosine Modulates Response to Checkpoint Inhibitor Immunotherapy.” *Science* 369 (6509): 1481–89. https://doi.org/10.1126/SCIENCE.ABC3421.

Malaguarnera, Lucia, Giuseppe Nunnari, and Michelino Di Rosa. 2016. “Nuclear Import Sequence Identification in HOAS3 Protein.” *Inflammation Research* 65 (11): 895–904. https://doi.org/10.1007/S00011-016-0972-8/FIGURES/6.

Manhas, Kavita R., Pamela A. Marshall, Carl E. Wagner, Peter W. Jurutka, Michelle V. Mancenido, Hannah Z. Debray, and Joseph N. Blattman. 2022. "Rexinoids Modulate Effector T Cell Expression of Mucosal Homing Markers CCR9 and A4β7 Integrin and Direct Their Migration In Vitro." *Frontiers in Immunology* 13 (January): 746484. https://doi.org/10.3389/FIMMU.2022.746484/BIBTEX.

Marques, Joao, Jangawar Anwar, Signe Eskildsen-Larsen, Dominique Rebouillat, Soren R. Paludan, Ganes Sen, Bryan R.G. Williams, and Rune Hartmann. 2008. "The P59 Oligoadenylate Synthetase-like Protein Possesses Antiviral Activity That Requires the C-Terminal Ubiquitin-like Domain." *Journal of General Virology* 89 (11): 2767–72. https://doi.org/10.1099/VIR.0.2008/003558-0.

Mashimo, Tomoji, Marianne Lucas, Dominique Simon-Chazottes, Marie Pascale Frenkiel, Xavier Montagutelli, Pierre Emmanuel Ceccaldi, Vincent Deubel, Jean Louis Guénet, and Philippe Desprès. 2002. "A Nonsense Mutation in the Gene Encoding 2′-5′-Oligoadenylate Synthetase/L1 Isoform Is Associated with West Nile Virus Susceptibility in Laboratory Mice." *Proceedings of the National Academy of Sciences* 99 (17): 11311–16. https://doi.org/10.1073/PNAS.172195399.

McCarthy, Edward F. 2006. "The Toxins of William B. Coley and the Treatment of Bone and Soft-Tissue Sarcomas." *The Iowa Orthopaedic Journal* 26: 154. /pmc/articles/PMC1888599/.

McCoy, Kathy D., Regula Burkhard, and Markus B. Geuking. 2019. "The Microbiome and Immune Memory Formation." *Immunology and Cell Biology* 97 (7): 625–35. https://doi.org/10.1111/IMCB.12273.

McCulloch, John A., Diwakar Davar, Richard R. Rodrigues, Jonathan H. Badger, Jennifer R. Fang, Alicia M. Cole, Ascharya K. Balaji, et al. 2022. "Intestinal Microbiota Signatures of Clinical Response and Immune-Related Adverse Events in Melanoma Patients Treated with Anti-PD-1." *Nature Medicine 2022 28:3* 28 (3): 545–56. https://doi.org/10.1038/s41591-022-01698-2.

Mehta, Amit K., Donald T. Gracias, and Michael Croft. 2018. "TNF Activity and T Cells." *Cytokine* 101 (January): 14–18. https://doi.org/10.1016/j.cyto.2016.08.003.

Meloen, R H, W C Puijk, and J W Slootstra. n.d. "Mimotopes: Realization of an Unlikely Concept." Accessed May 28, 2023. https://doi.org/10.1002/1099-1352.

Metchnikoff, E. 1882. "Über Die Intracelluläre Verdauung Bei Phagocyten." *Archiv Für Pathologische Anatomie Und Physiologie Und Für Klinische Medicin* 87 (2): 249–86. https://doi.org/10.3989/ASCLEPIO.2022.17.

Methé, Barbara A., Karen E. Nelson, Mihai Pop, Heather H. Creasy, Michelle G. Giglio, Curtis Huttenhower, Dirk Gevers, et al. 2012. "A Framework for Human

Microbiome Research." *Nature 2012 486:7402* 486 (7402): 215–21. https://doi.org/10.1038/nature11209.

Miller, J. F., and G. F. Mitchell. 1968. "Cell to Cell Interaction in the Immune Response I. Hemolysin-Forming Cells in Neonatally Thymectomized Mice Reconstituted With Thymus or Thoracic Duct Lymphocytes." *Journal of Experimental Medicine* 128 (4): 801–20. https://doi.org/10.1084/JEM.128.4.801.

Mitchison, N. A. 1971. "The Carrier Effect in the Secondary Response to Hapten-Protein Conjugates. I. Measurement of the Effect with Transferred Cells and Objections to the Local Environment Hypothesis." *European Journal of Immunology* 1 (1): 10–17. https://doi.org/10.1002/EJI.1830010103.

Moloney, Rachel D., Lieve Desbonnet, Gerard Clarke, Timothy G. Dinan, and John F. Cryan. 2014. "The Microbiome: Stress, Health and Disease." *Mammalian Genome*. Springer New York LLC. https://doi.org/10.1007/s00335-013-9488-5.

Mostashari, Farzad, Michel L. Bunning, Paul T. Kitsutani, Daniel A. Singer, Denis Nash, Michael J. Cooper, Naomi Katz, et al. 2001. "Epidemic West Nile Encephalitis, New York, 1999: Results of a Household-Based Seroepidemiological Survey." *The Lancet* 358 (9278): 261–64. https://doi.org/10.1016/S0140-6736(01)05480-0.

Motta, Vinicius, Fraser Soares, Tian Sun, and Dana J. Philpott. 2015. "Nod-like Receptors: Versatile Cytosolic Sentinels." *Physiological Reviews* 95 (1): 149–78. https://doi.org/10.1152/PHYSREV.00009.2014/ASSET/IMAGES/LARGE/Z9J0011527160005.JPEG.

Muktar, Yimer, Nateneal Tamerat, and Abnet Shewafera. 2016. "Aedes Aegypti as a Vector of Flavivirus." https://doi.org/10.4172/2329-891X.1000223.

Murphy, Kenneth, and Casey Weaver. 2016. *Janeway's Immunobiology*. Garland Science. https://doi.org/10.1201/9781315533247.

Nakanishi, Juro, Yoshihiro Wada, Koichiro Matsumoto, Miyuki Azuma, Ken Kikuchi, and Shoichi Ueda. 2006. "Overexpression of B7-H1 (PD-L1) Significantly Associates with Tumor Grade and Postoperative Prognosis in Human Urothelial Cancers." *Cancer Immunology, Immunotherapy : CII* 56 (8): 1173–82. https://doi.org/10.1007/S00262-006-0266-Z.

Narang, Pooja, Meixuan Chen, Amit A. Sharma, Karen S. Anderson, and Melissa A. Wilson. 2019. "The Neoepitope Landscape of Breast Cancer: Implications for Immunotherapy." *BMC Cancer* 19 (1): 1–10. https://doi.org/10.1186/S12885-019-5402-1/FIGURES/4.

Nath, Artika P., Asolina Braun, Scott C. Ritchie, Francis R. Carbone, Laura K. Mackay,

Thomas Gebhardt, and Michael Inouye. 2019. “Comparative Analysis Reveals a Role for TGF-β in Shaping the Residency-Related Transcriptional Signature in Tissue-Resident Memory CD8+ T Cells.” *PLOS ONE* 14 (2): e0210495. https://doi.org/10.1371/JOURNAL.PONE.0210495.

Nejman, Deborah, Ilana Livyatan, Garold Fuks, Nancy Gavert, Yaara Zwang, Leore T. Geller, Aviva Rotter-Maskowitz, et al. 2020. “The Human Tumor Microbiome Is Composed of Tumor Type-Specific Intracellular Bacteria.” *Science* 368 (6494): 973–80. https://doi.org/10.1126/SCIENCE.AAY9189/SUPPL_FILE/AAY9189_NEJMAN_SM.PDF.

Ni, Guoxin, Zhe Ma, and Blossom Damania. 2018. “CGAS and STING: At the Intersection of DNA and RNA Virus-Sensing Networks.” *PLoS Pathogens* 14 (8). https://doi.org/10.1371/JOURNAL.PPAT.1007148.

O’Garra, Anne. 1998. “Cytokines Induce the Development of Functionally Heterogeneous T Helper Cell Subsets.” *Immunity* 8 (3): 275–83. https://doi.org/10.1016/S1074-7613(00)80533-6.

Oates, John A., Alastair J.J. Wood, Gerald R. Donowitz, and Gerald L. Mandell. 2010. “Beta-Lactam Antibiotics.” *Http://Dx.Doi.Org/10.1056/NEJM198802183180706* 318 (7): 419–26. https://doi.org/10.1056/NEJM198802183180706.

Ossendorp, Ferry, Erica Mengedé, Marcel Camps, Rian Filius, and Cornelis J.M. Melief. 1998. “Specific T Helper Cell Requirement for Optimal Induction of Cytotoxic T Lymphocytes against Major Histocompatibility Complex Class II Negative Tumors.” *Journal of Experimental Medicine* 187 (5): 693–702. https://doi.org/10.1084/JEM.187.5.693.

Overacre-Delgoffe, Abigail E., and Timothy W. Hand. 2022. “Regulation of Tissue-Resident Memory T Cells by the Microbiota.” *Mucosal Immunology* 15 (3): 408–17. https://doi.org/10.1038/S41385-022-00491-1.

Page, D. L. 1953. “Thucydides’ Description of the Great Plague at Athens.” *The Classical Quarterly* 3 (3–4): 97–119. https://doi.org/10.1017/S0009838800003050.

Pardoll, Drew M., and Suzanne L. Topalian. 1998. “The Role of CD4+ T Cell Responses in Antitumor Immunity.” *Current Opinion in Immunology* 10 (5): 588–94. https://doi.org/10.1016/S0952-7915(98)80228-8.

Park, J., M. Kim, S. G. Kang, A. H. Jannasch, B. Cooper, J. Patterson, and C. H. Kim. 2014. “Short-Chain Fatty Acids Induce Both Effector and Regulatory T Cells by Suppression of Histone Deacetylases and Regulation of the MTOR–S6K Pathway.” *Mucosal Immunology 2015 8:1* 8 (1): 80–93. https://doi.org/10.1038/mi.2014.44.

Park, Young Jun, Da Sol Kuen, and Yeonseok Chung. 2018. "Future Prospects of Immune Checkpoint Blockade in Cancer: From Response Prediction to Overcoming Resistance." *Experimental and Molecular Medicine*. Nature Publishing Group. https://doi.org/10.1038/s12276-018-0130-1.

Pasteur, L. 1885. "Études Sur La Rage." *Journal de Microbiologie: Annales de l'Institut Pasteur* 19 (6): 425–588.

Paul, Sinu, Nathan P. Croft, Anthony W. Purcell, David C. Tscharke, Alessandro Sette, Morten Nielsen, and Bjoern Peters. 2020. "Benchmarking Predictions of MHC Class I Restricted T Cell Epitopes in a Comprehensively Studied Model System." *PLOS Computational Biology* 16 (5): e1007757. https://doi.org/10.1371/JOURNAL.PCBI.1007757.

Perry, Andrea K., Gang Chen, Dahai Zheng, Hong Tang, and Genhong Cheng. 2005. "The Host Type I Interferon Response to Viral and Bacterial Infections." *Cell Research* 15 (6): 407–22. https://doi.org/10.1038/SJ.CR.7290309.

Petersen, Charisse, and June L. Round. 2014. "Defining Dysbiosis and Its Influence on Host Immunity and Disease." *Cellular Microbiology* 16 (7): 1024–33. https://doi.org/10.1111/CMI.12308.

Petitprez, Florent, Aurélien de Reyniès, Emily Z. Keung, Tom Wei Wu Chen, Cheng Ming Sun, Julien Calderaro, Yung Ming Jeng, et al. 2020. "B Cells Are Associated with Survival and Immunotherapy Response in Sarcoma." *Nature* 577 (7791): 556–60. https://doi.org/10.1038/s41586-019-1906-8.

Pham, Tu Anh N., and Trevor D. Lawley. 2014. "Emerging Insights on Intestinal Dysbiosis during Bacterial Infections." *Current Opinion in Microbiology* 17 (1): 67–74. https://doi.org/10.1016/J.MIB.2013.12.002.

Pietrocola, Federico, and Guido Kroemer. 2019. "Caloric Restriction Promotes the Stemness and Antitumor Activity of T Lymphocytes." *Https://Doi.Org/10.1080/2162402X.2019.1616153* 8 (10). https://doi.org/10.1080/2162402X.2019.1616153.

Poore, Gregory D., Evguenia Kopylova, Qiyun Zhu, Carolina Carpenter, Serena Fraraccio, Stephen Wandro, Tomasz Kosciolek, et al. 2020. "Microbiome Analyses of Blood and Tissues Suggest Cancer Diagnostic Approach." *Nature 2020 579:7800* 579 (7800): 567–74. https://doi.org/10.1038/s41586-020-2095-1.

Postow, Michael A., Jason Chesney, Anna C. Pavlick, Caroline Robert, Kenneth Grossmann, David McDermott, Gerald P. Linette, et al. 2015. "Nivolumab and Ipilimumab versus Ipilimumab in Untreated Melanoma." *New England Journal of*

Medicine 372 (21): 2006–17. https://doi.org/10.1056/nejmoa1414428.

Qin, Junjie, Ruiqiang Li, Jeroen Raes, Manimozhiyan Arumugam, Kristoffer Solvsten Burgdorf, Chaysavanh Manichanh, Trine Nielsen, et al. 2010. "A Human Gut Microbial Gene Catalogue Established by Metagenomic Sequencing." *Nature 2010 464:7285* 464 (7285): 59–65. https://doi.org/10.1038/nature08821.

Raftery, Nicola, and Nigel J. Stevenson. 2017. "Advances in Anti-Viral Immune Defence: Revealing the Importance of the IFN JAK/STAT Pathway." *Cellular and Molecular Life Sciences* 74 (14): 2525–35. https://doi.org/10.1007/S00018-017-2520-2/FIGURES/3.

Rangan, Priya, and Anna Mondino. 2022. "Microbial Short-Chain Fatty Acids: A Strategy to Tune Adoptive T Cell Therapy." *Journal for Immunotherapy of Cancer* 10 (7): 4147. https://doi.org/10.1136/JITC-2021-004147.

Ranjan, Ravi, Asha Rani, Ahmed Metwally, Halvor S. McGee, and David L. Perkins. 2016. "Analysis of the Microbiome: Advantages of Whole Genome Shotgun versus 16S Amplicon Sequencing." *Biochemical and Biophysical Research Communications* 469 (4): 967–77. https://doi.org/10.1016/J.BBRC.2015.12.083.

Restrepo, Lucas, Phillip Stafford, and Stephen Albert Johnston. 2013. "Feasibility of an Early Alzheimer's Disease Immunosignature Diagnostic Test." *Journal of Neuroimmunology* 254 (1–2): 154–60. https://doi.org/10.1016/j.jneuroim.2012.09.014.

Rezasoltani, Sama, Abbas Yadegar, Hamid Asadzadeh Aghdaei, and Mohammad Reza Zali. 2021. "Modulatory Effects of Gut Microbiome in Cancer Immunotherapy: A Novel Paradigm for Blockade of Immune Checkpoint Inhibitors." *Cancer Medicine* 10 (3): 1141–54. https://doi.org/10.1002/CAM4.3694.

Riiser, Amund. 2015. "The Human Microbiome, Asthma, and Allergy." *Allergy, Asthma & Clinical Immunology 2015 11:1* 11 (1): 1–7. https://doi.org/10.1186/S13223-015-0102-0.

Riquelme, E., Yu Zhang, Liangliang Zhang, M. Montiel, Michelle Zoltan, W. Dong, Pompeyo Quesada, et al. 2019. "Tumor Microbiome Diversity and Composition Influence Pancreatic Cancer Outcomes." *Cell* 178 (4): 795-806.e12. https://doi.org/10.1016/J.CELL.2019.07.008.

Roberts, Walden K., Ara Hovanessian, Ronald E. Brown, Michael J. Clemens, and Ian M. Kerr. 1976. "Interferon-Mediated Protein Kinase and Low-Molecular-Weight Inhibitor of Protein Synthesis." *Nature 1976 264:5585* 264 (5585): 477–80. https://doi.org/10.1038/264477a0.

Roncarolo, Maria Grazia, Megan K. Levings, and Catia Traversari. 2001. "Differentiation of T Regulatory Cells by Immature Dendritic Cells." *Journal of Experimental Medicine* 193 (2): F5–10. https://doi.org/10.1084/JEM.193.2.F5.

Rooks, Michelle G., and Wendy S. Garrett. 2016. "Gut Microbiota, Metabolites and Host Immunity." *Nature Reviews Immunology 2016 16:6* 16 (6): 341–52. https://doi.org/10.1038/nri.2016.42.

Rothan, Hussin A., and Mukesh Kumar. 2019. "Role of Endoplasmic Reticulum-Associated Proteins in Flavivirus Replication and Assembly Complexes." *Pathogens* 8 (3). https://doi.org/10.3390/PATHOGENS8030148.

Routy, Bertrand, Emmanuelle Le Chatelier, Lisa Derosa, Connie P.M. Duong, Maryam Tidjani Alou, Romain Daillère, Aurélie Fluckiger, et al. 2018a. "Gut Microbiome Influences Efficacy of PD-1-Based Immunotherapy against Epithelial Tumors." *Science* 359 (6371): 91–97. https://doi.org/10.1126/science.aan3706.
———. 2018b. "Gut Microbiome Influences Efficacy of PD-1-Based Immunotherapy against Epithelial Tumors." *Science* 359 (6371): 91–97. https://doi.org/10.1126/SCIENCE.AAN3706/SUPPL_FILE/AAN3706_ROUTY_SM.PDF.

Russo, Edda, Antonio Taddei, Maria Novella Ringressi, Federica Ricci, and Amedeo Amedei. 2016. "The Interplay between the Microbiome and the Adaptive Immune Response in Cancer Development." *Therapeutic Advances in Gastroenterology* 9 (4): 594–605. https://doi.org/10.1177/1756283X16635082/ASSET/IMAGES/LARGE/10.1177_1756283X16635082-FIG1.JPEG.

Ryu, Tae Young, Kwangho Kim, Tae Su Han, Mi Ok Lee, Jinkwon Lee, Jinhyeon Choi, Kwang Bo Jung, et al. 2022. "Human Gut-Microbiome-Derived Propionate Coordinates Proteasomal Degradation via HECTD2 Upregulation to Target EHMT2 in Colorectal Cancer." *The ISME Journal 2021 16:5* 16 (5): 1205–21. https://doi.org/10.1038/s41396-021-01119-1.

Saleh, Anthony D., Brittany A. Simone, Juan Palazzo, Jason E. Savage, Yuri Sano, Tu Dan, Lianjin Jin, et al. 2013. "Caloric Restriction Augments Radiation Efficacy in Breast Cancer." *Http://Dx.Doi.Org/10.4161/Cc.25016* 12 (12): 1955–63. https://doi.org/10.4161/CC.25016.

Sallusto, Federica, Jens Geginat, and Antonio Lanzavecchia. 2004. "Central Memory and Effector Memory T Cell Subsets: Function, Generation, and Maintenance." *Https://Doi.Org/10.1146/Annurev.Immunol.22.012703.104702* 22 (March): 745–63. https://doi.org/10.1146/ANNUREV.IMMUNOL.22.012703.104702.

Sallusto, Federica, Danielle Lenig, Reinhold Förster, Martin Lipp, and Antonio

Lanzavecchia. 1999. “Two Subsets of Memory T Lymphocytes with Distinct Homing Potentials and Effector Functions.” *Nature 1999 401:6754* 401 (6754): 708–12. https://doi.org/10.1038/44385.

Salvo, Mauricio A., Brock Kingstad-Bakke, Cristhian Salas-Quinchucua, Erwin Camacho, and Jorge E. Osorio. 2018. “Zika Virus like Particles Elicit Protective Antibodies in Mice.” Edited by William B. Messer. *PLOS Neglected Tropical Diseases* 12 (2): e0006210. https://doi.org/10.1371/journal.pntd.0006210.

Sambi, Manpreet, Leila Bagheri, and Myron R. Szewczuk. 2019. “Current Challenges in Cancer Immunotherapy: Multimodal Approaches to Improve Efficacy and Patient Response Rates.” *Journal of Oncology*. Hindawi Limited. https://doi.org/10.1155/2019/4508794.

Sánchez-Alcoholado, Lidia, Bruno Ramos-Molina, Ana Otero, Aurora Laborda-Illanes, Rafael Ordóñez, José Antonio Medina, Jaime Gómez-Millán, and María Isabel Queipo-Ortuño. 2020. “The Role of the Gut Microbiome in Colorectal Cancer Development and Therapy Response.” *Cancers 2020, Vol. 12, Page 1406* 12 (6): 1406. https://doi.org/10.3390/CANCERS12061406.

Sarkizova, Siranush, Susan Klaeger, Phuong M. Le, Letitia W. Li, Giacomo Oliveira, Hasmik Keshishian, Christina R. Hartigan, et al. 2019. “A Large Peptidome Dataset Improves HLA Class I Epitope Prediction across Most of the Human Population.” *Nature Biotechnology 2019 38:2* 38 (2): 199–209. https://doi.org/10.1038/s41587-019-0322-9.

Sarma, J. Vidya, and Peter A. Ward. 2011. “The Complement System.” *Cell and Tissue Research* 343 (1): 227–35. https://doi.org/10.1007/S00441-010-1034-0/TABLES/1.

Schaupp, Laura, Sabine Muth, Leif Rogell, Michael Kofoed-Branzk, Felix Melchior, Stefan Lienenklaus, Stephanie C. Ganal-Vonarburg, et al. 2020. “Microbiota-Induced Type I Interferons Instruct a Poised Basal State of Dendritic Cells.” *Cell* 181 (5): 1080-1096.e19. https://doi.org/10.1016/J.CELL.2020.04.022.

Schiweck, Carmen, Sharmili Edwin Thanarajah, Mareike Aichholzer, Silke Matura, Andreas Reif, Elske Vrieze, Andreas Weigert, and Alexander Visekruna. 2022. “Regulation of CD4+ and CD8+ T Cell Biology by Short-Chain Fatty Acids and Its Relevance for Autoimmune Pathology.” *International Journal of Molecular Sciences 2022, Vol. 23, Page 8272* 23 (15): 8272. https://doi.org/10.3390/IJMS23158272.

Schneider, Josef Johann, Angela Unholzer, Martin Schaller, Monika Schäfer-Korting, and Hans Christian Korting. 2005. “Human Defensins.” *Journal of Molecular Medicine* 83 (8): 587–95. https://doi.org/10.1007/S00109-005-0657-1/METRICS.

Schoggins, John W. 2014. "Interferon-Stimulated Genes: Roles in Viral Pathogenesis." *Current Opinion in Virology* 6 (1): 40–46. https://doi.org/10.1016/J.COVIRO.2014.03.006.

Schoggins, John W., Donna A. MacDuff, Naoko Imanaka, Maria D. Gainey, Bimmi Shrestha, Jennifer L. Eitson, Katrina B. Mar, et al. 2013. "Pan-Viral Specificity of IFN-Induced Genes Reveals New Roles for CGAS in Innate Immunity." *Nature 2013 505:7485* 505 (7485): 691–95. https://doi.org/10.1038/nature12862.

Sender, Ron, Shai Fuchs, and Ron Milo. 2016. "Revised Estimates for the Number of Human and Bacteria Cells in the Body." *PLOS Biology* 14 (8): e1002533. https://doi.org/10.1371/JOURNAL.PBIO.1002533.

Sfanos, Karen S., Jurga Sauvageot, Helen L. Fedor, James D. Dick, Angelo M. De Marzo, and William B. Isaacs. 2008. "A Molecular Analysis of Prokaryotic and Viral DNA Sequences in Prostate Tissue from Patients with Prostate Cancer Indicates the Presence of Multiple and Diverse Microorganisms." *The Prostate* 68 (3): 306–20. https://doi.org/10.1002/PROS.20680.

Shemesh, Colby S., Joy C. Hsu, Iraj Hosseini, Ben Quan Shen, Anand Rotte, Patrick Twomey, Sandhya Girish, and Benjamin Wu. 2021. "Personalized Cancer Vaccines: Clinical Landscape, Challenges, and Opportunities." *Molecular Therapy* 29 (2): 555–70. https://doi.org/10.1016/J.YMTHE.2020.09.038.

Silva Antunes, Ricardo da, Ferran Soldevila, Mikhail Pomaznoy, Mariana Babor, Jason Bennett, Yuan Tian, Natalie Khalil, et al. 2021. "A System-View of Bordetella Pertussis Booster Vaccine Responses in Adults Primed with Whole-Cell versus Acellular Vaccine in Infancy." *JCI Insight* 6 (7). https://doi.org/10.1172/JCI.INSIGHT.141023.

Silverman, Robert H., and Susan R. Weiss. 2014. "Viral Phosphodiesterases That Antagonize Double-Stranded RNA Signaling to RNase L by Degrading 2-5A." *Https://Home.Liebertpub.Com/Jir* 34 (6): 455–63. https://doi.org/10.1089/JIR.2014.0007.

Sivan, Ayelet, Leticia Corrales, Nathaniel Hubert, Jason B. Williams, Keston Aquino-Michaels, Zachary M. Earley, Franco W. Benyamin, et al. 2015. "Commensal Bifidobacterium Promotes Antitumor Immunity and Facilitates Anti-PD-L1 Efficacy." *Science* 350 (6264): 1084–89. https://doi.org/10.1126/science.aac4255.

Smith, Christof C., Sara R. Selitsky, Shengjie Chai, Paul M. Armistead, Benjamin G. Vincent, and Jonathan S. Serody. 2019. "Alternative Tumour-Specific Antigens." *Nature Reviews Cancer 2019 19:8* 19 (8): 465–78. https://doi.org/10.1038/s41568-019-0162-4.

Smith, Melody, Anqi Dai, Guido Ghilardi, Kimberly V. Amelsberg, Sean M. Devlin, Raymone Pajarillo, John B. Slingerland, et al. 2022. “Gut Microbiome Correlates of Response and Toxicity Following Anti-CD19 CAR T Cell Therapy.” *Nature Medicine 2022 28:4* 28 (4): 713–23. https://doi.org/10.1038/s41591-022-01702-9.

Smyth, Mark J., Erika Cretney, Janice M. Kelly, Jennifer A. Westwood, Shayna E.A. Street, Hideo Yagita, Kazuyoshi Takeda, Serani L.H.Van Dommelen, Mariapia A. Degli-Esposti, and Yoshihiro Hayakawa. 2005. “Activation of NK Cell Cytotoxicity.” *Molecular Immunology* 42 (4): 501–10. https://doi.org/10.1016/J.MOLIMM.2004.07.034.

Soria-Guerra, Ruth E., Ricardo Nieto-Gomez, Dania O. Govea-Alonso, and Sergio Rosales-Mendoza. 2015. “An Overview of Bioinformatics Tools for Epitope Prediction: Implications on Vaccine Development.” *Journal of Biomedical Informatics* 53 (February): 405–14. https://doi.org/10.1016/J.JBI.2014.11.003.

Soveg, Frank W., Johannes Schwerk, Nandan S. Gokhale, Karen Cerosaletti, Julian R. Smith, Erola Pairo-Castineira, Alison M. Kell, et al. 2021. “Endomembrane Targeting of Human OAS1 P46 Augments Antiviral Activity.” *ELife* 10. https://doi.org/10.7554/ELIFE.71047.

Spencer, Christine N., Vancheswaran Gopalakrishnan, Jennifer McQuade, Miles C. Andrews, Beth Helmink, M.A. Wadud Khan, Elizabeth Sirmans, et al. 2019. “Abstract 2838: The Gut Microbiome (GM) and Immunotherapy Response Are Influenced by Host Lifestyle Factors.” *Cancer Research* 79 (13_Supplement): 2838–2838. https://doi.org/10.1158/1538-7445.AM2019-2838.

Stafford, Phillip, Zbigniew Cichacz, Neal W. Woodbury, and Stephen Albert Johnston. 2014a. “Immunosignature System for Diagnosis of Cancer.” *Proceedings of the National Academy of Sciences* 111 (30): E3072–80. https://doi.org/10.1073/PNAS.1409432111.

———. 2014b. “Immunosignature System for Diagnosis of Cancer.” *Proceedings of the National Academy of Sciences of the United States of America* 111 (30): E3072–80. https://doi.org/10.1073/pnas.1409432111.

Stafford, Phillip, Rebecca Halperin, Joseph Bart Legutki, Dewey Mitchell Magee, John Galgiani, and Stephen Albert Johnston. 2012. “Physical Characterization of the ‘Immunosignaturing Effect.’” *Molecular and Cellular Proteomics* 11 (4): M111.011593. https://doi.org/10.1074/mcp.M111.011593.

Stafford, Phillip, Stephen Albert Johnston, Orhun H. Kantarci, Ameneh Zare-Shahabadi, Arthur Warrington, and Moses Rodriguez. 2020. “Antibody Characterization Using Immunosignatures.” *PLOS ONE* 15 (3): e0229080. https://doi.org/10.1371/JOURNAL.PONE.0229080.

Statovci, Donjete, Mònica Aguilera, John MacSharry, and Silvia Melgar. 2017. "The Impact of Western Diet and Nutrients on the Microbiota and Immune Response at Mucosal Interfaces." *Frontiers in Immunology* 8 (JUL): 838. https://doi.org/10.3389/FIMMU.2017.00838/BIBTEX.

Stewart, Alexandra J., and Phillip M. Devlin. 2006. "The History of the Smallpox Vaccine." *Journal of Infection* 52 (5): 329–34. https://doi.org/10.1016/J.JINF.2005.07.021.

Stone, Kelly D., Calman Prussin, and Dean D. Metcalfe. 2010. "IgE, Mast Cells, Basophils, and Eosinophils." *Journal of Allergy and Clinical Immunology* 125 (2): S73–80. https://doi.org/10.1016/J.JACI.2009.11.017.

Sun, Bo, Karin B Sundström, Jun Jie Chew, Pradeep Bist, Esther S Gan, Hwee Cheng Tan, Kenneth C Goh, et al. n.d. "Dengue Virus Activates CGAS through the Release of Mitochondrial DNA OPEN." Accessed May 17, 2023. https://doi.org/10.1038/s41598-017-03932-1.

Sun, Mingming, Wei Wu, Liang Chen, Wenjing Yang, Xiangsheng Huang, Caiyun Ma, Feidi Chen, et al. 2018. "Microbiota-Derived Short-Chain Fatty Acids Promote Th1 Cell IL-10 Production to Maintain Intestinal Homeostasis." *Nature Communications 2018 9:1* 9 (1): 1–15. https://doi.org/10.1038/s41467-018-05901-2.

Swann, Jeremy B., and Mark J. Smyth. 2007. "Immune Surveillance of Tumors." *The Journal of Clinical Investigation* 117 (5): 1137–46. https://doi.org/10.1172/JCI31405.

Tabassum, Doris P., and Kornelia Polyak. 2015. "Tumorigenesis: It Takes a Village." *Nature Reviews Cancer 2015 15:8* 15 (8): 473–83. https://doi.org/10.1038/nrc3971.

Tan, Jian, Craig McKenzie, Maria Potamitis, Alison N. Thorburn, Charles R. Mackay, and Laurence Macia. 2014. "The Role of Short-Chain Fatty Acids in Health and Disease." *Advances in Immunology* 121 (January): 91–119. https://doi.org/10.1016/B978-0-12-800100-4.00003-9.

Tan, Rebecca S.T., Bow Ho, Bernard P. Leung, and Jeak L. Ding. 2014. "TLR Cross-Talk Confers Specificity to Innate Immunity." *International Reviews of Immunology* 33 (6): 443–53. https://doi.org/10.3109/08830185.2014.921164.

Tanjo, Tomoya, Yosuke Kawai, Katsushi Tokunaga, Osamu Ogasawara, and Masao Nagasaki. 2020. "Practical Guide for Managing Large-Scale Human Genome Data in Research." *Journal of Human Genetics 2020 66:1* 66 (1): 39–52. https://doi.org/10.1038/s10038-020-00862-1.

Taschuk, Frances, Iulia Tapescu, Ryan H. Moy, and Sara Cherry. 2020. "Ddx56 Binds to

Chikungunya Virus Rna to Control Infection." *MBio* 11 (5): 1–16. https://doi.org/10.1128/MBIO.02623-20/SUPPL_FILE/MBIO.02623-20-ST004.DOCX.

Tawbi, Hussein A., Melissa Burgess, Vanessa Bolejack, Brian A. Van Tine, Scott M. Schuetze, James Hu, Sandra D'Angelo, et al. 2017. "Pembrolizumab in Advanced Soft-Tissue Sarcoma and Bone Sarcoma (SARC028): A Multicentre, Two-Cohort, Single-Arm, Open-Label, Phase 2 Trial." *The Lancet Oncology* 18 (11): 1493–1501. https://doi.org/10.1016/S1470-2045(17)30624-1.

Taylor, Brandie C., and Justin M. Balko. 2022. "Mechanisms of MHC-I Downregulation and Role in Immunotherapy Response." *Frontiers in Immunology* 13 (February): 771. https://doi.org/10.3389/FIMMU.2022.844866/BIBTEX.

Taylor, Rebecca C., Ami Patel, Katherine S. Panageas, Klaus J. Busam, and Mary S. Brady. 2007. "Tumor-Infiltrating Lymphocytes Predict Sentinel Lymph Node Positivity in Patients with Cutaneous Melanoma." *Journal of Clinical Oncology* 25 (7): 869–75. https://doi.org/10.1200/JCO.2006.08.9755.

Terabe, Masaki, and Jay A. Berzofsky. 2008. "Chapter 8 The Role of NKT Cells in Tumor Immunity." *Advances in Cancer Research* 101 (January): 277–348. https://doi.org/10.1016/S0065-230X(08)00408-9.
———. 2014. "The Immunoregulatory Role of Type i and Type II NKT Cells in Cancer and Other Diseases." *Cancer Immunology, Immunotherapy* 63 (3): 199–213. https://doi.org/10.1007/S00262-013-1509-4/FIGURES/3.

Thaiss, Christoph A., Niv Zmora, Maayan Levy, and Eran Elinav. 2016. "The Microbiome and Innate Immunity." *Nature 2016 535:7610* 535 (7610): 65–74. https://doi.org/10.1038/nature18847.

Thomas, Sunil, and George C. Prendergast. 2016. "Cancer Vaccines: A Brief Overview." *Methods in Molecular Biology* 1403 (April): 755–61. https://doi.org/10.1007/978-1-4939-3387-7_43/COVER.

Thompson, Kevin J., James N. Ingle, Xiaojia Tang, Nicholas Chia, Patricio R. Jeraldo, Marina R. Walther-Antonio, Karunya K. Kandimalla, et al. 2017. "A Comprehensive Analysis of Breast Cancer Microbiota and Host Gene Expression." *PLOS ONE* 12 (11): e0188873. https://doi.org/10.1371/JOURNAL.PONE.0188873.

Till, J. E. & McCulloch E. A. 1961. "A Direct Measurement of the Radiation Sensitivity of Normal Mouse Bone Marrow Cells." *Radiation Research* 14 (2): 213–22. https://meridian.allenpress.com/radiation-research/article-abstract/175/2/145/162/A-Direct-Measurement-of-the-Radiation-Sensitivity.

Tonegawa, Susumu. 1983. "Somatic Generation of Antibody Diversity." *Nature 1983*

302:5909 302 (5909): 575–81. https://doi.org/10.1038/302575a0.

Topalian, Suzanne L., F. Stephen Hodi, Julie R. Brahmer, Scott N. Gettinger, David C. Smith, David F. McDermott, John D. Powderly, et al. 2012. "Safety, Activity, and Immune Correlates of Anti–PD-1 Antibody in Cancer." *New England Journal of Medicine* 366 (26): 2443–54. https://doi.org/10.1056/NEJMOA1200690/SUPPL_FILE/NEJMOA1200690_DISCLOSURES.PDF.

Topalian, Suzanne L., Mario Sznol, David F. McDermott, Harriet M. Kluger, Richard D. Carvajal, William H. Sharfman, Julie R. Brahmer, et al. 2014. "Survival, Durable Tumor Remission, and Long-Term Safety in Patients with Advanced Melanoma Receiving Nivolumab." *Journal of Clinical Oncology* 32 (10): 1020–30. https://doi.org/10.1200/JCO.2013.53.0105.

Trapani, Joseph A., and Mark J. Smyth. 2002. "Functional Significance of the Perforin/Granzyme Cell Death Pathway." *Nature Reviews Immunology 2002 2:10* 2 (10): 735–47. https://doi.org/10.1038/nri911.

Trinchieri, Giorgio. 1993. "Interleukin-12 and Its Role in the Generation of TH1 Cells." *Immunology Today* 14 (7): 335–38. https://doi.org/10.1016/0167-5699(93)90230-I.

Trujillo, Jonathan A., Randy F. Sweis, Riyue Bao, and Jason J. Luke. 2018. "T Cell–Inflamed versus Non-T Cell–Inflamed Tumors: A Conceptual Framework for Cancer Immunotherapy Drug Development and Combination Therapy Selection." *Cancer Immunology Research* 6 (9): 990–1000. https://doi.org/10.1158/2326-6066.CIR-18-0277.

Tuddenham, Susan, and Cynthia L. Sears. 2015. "The Intestinal Microbiome and Health." *Current Opinion in Infectious Diseases* 28 (5): 464. https://doi.org/10.1097/QCO.0000000000000196.

Ubeda, Carles, and Eric G. Pamer. 2012. "Antibiotics, Microbiota, and Immune Defense." *Trends in Immunology* 33 (9): 459–66. https://doi.org/10.1016/J.IT.2012.05.003.

Uematsu, Satoshi, and Shizuo Akira. 2007. "Toll-like Receptors and Type I Interferons." *The Journal of Biological Chemistry* 282 (21): 15319–23. https://doi.org/10.1074/jbc.R700009200.

Uribe-Herranz, Mireia, Kyle Bittinger, Stavros Rafail, Sonia Guedan, Stefano Pierini, Ceylan Tanes, Alex Ganetsky, et al. 2018. "Gut Microbiota Modulates Adoptive Cell Therapy via CD8α Dendritic Cells and IL-12." *JCI Insight* 3 (4). https://doi.org/10.1172/JCI.INSIGHT.94952.

Vétizou, Marie, Jonathan M. Pitt, Romain Daillère, Patricia Lepage, Nadine Waldschmitt, Caroline Flament, Sylvie Rusakiewicz, et al. 2015. “Anticancer Immunotherapy by CTLA-4 Blockade Relies on the Gut Microbiota.” *Science* 350 (6264): 1079–84. https://doi.org/10.1126/science.aad1329.

Vivarelli, Silvia, Rossella Salemi, Saverio Candido, Luca Falzone, Maria Santagati, Stefania Stefani, Francesco Torino, Giuseppe Luigi Banna, Giuseppe Tonini, and Massimo Libra. 2019. “Gut Microbiota and Cancer: From Pathogenesis to Therapy.” *Cancers*. MDPI AG. https://doi.org/10.3390/cancers11010038.

Wabl, Matthias, and Charles Steinberg. 1996. “Affinity Maturation and Class Switching.” *Current Opinion in Immunology* 8 (1): 89–92. https://doi.org/10.1016/S0952-7915(96)80110-5.

Waldhauer, I., and A. Steinle. 2008. “NK Cells and Cancer Immunosurveillance.” *Oncogene 2008 27:45* 27 (45): 5932–43. https://doi.org/10.1038/onc.2008.267.

Walker, Jennifer A., and Andrew N.J. McKenzie. 2017. “TH2 Cell Development and Function.” *Nature Reviews Immunology 2017 18:2* 18 (2): 121–33. https://doi.org/10.1038/nri.2017.118.

Walunas, Theresa L., Deborah J. Lenschow, Christina Y. Bakker, Peter S. Linsley, Gordon J. Freeman, Jonathan M. Green, Craig B. Thompson, and Jeffrey A. Bluestone. 1994. “CTLA-4 Can Function as a Negative Regulator of T Cell Activation.” *Immunity* 1 (5): 405–13. https://doi.org/10.1016/1074-7613(94)90071-X.

Wang, Yinbiao, Andreas Holleufer, Hans Henrik Gad, and Rune Hartmann. 2020. “Length Dependent Activation of OAS Proteins by DsRNA.” *Cytokine* 126 (February): 154867. https://doi.org/10.1016/J.CYTO.2019.154867.

Warfel, Jason M., and Kathryn M. Edwards. 2015. “Pertussis Vaccines and the Challenge of Inducing Durable Immunity.” *Current Opinion in Immunology* 35 (August): 48–54. https://doi.org/10.1016/J.COI.2015.05.008.

West, Erin E., Martin Kolev, and Claudia Kemper. 2018. “Complement and the Regulation of T Cell Responses.” *Annual Review of Immunology* 36 (1): 309–38. https://doi.org/10.1146/annurev-immunol-042617-053245.

Wickenhagen, Arthur, Elena Sugrue, Spyros Lytras, Srikeerthana Kuchi, Marko Noerenberg, Matthew L. Turnbull, Colin Loney, et al. 2021. “A Prenylated DsRNA Sensor Protects against Severe COVID-19.” *Science* 374 (6567). https://doi.org/10.1126/SCIENCE.ABJ3624/SUPPL_FILE/SCIENCE.ABJ3624_MDAR_REPRODUCIBILITY_CHECKLIST.PDF.

Willing, Ben P., Johan Dicksved, Jonas Halfvarson, Anders F. Andersson, Marianna Lucio, Zongli Zheng, Gunnar Järnerot, Curt Tysk, Janet K. Jansson, and Lars Engstrand. 2010. "A Pyrosequencing Study in Twins Shows That Gastrointestinal Microbial Profiles Vary With Inflammatory Bowel Disease Phenotypes." *Gastroenterology* 139 (6): 1844-1854.e1. https://doi.org/10.1053/J.GASTRO.2010.08.049.

Wilson, Eric A., Gabrielle Hirneise, Abhishek Singharoy, and Karen S. Anderson. 2020. "Total Predicted MHC-I Epitope Load Is Inversely Associated with Population Mortality from SARS-COV-2." *MedRxiv*. medRxiv. https://doi.org/10.1101/2020.05.08.20095430.
———. 2021a. "Total Predicted MHC-I Epitope Load Is Inversely Associated with Population Mortality from SARS-CoV-2." *Cell Reports Medicine* 2 (3): 100221. https://doi.org/10.1016/j.xcrm.2021.100221.
———. 2021b. "Total Predicted MHC-I Epitope Load Is Inversely Associated with Population Mortality from SARS-CoV-2." *Cell Reports Medicine* 2 (3): 100221. https://doi.org/10.1016/J.XCRM.2021.100221.

Wolchok, Jedd D., Harriet Kluger, Margaret K. Callahan, Michael A. Postow, Naiyer A. Rizvi, Alexander M. Lesokhin, Neil H. Segal, et al. 2013. "Nivolumab plus Ipilimumab in Advanced Melanoma." *New England Journal of Medicine* 369 (2): 122–33. https://doi.org/10.1056/nejmoa1302369.

Wong, Brian, and Yongwon Choi. 1997. "Pathways Leading to Cell Death in T Cells." *Current Opinion in Immunology* 9 (3): 358–64. https://doi.org/10.1016/S0952-7915(97)80082-9.

Woo, Seng Ryong, Leticia Corrales, and Thomas F. Gajewski. 2015. "Innate Immune Recognition of Cancer." *Annual Review of Immunology* 33 (March): 445–74. https://doi.org/10.1146/ANNUREV-IMMUNOL-032414-112043.

Wu, Jiaxi, and Zhijian J. Chen. 2014. "Innate Immune Sensing and Signaling of Cytosolic Nucleic Acids." *Https://Doi.Org/10.1146/Annurev-Immunol-032713-120156* 32 (March): 461–88. https://doi.org/10.1146/ANNUREV-IMMUNOL-032713-120156.

Xin Yu, Jia, Vanessa M. Hubbard-Lucey, and Jun Tang. 2019. "Immuno-Oncology Drug Development Goes Global." *Nature Reviews Drug Discovery* 18 (12): 899–900. https://doi.org/10.1038/D41573-019-00167-9.

Yang, Jason H., Prerna Bhargava, Douglas McCloskey, Ning Mao, Bernhard O. Palsson, and James J. Collins. 2017. "Antibiotic-Induced Changes to the Host Metabolic Environment Inhibit Drug Efficacy and Alter Immune Function." *Cell Host and Microbe* 22 (6): 757-765.e3. https://doi.org/10.1016/j.chom.2017.10.020.

Yang, Juan, Xianzhi Yang, Wenfeng Pan, Mingshuo Wang, Yuxiong Lu, Jianeng Zhang, Ziqian Fang, et al. 2021. "Fucoidan-Supplemented Diet Potentiates Immune Checkpoint Blockage by Enhancing Antitumor Immunity." *Frontiers in Cell and Developmental Biology* 9 (August): 2198. https://doi.org/10.3389/FCELL.2021.733246/BIBTEX.

Yang, Wenjing, and Yingzi Cong. 2021. "Gut Microbiota-Derived Metabolites in the Regulation of Host Immune Responses and Immune-Related Inflammatory Diseases." *Cellular & Molecular Immunology 2021 18:4* 18 (4): 866–77. https://doi.org/10.1038/s41423-021-00661-4.

Yang, Wenjing, Tianming Yu, Xiangsheng Huang, Anthony J. Bilotta, Leiqi Xu, Yao Lu, Jiaren Sun, et al. 2020. "Intestinal Microbiota-Derived Short-Chain Fatty Acids Regulation of Immune Cell IL-22 Production and Gut Immunity." *Nature Communications 2020 11:1* 11 (1): 1–18. https://doi.org/10.1038/s41467-020-18262-6.

Yang, Yichun, Lei Han, Zewei He, Xiaojuan Li, Suping Yang, Jifei Yang, Ya Zhang, Dongqi Li, Yihaho Yang, and Zuozhang Yang. 2018. "Advances in Limb Salvage Treatment of Osteosarcoma." *Journal of Bone Oncology* 10 (March): 36–40. https://doi.org/10.1016/J.JBO.2017.11.005.

Yaron, Jordan R., Sriram Ambadapadi, Liqiang Zhang, Ramani N. Chavan, Scott A. Tibbetts, Shahar Keinan, Arvind Varsani, et al. 2020. "Immune Protection Is Dependent on the Gut Microbiome in a Lethal Mouse Gammaherpesviral Infection." *Scientific Reports* 10 (1): 1–13. https://doi.org/10.1038/s41598-020-59269-9.

Young, Lesley, Julia Sung, and John R. Masters. 2010. "Detection of Mycoplasma in Cell Cultures." *Nature Protocols 2010 5:5* 5 (5): 929–34. https://doi.org/10.1038/nprot.2010.43.

Zhang, Jindong, Lijin Song, Yujing Wang, Chang Liu, Lu Zhang, Shiwei Zhu, Shuangjiang Liu, and Liping Duan. 2019. "Beneficial Effect of Butyrate-Producing Lachnospiraceae on Stress-Induced Visceral Hypersensitivity in Rats." *Journal of Gastroenterology and Hepatology* 34 (8): 1368–76. https://doi.org/10.1111/JGH.14536.

Zhang, Lin, Jose R. Conejo-Garcia, Dionyssios Katsaros, Phyllis A. Gimotty, Marco Massobrio, Giorgia Regnani, Antonis Makrigiannakis, et al. 2003. "Intratumoral T Cells, Recurrence, and Survival in Epithelial Ovarian Cancer." *New England Journal of Medicine* 348 (3): 203–13. https://doi.org/10.1056/NEJMOA020177/SUPPL_FILE/NEJM_ZHANG_203SA1-4.PDF.

Zhang, Xiuli, Piyush K. Sharma, S. Peter Goedegebuure, and William E. Gillanders.

2017. “Personalized Cancer Vaccines: Targeting the Cancer Mutanome.” *Vaccine* 35 (7): 1094–1100. https://doi.org/10.1016/J.VACCINE.2016.05.073.

Zhao, Sha, Guanghui Gao, Wei Li, Xuefei Li, Chao Zhao, Tao Jiang, Yijun Jia, et al. 2019. “Antibiotics Are Associated with Attenuated Efficacy of Anti-PD-1/PD-L1 Therapies in Chinese Patients with Advanced Non-Small Cell Lung Cancer.” *Lung Cancer* 130 (April): 10–17. https://doi.org/10.1016/j.lungcan.2019.01.017.

Zhao, Ye, Feidi Chen, Wei Wu, Mingming Sun, Anthony J. Bilotta, Suxia Yao, Yi Xiao, et al. 2018. “GPR43 Mediates Microbiota Metabolite SCFA Regulation of Antimicrobial Peptide Expression in Intestinal Epithelial Cells via Activation of MTOR and STAT3.” *Mucosal Immunology* 11 (3): 752–62. https://doi.org/10.1038/MI.2017.118.

Zhou, Aimin, Jayashree Paranjape, Thomas L. Brown, Huiqin Nie, Sharon Naik, Beihua Dong, Ansi Chang, et al. 1997. “Interferon Action and Apoptosis Are Defective in Mice D1. A. Zhou, et Al., Interferon Action and Apoptosis Are Defective in Mice Devoid of 2′,5′-Oligoadenylate-Dependent RNase L. EMBO J. 16, 6355–6363 (1997).Evoid of 2′,5′-Oligoadenylate-Dependent RNase L.” *The EMBO Journal* 16 (21): 6355–63. https://doi.org/10.1093/EMBOJ/16.21.6355.

Zhou, Xuefei, Xiangrui Liu, Leaf Huang, X Zhou, L Huang, and X Liu. 2021. “Macrophage-Mediated Tumor Cell Phagocytosis: Opportunity for Nanomedicine Intervention.” *Advanced Functional Materials* 31 (5): 2006220. https://doi.org/10.1002/ADFM.202006220.

Zhuang, He, Liang Cheng, Yao Wang, Yu Kun Zhang, Man Fei Zhao, Gong Da Liang, Meng Chun Zhang, et al. 2019. “Dysbiosis of the Gut Microbiome in Lung Cancer.” *Frontiers in Cellular and Infection Microbiology* 9 (APR). https://doi.org/10.3389/FCIMB.2019.00112.

Zinkernagel, R. M., and P. C. Doherty. 1974. “Restriction of in Vitro T Cell-Mediated Cytotoxicity in Lymphocytic Choriomeningitis within a Syngeneic or Semiallogeneic System.” *Nature 1974 248:5450* 248 (5450): 701–2. https://doi.org/10.1038/248701a0.

APPENDIX A

ASU IACUC APPROVAL/ EXEMPTION

Institutional Animal Care and Use Committee (IACUC)
Office of Research Integrity and Assurance
Arizona State University
660 South Mill Avenue, Suite 312
Tempe, Arizona 85287-6111
Phone: (480) 965-6788 *FAX:* (480) 965-7772

Animal Protocol Review

ASU Protocol Number:	**22-1885R RFC 2**
Protocol Title:	**Overcoming Inhibition of T Cell Control of Osteosarcoma**
Principal Investigator:	**Joseph Blattman**
Date of Action:	**3/18/2022**

The animal protocol review was considered by the Committee and the following decisions were made:

The request for changes was approved by Designated Review to add additional procedures, 70 mice and update funding source information on the protocol.

NOTE: If you have not already done so, documentation of Level III Training (i.e., procedure-specific training) will need to be provided to the IACUC office before participants can perform procedures without supervision. For more information on Level III requirements see https://researchintegrity.asu.edu/animals/training, or contact Research Support Services within DACT at dactrss@asu.edu.

Additional requirements:

☐ This protocol requires that Research Support Services group within DACT provide supervision for the first time a procedure is conducted. Contact dactrss@asu.edu to schedule.
☐ This protocol indicates that there are surgical procedures. A surgical checklist may be required to be submitted to Research Support Services within DACT (dactrss@asu.edu), prior to starting surgeries.
☐ Other requirements:

Total # of Animals:	**1545**	
Species:	**Mice**	**Unalleviated Pain/Distress: No**

Protocol Approval Period: **10/28/2021 – 10/27/2024**

Sponsor:	**Jeff Gordon Children's Foundation**
ASU Proposal/Award #:	**FP00032109 (AWD00036966)**
Title:	**Identifying Key Differences Between Immune Checkpoint Blockade Responders vs. Non-Responders in Metastatic Osteosarcoma**

Signature: _Nicole Shepherd_ Date: 3/22/2022
IACUC Chair or **Designee**

Cc: IACUC Office
IACUC Chair