Phosphopeptide Immunity in Healthy Donors and Cancer Patients

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Dissertation Abstract

MHC-I-presented phosphopeptides have been identified as cancer-expressed antigens and sometimes as targets of immune responses in healthy donors. It is unknown to what extent responses reflect memory or effector responses, and from what types of immunogenic exposures MHC-phosphopeptides arise in healthy donors. We characterized responses in 15 healthy donors to a total of 205 HLA-A2 or HLA-B7-restricted phosphopeptides previously identified on one or more cancer types. Most healthy donors demonstrated some pre-existing immune memory to MHC-phosphopeptides. Wide heterogeneity in the specific phosphopeptides recognized in each donor suggested that most immunity arose due to encountering uncommon immunogens, such as rarer infectious agents or transformed cells. Donor-to-donor variability in the number of phosphopeptides recognized suggested variations in exposure histories, tolerance, and/or antigen presentation. Despite this great heterogeneity, we identified three immunodominant phosphopeptides that were recognized by most HLA-A2$^+$ healthy donors, suggesting a common source of exposure such as an infectious agent. Analyses in two healthy donors demonstrated predominantly resting T_{CM} memory, as well as incidents of active responses to a subset of phosphopeptides, consistent with recent re-exposures to the immunogen. In the absence of evident illness at the time T cells were collected from these donors, active responses may suggest that the phosphopeptides arose on transformed cells.

We also examined responses to immunodominant phosphopeptides and tumor-expressed phosphopeptides in a cohort of 10 HLA-A2$^+$ ovarian cancer patients. Responses to all phosphopeptides were negligible, regardless of whether they were expressed on the patient's tumor and including 2 of the 3 immunodominant phosphopeptides. Despite this,

4 of the 10 patients responded to the immunodominant, $pIRS2_{1098-1105}$, including VTB239, whose tumor expressed this phosphopeptide. VTB239's response was skewed toward an active T_{EM}/T_{EMRA} response and was seen in both PBMCs and TILs, suggesting an active response and demonstrating the ability of phosphopeptide-specific T cells to infiltrate a tumor. Melanoma patients were also impaired in responding to a subset of phosphopeptides. However, vaccination was able to induce responses in most patients. These data support continued investigation into treatments that induce or augment T cell targeting of MHC-phosphopeptides as a part of cancer immunotherapy. Therapies that boost phosphopeptide expression on patients' tumor may also be considered as part of a combinatorial approach.

Dedication

To the women who generously contributed to this study, in an effort to support finding cancer treatments for future generations, while suffering through their own personal battles.

To all the young people who think, “Who am I to be…?” Who are you *not to be*? Don’t be afraid. Take the leap and make mistakes. Lots of them. The worst that could happen is you learn more about yourself, who you are, and who you want to be. And it’s okay for that to change and grow with you.

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List of Abbreviations

Abbreviation	Full Name
184B5	Human breast epithelial cell line transformed by exposure to benzo(a)pyrene
ACT	Adoptive cell therapy
AJCC	American Joint Committee on Cancer
AKT	Ak strain transforming kinase, also known as Protein kinase B (PKB)
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
ANA	Anti-nuclear antibody
APC	Adenomatous polyposis coli
APC	Allophycocyanin
APCs	Antigen presenting cells
ATP	Adenosine triphosphate
B-LCL	EBV-transformed lymphoblastoid B-cell lines
B7-H1	PD-L1
BRAF	Protein belonging to the RAF family of serine/threonine protein kinases
BRAFV600E	Mutation in the gene for BRAF that is common in melanoma
BRCA1	Breast cancer type 1 susceptibility protein
BRCA2	Breast cancer type 2 susceptibility protein
BRIP1	BRCA1 Interacting Helicase 1
BT-20	Human breast cancer cell line
BTAK	serine/threonine kinase
MYC	Myc proto-oncogene
CCR7	Chemokine receptor 7
CD62L	L-selectin
CDR3	Complementarity determining region 3
CEA	Carcinoembryonic antigen
CLL	Chronic lymphocytic leukemia
CM	Central memory
CMV	Cytomegalovirus
CNAs	Copy number alterations
CNS	Central nervous system
COV413	Human ovarian cancer cell line derived from omentum metastasis
CR	Complete Response
CTAs	Cancer testis antigens
CTCAE	Common Terminology Criteria for Adverse Events
cTECs	Cortical thymic epithelial cells
CTLA-4	Cytotoxic T lymphocyte associated protein 4
CTLs	Cytotoxic T Lymphocytes
CVs	Coefficients of variation
DAAs	Disease-associated antigens

DCs	Dendritic cells
DEP1	Density-enhanced phosphatase 1
DFS	Disease-free survival
DLTs	Dose-limiting toxicity
DNA	Deoxyribonucleic acid
DNMTi	DNA Methyltransferase Inhibitors
DPBA	Decorin-binding protein A precursor
DPBS	Dulbecco's Phosphate Buffer Solution
EBV	Epstein-Barr Virus
ECM	Extracellular matrix
ECOG	Eastern Cooperative Oncology Group
EGFR	Epidermal growth factor receptor
EM	Effector memory
EMT	Epithelial-to-mesenchymal transition
EOC	Epithelial ovarian cancer
EPCAM	Epithelial Cell Adhesion Molecule
ER	Endoplasmic reticulum
ERK1/2	Extracellular-regulated kinase
FACS	Fluorescence activated cell sorting
Fas	CD95
FasL	CD95 ligand
FAT1	FAT Atypical Cadherin 1
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FGFR	Fibroblast growth factor receptor
FGFR2	Fibroblast growth factor receptor 2
FLT3	Fms Related Receptor Tyrosine Kinase 3
FWT1	Wilm's Tumor Protein 1
FWT2	Wilm's Tumor Protein 2
G-CSF	Granulocyte colony-stimulating factor
GLEPP1	Renal glomerular epithelial cell (podocyte) membrane protein-tyrosine phosphatase
GM-CSF	Granulocyte-macrophage colony-stimulating factor
gp100	Glycoprotein 100
HBOC	Hereditary Breast and Ovarian Cancer
HBV	Hepatitis B Virus
HCC	Hepatocellular carcinoma
HCL	Hairy cell leukemia
HCV	Hepatitis C Virus
HD	Healthy donor
HER2/neu	Erb-B2 Receptor Tyrosine Kinase 3 (ERBB3)
HIV	Human Immunodeficiency Virus
HLA	Human leukocyte antigen
HLA-A2	Human leukocyte antigen gene A2 subtype 01

HLA-B7	Human leukocyte antigen gene B7 subtype 02
HNPCC	Hereditary Non-Polyposis Colon Cancer
HPV	Human Papillomavirus
hTERT	Human telomerase reverse transcriptase
ICD	Immunogenic cell death
IFA	Incomplete Freund's adjuvant
IFNα	Interferon alpha
IFNγ	Interferon gamma
IFNγR	Interferon gamma receptor
IL-1	Interleukin 1
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-15	Interleukin 15
IL-1β	Interleukin 1 beta
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-6	Interleukin 6
IL-7	Interleukin 7
IMAC	Immobilized Metal Ion Affinity Chromatography
IRS2	Insulin receptor substrate 2
JNK	Jun N-terminal kinase
JY	EBV-immortalized B cell lymphoblastoid cell line
K562	Human cell line derived from a patient with chronic myeloid leukemia
LAG3	Lymphocyte activating 3
LLECs	Long-lived effector cells
M-CSF	Macrophage colony stimulating factor
M1	Matrix 1 protein
M2	Matrix 2 protein
MAGE-A	Melanoma Antigen Gene Protein
MAGE-A10	Melanoma Antigen Gene Protein
MAGE-A6	Melanoma Antigen Gene Protein
MAGE-C2	Melanoma Antigen Gene Protein
MALT	Mucosa-associated lymphoid tissue
MAP3K1	Mitogen-activated protein kinase kinase kinase 1
MAP3K11	Mitogen-activated protein kinase kinase kinase 11
MAPK	Mitogen-activated protein kinases
MART-1	Melanoma antigen recognized by T cells 1 protein; also known as protein Melan-A
mDCs	Monocyte-derived mature dendritic cells
MDSCs	Myeloid-derived suppressor cells
MEK	Mitogen-activated protein kinase kinase; also known as MAP2K
MHC	Major Histocompatibility Complex
MHC-I	Major Histocompatibility Complex-Class I
MHC-II	Major Histocompatibility Complex-Class II

miRNA	Micro-ribonucleic acid
MLH1	MutL homolog 1
MLL	Also known as KMT2A
MPECs	Memory precursor effector cells
MRI	Magnetic resonance imaging
MSH2	MutS homolog 2
MSH6	MutS homolog 6
MSS	Microsatellite stable
mTECs	Medullary thymic epithelial cells
mTOR	Molecular target of rapamycin
MUC1	Mucin 1
N	Naïve
ncRNA	Non-coding ribonucleic acid
NCI	National Cancer Institute
NF1	Neurofibromin 1
NF2	Neurofibromin 2
NK cells	Natural killer cells
NLRC5	NLR Family CARD Domain Containing 5
NOTCH1	Neurogenic locus notch homolog protein 2
NSG	NOD/SCID/IL-2Rγc-/- mice
NY-ESO-1	New York Esophageal Squamous Cell Carcinoma 1
OS	Overall survival
P1, P2, P3, …	Designation for the position (P) of the amino acid in the peptide sequence
P2X7R	Purinoreceptor 7
p38	Mitogen-activated protein kinase p38
PALB2	Partner and localizer of BRCA2
PBLs	Peripheral blood-derived lymphocytes
PBMCs	Peripheral blood-derived mononuclear cells
PD-1	Programmed cell death protein 1
PD-L1	Programmed cell death 1 ligand
PDAC	Pancreatic ductal adenocarcinoma
PE	Phycoerythrin
PFS	Progression-free survival
PGE2	Prostaglandin E2
PI3K	Phosphatidylinositol 3 kinase
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Peptide loading complex
PMS2	PMS1 homolog 2, Mismatch repair system component
poly-ICLC	A synthetic complex of carboxymethylcellulose, polyinosinic-polycytidylic acid, and poly-L-lysine double-stranded RNA
PP2A	Protein phosphatase 2
PTEN	Phosphatase And Tensin Homolog
PTP1B	Protein-tyrosine phosphatase 1B

PTPBAS	Tyrosine-protein phosphatase non-receptor type 13
PTPD2	Protein tyrosine phosphatase
PTPH1	Protein tyrosine phosphatase
PTPp	Protein tyrosine phosphatase
PTPγ	Protein tyrosine phosphatase
RAD51C	DNA repair protein RAD51 homolog 3
RAD51D	DNA repair protein RAD51 homolog 4
RAF	Raf-1 proto-oncogene, serine/threonine kinase
RAS	Kinase family containing K-ras, N-ras and H-ras
RB1	Retinoblastoma protein 1
RBM15	RNA binding motif protein 15
RNA	Ribonucleic acid
ROS	Reactive oxygen species
S100	S100 protein
SCAP	Sterol regulatory element-binding protein
SCM	Memory stem cells
SFCs	Spot-forming cells
SHP1	Protein tyrosine phosphatase
SKOV3	Human ovarian cancer cell line derived from ascites
T2	Human lymphocyte cell line deficient in antigen processing, CEMx721.174.T2, expresses HLA-A2:01
T2-B7	Human lymphocyte cell line deficient in antigen processing, CEMx721.174.T2, expresses HLA-A2:01, engineered to also express HLA-B7:02
TAP	Transporter of antigen presentation
TAP1/2	TAP 1 and TAP2 heterodimeric complex involved in transporting peptides into the endoplasmic reticulum for loading onto the MHC-complex
TCGA	The Cancer Genome Atlas
TCR	T cell receptor
TDLN	Tumor draining lymph node
TEMRA	$CD45RA^+$ effector memory and effectors
TGFβ	Transforming growth factor beta
TILs	Tumor-infiltrating lymphocytes
TLR	Toll-like receptor
TME	Tumor microenvironment
TNFα	Tumor necrosis factor alpha
TP53	Tumor protein p53
Tregs	Regulatory T cells
TSG	Tumor suppressor gene
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
WWP1	WW Domain Containing E3 Ubiquitin Protein Ligase 1
β2m	Beta-2-microglobulin

Chapter 1: Background & Introduction

1.1. Preface

Cancer immunotherapies, such as vaccines and adoptive T cell therapy, require identification of appropriate antigens to target. Targeting antigens that are involved in malignant transformation may reduce the risk of tumor cell escape by immune editing. In cancer cells, dysregulated signaling pathways result in increased proliferation, survival, invasion and metastasis. Therefore, one class of antigens that potentially contribute to malignant transformation are those derived from proteins involved in or driving these dysregulated signaling pathways. One of the predominant modifications contributing to dysregulated signaling in cancer cells is phosphorylation, which is often a consequence of either (1) a kinase that is upregulated or constitutively activated or (2) a phosphatase that is downregulated or inhibited, resulting in an overabundance of intracellular phosphorylated proteins. These phosphorylated proteins become candidates for proteasomal degradation, processing by Major Histocompatibility Complex (MHC) machinery, and expression on MHC-I molecules on the surface of cancer cells. Thus, these so-called “phosphopeptides” are potential targets for anti-cancer cellular immunity.

Our lab has identified over 2000 phosphopeptides, many of which are presented uniquely on transformed cells and are derived from proteins known to be involved in dysregulated cell signaling. The evidence thus far suggests that phosphopeptides may serve as good, targetable cancer antigens. Phosphopeptide-specific $CD8^+$ T cells from healthy donors are cytolytic, specifically targeting cells pulsed with phosphopeptides but not their unphosphorylated counterparts or peptides with a single amino acid substitution, demonstrating their sequence- and phosphate-specificity [1, 2]. Critically, phosphopeptide-

specific CD8$^+$ T cells from healthy donors are also able to kill cancer cell lines and AML and CLL patient-derived cells [2]. In humanized murine models phosphopeptide-specific T cells were able to delay tumor outgrowth of melanoma and lung tumors [3–5]. Together, these data suggest that phosphopeptide-specific immunity can contribute to tumor control. The overall goal of this project is to characterize the immune response against cancer-expressed phosphopeptide antigens in healthy donors and in patients with solid malignancies.

1.2. MHC-I antigen processing and presentation in health and in cancer

1.2.1 Pathway

The MHC-I antigen processing and presentation pathway presents peptides on the cell surface as a means for CD8$^+$ T cells to surveil for evidence of either infection or cellular transformation that can trigger the T cell to target the cell for elimination. The MHC class I molecules on healthy cells present autologous peptides derived from normal proteins involved in homeostatic cell functioning.

Peptides that enter the MHC-I antigen processing and presentation pathway are derived from proteasomally-degraded proteins [6–10]. Improperly folded, incompletely translated, or damaged cytosolic proteins are quickly degraded by the ubiquitin-proteasome pathway [11]. Proteins that are closely regulated, such as those involved in gene transcription and cell cycle control, are also rapidly degraded [8]. Enzymes facilitate the tagging of proteins for degradation with chains of ubiquitin, which are recognized by the proteasome [11]. Once a peptide has been generated, it enters the endoplasmic reticulum (ER) through the transporter of antigen presentation (TAP), a heterodimeric transporter made up of TAP1 and TAP2 [12]. There, the peptide is loaded onto an MHC-I molecule,

forming the peptide loading complex (PLC). The MHC-peptide complex then passes through the Golgi, fuses with the cell membrane, and is presented on the extracellular side of the membrane to be surveilled by $CD8^+$ T cells.

1.2.2 Categories of MHC-I cancer antigens

Strategies to induce anti-tumor T cell responses, including vaccination or adoptive cell therapy, are improved by the identification of MHC-presented peptide antigens that are associated with or specific to cancer cells, and not expressed or expressed at low levels on healthy cells.

Cancer testis antigens (CTAs) are derived from proteins that are expressed during *in utero* development and only in male germ cells in healthy adults, but are re-expressed in numerous types of cancer [13–15]. Some of the CTA proteins promote proliferation, migration, and meiosis/genomic instability in both germ cells and cancer cells [13]. Many cancer patients have T cell and antibody responses against CTAs, demonstrating that CTAs are immunogenic [16]. Vaccines have been shown to induce or enhance responses against CTAs in patients and they have been well-tolerated, but clinical success has been variable. Some studies have demonstrated association between CTA-specific T cell responses and survival, while others have not (reviewed in [13]). It has been proposed that vaccines targeting multiple CTAs could be more effective [13]. Similarly, adoptive T cell therapy with CTA-specific T cells has had some clinical success, although severe toxicity has occurred in some clinical trials [13]. One possible future direction utilizing CTA-specific T cell responses is in combination with immunotherapy or chemotherapy, since some CTAs have been shown to possibly contribute to tumor malignancy [17–20].

Differentiation antigens are derived from proteins that are expressed by the cancer cell as part of its tissue-specific function and phenotype. These were first identified with melanocyte differentiation antigens, such as MART1, gp100, tyrosinase, and CEA, due to their high over-expression in melanoma cells [15]. Melanocyte differentiation antigens were some of the first targeted tumor antigens [15, 21]. Additional differentiation antigens that are often over-expressed in many epithelial tumors include MUC1, cyclin B1, Her-2/neu, Survivin, hTERT, and mesothelin [21]. However, there were real issues of on-target, off-tumor toxicities observed when responses to some differentiation antigens were induced due to their expression on healthy cells. Autoimmune reactions such as vitiligo often correlate with the anti-tumor response, but more serious toxicities such as eye inflammation and life-threatening colitis are limitations [22].

Proteins from oncogenic viruses are a strongly immunogenic category of tumor antigens with little risk of off-tumor toxicity [15]. Chronic infection with EBV can cause lymphomas, including Burkitt's, Hodgkin's, and diffuse large B-cell, in addition to epithelial cell-derived cancers, including nasopharyngeal, gastric, and breast cancers [23, 24]. EBV vaccines have demonstrated very limited success in inducing humoral responses or clinical benefit, suggesting that better antigens need to be identified [25]. However, clinical trials using adoptive cell therapy in EBV-associated cancers have been very successful [25]. Human Papillomavirus (HPV) causes most cases of cervical cancer and a subset of head and neck squamous cell carcinomas [26]. The first preventative cancer vaccine was developed against HPV, and has been successful in decreasing the rates of HPV-driven cancers [27]. However, these HPV vaccine can only protect against infection and are not effective at eliminating established HPV-driven cancers since the vaccine does

not contain the two oncogenic HPV proteins [25]. Therapeutic cancer vaccines targeting the highly expressed oncogenic HPV E6 and E7 proteins, in combination with checkpoint blockade inhibitors, are currently under investigation [28–30]. Chronic infection with Hepatitis B Virus (HBV) or Hepatitis C Virus (HCV) can cause hepatocellular carcinoma (HCC) [31–34]. However, HCCs do not express viral antigens, and, consequently, virally-targeted immunotherapy is not possible [25]. Despite the few successes of immunotherapies targeting virally-derived tumors, the major limitation is the relative infrequency of viral-driven tumors, estimated at ~12% of cancers worldwide [25].

Patient tumor-specific neoantigens arise from somatic non-synonymous mutations. Potential MHC-presented peptides derived from the mutated proteins can be identified by whole exome sequencing and improved peptide generation and HLA binding prediction algorithms [35]. Patient T cell reactivity can then be assessed *in vitro* to find neoantigen-specific T cells that can be expanded with peptide stimulation or used to generate T cells with recombinant T cell receptors (TCRs) [36]. Vaccine strategies have also been shown to induce or augment neoantigen-specific T cell responses, to diversify the neoantigen-specific TCR repertoire, to induce epitope spreading, and to induce memory T cell responses [37–40]. Anti-PD-1 therapy following neoantigen vaccination drove the expansion and diversification of neoantigen-specific T cell reactivity [37]. The possibility of identifying neoantigen-specific TCRs from healthy donors has also demonstrated promise in terms of being able to create patient T cells with recombinant TCRs when they cannot be found in high enough numbers in the patients [41]. Therefore, there have been quite promising results with immunotherapies targeting patient-specific neoantigens. However, neoantigens derived from somatic mutations are generally not publicly shared

across patients and, therefore, must be identified on a patient-by-patient basis, making this an expensive and time-consuming therapy that is only available to some patients and not available in a timely manner for patients with aggressive disease.

Post-translationally modified proteins are a more recently investigated class of cancer antigens. Changes in post-translational modifications can reflect alterations in cell signaling. Circumstantial data suggest that T cells are not selected against post-translationally modified peptides in the thymus, meaning they escape central tolerance and may be strongly immunogenic [42]. Numerous groups have demonstrated that tumor cells contain proteins with post-translational modifications that are either low or absent under normal conditions. This includes citrullination, glycosylation, and phosphorylation of cytosolic proteins [1–3, 5, 43–45]. My work has investigated immune responses in healthy individuals and in cancer patients to phosphopeptides derived from phosphorylated proteins and presented by MHC-I molecules on tumor cells.

1.2.3 Evasion of T cell targeting

Losses or inhibitions in any part of the antigen presentation and processing pathway limit the ability of $CD8^+$ T cells to properly surveil cancer cells and are a predominant mechanism of immune evasion [46–50]. This has been demonstrated by tumor cells down-regulating proteasomal proteins, TAP1/2, beta-2-microglobulin (β2m), and MHC-I [51–54]. For example, loss of responsiveness to IFNγ, the predominant driving force behind upregulated expression of MHC-related genes, is associated with non-responsiveness to immunotherapy and with poorer patient survival [47]. Patients' tumors progressively become less responsive to IFNγ, down-regulating the expression of IFNγR on their cell surface [55]. The losses of expression of IFNγR, MHC-I, or β2m, all essential components

of MHC-I processing and presentation, have been correlated with tumor cell escape [51–54]. Genetic alterations resulting in loss of *NLRC5* expression, including promoter methylation, copy number loss, and somatic mutations, impaired the expression of parts of the MHC-I pathway and anti-correlated with the activation of CD8+ T cells and patient survival in several types of cancer [56]. Tumor cells can also become less susceptible to direct Fas-FasL cell killing by down-regulating expression of the Fas receptor [57]. Responses to both anti-CTLA-4 and anti-PD-1 therapies have been shown to require intact IFNγ signaling [47, 48]. The loss of β2m expression has been shown to be associated with lack of response to anti-CTLA-4 or anti-PD-1 and with poorer overall survival [47]. These studies demonstrate the importance of MHC-I presented tumor antigens to the $CD8^+$ T cell anti-tumor response.

1.3 $CD8^+$ T cells

1.3.1 Role of $CD8^+$ T cells in immune responses

$CD8^+$ T cells are mediators of cellular adaptive immunity, directly recognizing and targeting infected or malignant cells in an antigen-specific manner. T cells are an essential component of immune memory and confer long-lived protection against repeat exposures. $CD8^+$ T cells recognize cells via their TCR, which recognizes a surface-expressed MHC-I molecule associated with a specific peptide, usually of 8-10 amino acids. Upon recognition, $CD8^+$ T cells become effectors, can proliferate, produce effector cytokines, and/or kill the recognized cell by release of cytotoxic granules. After clearance of the immunogenic insult, most of the effectors, the “short lived effector cells” (SLECs), die [58]. Some become “long-lived effectors” (LLECs) with low expression of the IL-7R memory marker yet are still capable of persistence, remaining poised and ready to react to a recurrence of the insult

[59–61]. The third possible fate is formation of memory T cells, which allow a rapid and robust response against re-exposures to the same immunogenic insults.

1.3.2 T cell development and T cell receptor repertoires

$CD8^+$ T cells develop in the thymus. Precursor $CD4^+CD8^+$ thymocytes that recognize self-peptides on MHC-I molecules with low affinity are positively selected [62]. Thymocytes that fail to recognize any MHC-peptide complexes die "by neglect" [63]. Positively-selected T cells down-regulate either CD4 or CD8, travel to the medulla, and are then subject to negative selection, in which thymocytes that strongly recognize any MHC-self peptide complex are deleted to limit peripheral self-reactivity. In contrast to the cortex, where cortical thymic epithelial cells (cTECs) are the primary presenters for positive selection, MHC-self peptides in the medulla can be presented by a number of tolerizing cell types, including medullary thymic epithelial cells (mTECs) and dendritic cells [63].

The repertoires of MHC-presented "private" and "public" self-peptides facilitate the positive selection of $CD4^+CD8^+$ double positive thymocytes. The constitutive 26S proteasome is expressed in cTECs, enabling "public" peptides to be presented for the positive selection process during T cell development [63]. The thymoproteasome is also expressed in cTECs and plays a critical role in positive selection, as demonstrated by serious deficiencies in the peripheral T cell repertoire of thymoproteasome-deficient mice [63–65]. The mechanism by which it positively selects for T cells is thought to be via its cleavage site preferences and generation of weakly MHC-binding peptides that select for TCRs with low-affinity for self-peptide-MHCs. The thymoproteasome is only expressed in cTECs, and generates a distinct repertoire of peptides from that of the constitutive 26S proteasome due to its different subunits [63, 65, 66]. As such, it is responsible for

generating "private" peptides [63–65]. Interestingly, the stochastic expression and presentation of "private" self-peptides can drive person-to-person variations in their T cell repertoires based on the selection of TCRs that recognize self-peptides with low-affinity in one person but are never selected against in another person [67–70]. This shaping of the TCR repertoire based on private self-peptides may explain some person-to-person variations in the ability to generate responses against a particular MHC-peptide epitope, particularly for MHC-peptides that may be expressed by some healthy cells such as phosphopeptides. If and how phosphopeptides are generated during thymic selection remains unknown. However, based on structural data and the dependency of T cell recognition on the phosphate (as will be discussed in **1.9.2**, **1.9.3**), it is likely that phosphopeptide-specific TCRs would require a phosphorylated peptide for selection. The inability to generate phosphopeptides in the thymus during positive selection could limit an individual's ability to respond to later exposures.

1.3.3 T cell differentiation

Naïve T cells express high levels of CCR7 and CD62L, which allow them to traffic to and enter the lymph nodes, where they surveil for their cognate antigen on a professional antigen presenting cell. Upon engagement with their cognate antigen, T cells undergo a massive proliferative burst, increasing ~1,000-fold [58, 71, 72]. They acquire effector functions and down-regulate CCR7 and CD62L so they can leave the lymph node to travel to the exposure site [73–75]. Terminally differentiated effectors (SLECs) have negligible capacity for proliferation or persistence, while long-lived effector cells (LLECs) survive and undergo homeostatic proliferation for months following clearance of an insult [60, 76]. LLECs have been described in mice and as being distinct from true memory cells due to

their low level IL-7R expression [60, 76]. However, the identification and characterization of possible human LLECs are still under investigation. After the initial proliferative burst, there is antigen clearance and contraction of the effector T cells [72, 75]. Most of the effectors, the SLECs, die by apoptosis, while a subset go on to form long-lived memory cells [72, 75]. Memory $CD8^+$ T cells originate from memory precursor effector cells (MPECs), cells that retain or re-express a high level of the IL-7 receptor after priming [58, 77]. After antigen clearance, it is believed that the MPECs preferentially differentiate into memory cells. IL-7 is required for the formation of memory cells [58, 78] and, along with IL-15, is a key homeostatic cytokine in the maintenance of long-lived memory cells [79–81]. The memory T cell compartment is broadly made up of four categories: memory stem (T_{SCM}), central memory (T_{CM}), effector memory (T_{EM}), and $CD45RA^+$ effector memory (T_{EMRA}). The hierarchy in terms of greatest multipotentiality to most differentiated is currently understood as follows: T_N (naïve) > T_{SCM} > T_{CM} > T_{EM} > T_{EMRA} > differentiated effectors [82, 83]. T_{SCM} have enhanced survival and proliferation over all other antigen-experienced subsets, and they are chemo-resistant [82, 84, 85]. They have been shown to be highly multi-potential in mice, non-human primates, and humans [82, 85–87]. T_{SCM} are the only T cell subset that, when transferred into a lymphodepleted mouse, are able to fully recapitulate all other memory and effector $CD8^+$ T cell subsets [82, 85–87]. Their pluripotency is due to maintained epigenetic permissibility, similar to that of naïve T cells [88]. Genetically-modified cells transferred into patients in adoptive cell therapy have been detected 2-14 years later, with the persistent clones derived from the originally transferred T_{SCM} and T_{CM} [89].

T_{CM}, like T_{SCM}, are also multi-potential, long-lived, and highly proliferative [90]. Some can produce effector function in less than 24 hours, but most T_{CM}, upon re-activation, proliferate, giving rise to daughter cells, some of which become T_{EM}, T_{EMRA}, or terminal effectors [90]. T_{CM} are predominantly localized to lymph nodes, due to their high expression of CCR7 and/or CD62L, where they can surveil antigens brought to the lymph nodes by antigen-presenting cells (APCs), and rapidly expand in response to antigen reencounters [91–93]. The T_{EM} subset captures a diversity of T cells with variations in durability and specific effector functions [94]. Overall, T_{EM} can persist for years and can quickly produce cytokine and effector function upon re-exposure [90, 90, 91, 95]. However, they have more limited proliferative capacity than T_{CM} and T_{SCM} and more limited potentiality, in that they can give rise to T_{EM}, T_{EMRA}, and differentiated effectors but not T_{CM} or T_{SCM} [90, 95]. They lack expression of CCR7 and CD62L, and, consequently, are predominantly localized to the blood, where they can immediately act at the site of antigen re-exposure [90, 91, 95].

Very much like T_{EM}, T_{EMRA} lack CCR7 and CD62L and, consequently, primarily circulate through the blood. They are transcriptionally and epigenetically-poised to quickly produce cytokines upon antigen re-exposure [90, 95, 96]. T_{EMRA} can persist for years, but, unlike T_{EM}, they have negligible proliferative capacity [90, 96].

During an encounter, T_{SCM} and T_{CM} proliferate and differentiate into T_{EM}, T_{EMRA}, and terminal effector cells. T_{EM} self-regenerate and also differentiate into terminal effector cells. In doing so, the antigen-specific pool of T cells becomes more highly populated by the two more effector-like subsets, T_{EM} and T_{EMRA}, during an exposure [94, 87, 97, 98, 59]. The two more durable and stem-like subsets, T_{SCM} and T_{CM}, are more abundant when an

antigen has not recently been encountered. Based on this information, we can form inferences about whether there had been a recent antigen exposure in the individual or whether there is a resting memory status by identifying the types and abundance of antigen-responsive T cells. By combining the characterization of the responsive T cell subsets to an antigen with longitudinal analyses, we can assess the frequency with which the antigen arises in the individual, which is particularly informative when the origin of the antigen is unknown.

While all subsets of T cells are needed to cover the range of T cell properties – immediate function as well as expansion and long-term persistence – some pathogens are better controlled by certain T cell features than others [60, 99]. In addition, some pathogen antigen-specific T cells acquire certain memory/effector characteristics due to the nature of that antigen [94, 98, 100]. For example, for antigens that arise transiently (as opposed to chronically), cognate T cells are more prevalently T_{CM} during periods of non-active response [97, 98, 101]. This phenotype is the same for T cells specific to antigens that are expressed at low-levels, even if they are expressed chronically, such as T cells specific to EBV LMP2A, an antigen that is expressed persistently at low levels during the latent phase of EBV chronic infection [94]. T cells that are specific to antigens that are frequently or persistently expressed at high levels, such as antigens expressed during the lytic phase of CMV or EBV, have a greater proportion of T_{EM} and effector cells and a smaller proportion of T_{CM} [87, 98, 101, 102], reflecting the ongoing effector response against infected cells and persistent inflammatory signaling. Thus, the repertoire of memory and effector subsets among antigen-specific T cells reflect the status of antigen exposure in that individual at the time blood is collected.

1.4 CD8$^+$ T Cells in Cancer

1.4.1 Cancer Immune Surveillance

The theory of cancer immune surveillance, that the immune system is constantly patrolling the body's tissues to seek out and destroy incipient cancer cells, was first proposed by Paul Ehrlich, and later elaborated on by Burnet and Thomas [103–106]. Studies over the last several decades have provided strong support for immunosurveillance.

Patient and post-mortem case studies suggest that incipient cancer cells may arise relatively frequently but never develop occult cancer and that this may be due to immune control [107, 106, 108–112]. In one study, as many as 25% of men deceased of non-cancer-related events showed cancer of the prostate [107, 108]. There are also a number of examples of compromised immunity resulting in higher risks of cancer. Organ transplant patients on long-term immunosuppressants develop cancer (derived from autologous cells) at a higher rate than age-matched non-transplant patients [106]. In a few cases, organ transplant patients' tumors spontaneously regressed upon removal of immunosuppressive treatments [106]. Additionally, common variable immunodeficiency, X-linked agammaglobulinaemia, IgA deficiency, and Severe Combined Immunodeficiency are associated with higher risks of childhood cancers, particularly lymphoma [109]. Furthermore, multiple groups have described pre-malignant lesions, benign masses that express some markers of transformation, containing immune cell infiltrates of tumor antigen-specific T cells as well as serum antibodies [110–112]. Patients with Monoclonal Gammopathy of Undetermined Significance frequently show CD4$^+$ and CD8$^+$ T-cell responses to myeloma-associated CTA prior to development of multiple myeloma [113]. There is also evidence of pre-existing immune responses to tumor-associated antigens in

healthy individuals with no evident pre-malignant lesions [1–6]. Therefore, the data from numerous studies are consistent with the presence of pre-malignant lesions kept under control by the immune system. In this work we studied the possibility that CD8^{+} T cell responses to some phosphopeptides are generated by exposures during immune surveillance, as suggested by two previous studies in our lab [2, 3].

Observations of increased cancer incidence in mice with IFNγ or lymphocyte deficiencies also suggest the involvement of the immune system in preventing cancer development [55, 120–122]. Furthermore, tumors grown in immune-compromised mice remained highly immunogenic when transferred into immune-competent mice, but tumors that grew out in immune-competent mice became less immunogenic over time and could not be controlled when transferred into naïve immune-competent mice [55, 121–124]. These results suggested that pressure from the immune system resulted in immune editing of the tumor cells, such that the susceptible tumor cells were pruned by the immune system, while the resistant tumor cells grew out [55, 120–124]. From these results, three stages of immunosurveillance of tumors have been proposed: elimination, equilibrium, and escape [123]. "Elimination" refers to the original meaning of immunosurveillance used by early scientists in the field: immune cells monitor, recognize, and eliminate incipient cancer cells, possibly developing immune memory in the process. "Equilibrium" refers to a dynamic balance between the immune system and pre-malignant or early-stage malignant cancer cells, which are controlled but not fully eliminated. Lastly, "Escape" refers to the stage in which some cells have acquired adaptations and lost expression of some antigens (immunoediting). These resistant cells are able to grow out and establish tumors.

1.4.2 T cell dysfunction in cancer

Broadly, three forms of CD8+ T cell dysfunction are found in cancer: tolerance, exhaustion, and senescence. Insufficient T cell priming results in T cell tolerance either by activation by immature or tolerogenic DCs or by exposure to insufficient or inappropriate cytokines during activation. [125–128]. Exhaustion, while still not fully understood, is thought to be due to chronic antigen exposure and/or to directly suppressive factors in the TME or on the tumor or stromal cells [125]. Exhausted T cells have low or impaired functionality relative to effector cells [129–131]. T cell senescence occurs with insufficient co-stimulation which results in stalled proliferation.

The TME contains a milieu of immunosuppressive factors and cells, including regulatory CD4+ T cells (Tregs), tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs), and the tumor cells and associated stromal cells themselves. Tregs directly suppress CTL activity [125]. Tregs also indirectly suppress CTL activation by influencing an immature or tolerogenic APC phenotype and by depleting the microenvironment of IL-2 [125, 132]. MDSCs induce T cell anergy by depleting the TME of necessary amino acids such as arginine, cysteine and tryptophan. They inhibit T cell function, proliferation, and survival by creating reactive oxygen species (ROS) and nitric oxide (NO), both of which are detrimental to T cells [133]. MDSCs are also directly immunosuppressive since they express PD-L1, secrete factors such as TGFβ and IL-10, and recruit Tregs to the TME [133]. Tumor-associated macrophages (TAMs) resemble an M2 pro-tumorigenic phenotype. TAMs promote tumor growth and also inhibit T cell proliferation and effector function by their production of IL-10, prostaglandins, TGFβ and ROS [125]. Stromal cells can limit the ability of T cells to enter the TME and can suppress

their function by production of TGFβ [134]. Inhibitory molecules expressed on the tumor or stromal cells engage with their ligands or receptors on the T cells, dampening the T cell's effector function.

1.4.3 Inducing or augmenting T cell responses as part of cancer therapy

Cytokine therapy is often used to supplement other immunotherapies. IL-2 was first administered in patients with metastatic disease, renal cell carcinoma or melanoma in 1985 to activate and expand the endogenous T cell response [132]. However, IL-2 alone was insufficient for most patients to revive their anti-tumor immune response, and IL-2 becomes toxic when administered at higher doses [132]. IFNα was the only other FDA approved cytokine standalone treatment. It was approved for the treatment of hairy cell leukemia, follicular non-Hodgkin lymphoma, melanoma, and AIDS-related Kaposi's sarcoma [135]. Additional cytokines, including IL-7, IL-15, GM-CSF, and IL-12, to name a few [135], have been investigated for their ability to induce, augment, or prolong the anti-tumor immune response when administered in combination with other immunotherapy modalities.

Vaccination strategies to activate and expand endogenous T cells *in vivo* include injecting irradiated cancer cells, tumor cell lysates, peptides, or peptide-loaded carriers with adjuvants. The formulations are highly varied, with some targeting $CD4^+$ T cells, others targeting $CD8^+$ T cells, and some targeting both. Different adjuvants, routes of administration, dosing, and timing are also being analyzed to enhance the vaccine's immunogenicity, overcome tolerance if needed, and induce a systemic response. The peptides included in vaccines tested in clinical trial include targeting CTAs, targeting patient-specific neoantigens, or targeting commonly over-expressed or post-translationally

modified tumor antigens. Other ongoing research is focused on improving APCs for proper T cell activation. DC-targeting vaccines often include the cytokine GM-CSF for DC maturation and proper upregulation of co-stimulatory molecules [136–138]. $CD4^+$ T cells are needed *in vivo* to license DCs so that they become fully mature and able to properly co-stimulate $CD8^+$ T cells upon activation. Furthermore, $CD4^+$ T cells are necessary for $CD8^+$ T cell memory formation [139, 140]. Therefore, including $CD4^+$ T cell epitopes (i.e., longer helper peptides) in vaccines can activate both $CD4^+$ and $CD8^+$ T cells, which may be synergistic [141, 142]. There is evidence that $CD4^+$ helper T cells support the expansion of intratumoral $CD8^+$ T cells and that vaccines with $CD4^+$ T cell helper epitopes induce epitope spreading [143].

During vaccination strategies, additional cytokine support, such as IL-12 or IL-2, can provide the signal 3 that is needed by T cells to properly expand and acquire effector and cytolytic functions. Alternatively, T cell-specific blockade of immunosuppressive cytokine signaling, such as through TGFβ or IL-10 signaling, could prevent the tolerization of tumor-specific T cells [144, 145]. Neoantigen discovery efforts have advanced in the last several years with the goal of identifying patient tumor-specific antigens and inducing neoantigen-specific T cell repertoires, either *in vitro* or *in vivo* by vaccination. Overall the success of vaccination has been limited, likely due to a combination of heterogeneous antigen expression across the tumor and tumor sites, loss of antigen expression due to immune editing, local immune suppression, and inadequate numbers of CTLs infiltrating the tumors.

Ex vivo T cell activation and expansion utilizes the patient's endogenous T cells, either from TILs or peripheral blood mononuclear cells (PBMCs), using APCs plus

peptides or tumor cell lysates and cytokines (often IL-2) [146–148]. Approximately 30% of patients respond initially to adoptive cell therapy (ACT), with response correlated to the *in vitro* tumor-specific cytotoxic activity of the transferred immune cells [148, 149]. However, *in vivo* persistence of the transferred cells is limited, and not all patients' TILs are able to be resuscitated and become cytotoxic *ex vivo*. The addition of cytokine support or TLR agonists upon transfusion are being analyzed for their ability to enhance the effector function and/or survival and persistence of the infused immune cells [150].

Engineered T cells with recombinant TCRs can also be created from patient PBMCs, expanded *in vitro*, and transferred into the patient as ACT. Ongoing research is analyzing the efficacy of genetically modifying the transferred cells to express recombinant high affinity TCRs against known tumor antigens and pro-survival and pro-persistence genes and co-stimulatory molecules, while eliminating inhibitory molecules to resist direct inhibition [150]. Furthermore, identifying highly conserved antigens with high affinity TCRs would enable recombinant T cells to be manufactured at high numbers and transferred into patients. Because tumor antigen-specific T cells from TILs often have higher avidity TCRs than tumor antigen-specific T cells among PBLs [151], the identification and isolation of TILs may provide optimal TCRs when engineering recombinant TCRs.

Oncolytic viruses have been used to prime the TME to be more immunogenic. Oncolytic viruses can act as *in situ* vaccinations, killing tumor cells and releasing antigens, while creating an immunogenic environment to stimulate CTLs [152–154].

Checkpoint blockade inhibitors have had remarkable success in many cancer types by blocking PD-1, PD-L1, or CTLA-4, or the combination of targeting the PD-1 pathway

and CTLA-4. As of January 2020, 17 advanced unresectable cancer types had FDA approval for at least one of the PD-1 pathway-targeting monoclonal antibody therapies [155]. Approximately 30% of melanoma patients have long-term response to checkpoint inhibition. However, it is ineffective in some patients through intrinsic resistance and later in other patients through acquired resistance [156]. Individual factors, including the cancer type, the tumor mutational burden, high number of $CD8^+$ TILs, expression of PD-1 on the immune cells and/or PD-L1 on tumor cells or on APCs, and disruption of the DNA damage repair machinery, as well as certain microbiome compositions, correlate with better response to anti-PD-1 therapy [155, 157–167]. It is likely that additional biomarkers will continue to be identified. In any given cancer type, the response rates are between approximately 15 and 65% [155]. PD-1 blockade increases T cell activation by APCs and by inhibiting the suppression by tumor cells when T cells try to engage (reviewed in [158]). CTLA-4 blockade, depending on the antibody isotype, works by depleting Tregs or by inhibiting initial T cell activation and priming (reviewed in [158, 168]). Checkpoint inhibitors have been shown to increase the intratumoral TCR clonality, suggesting that either these new clones were previously unable to infiltrate the tumor or that they were present below the level of detection prior to therapy [169]. The use of checkpoint inhibitors as adjuvants to prime T cells for additional re-stimulation or activation by vaccination is promising [125], albeit requires additional co-therapies to take advantage of the activated T cells.

DNA Methyltransferase Inhibitors (DNMTi) have been used to overcome tumor cell evasion of T cell targeting. The predominant source of immune evasion by tumor cells is the loss of antigen expression or overall loss of MHC-I peptide presentation, such as by

the loss or silencing of expression of proteasomal proteins, TAP1/2, β2m, and MHC-I [51–54]. Treatment with DNMTi has been shown to upregulate the expression of genes that have been epigenetically silenced, such as MHC-I, as well as to increase the expression of CTAs and possibly other antigens, consequently making the tumor more immunogenic [13, 170]. Viral or nanoparticle-based therapy could potentially be used to transfect tumor cells to induce higher expression of MHC, TAP, and FasL to make them more visible and susceptible to T cell targeting and killing, although this requires investigation. The presence of a target is absolutely essential for effective anti-tumor CD8^{+} T cell responses. Therapeutic interventions to overcome these evasion strategies are crucial.

1.5 Cancer

1.5.1 Early transformation events

Cancer is the second most prevalent cause of death in the United States, after cardiovascular disease. Despite humans having redundant DNA repair mechanisms [171], mutations accumulate over time due to errors during DNA synthesis. These errors may be statistically-inevitable due to the mechanisms of DNA synthesis. They may also be environmentally-induced. Some of these errors, in combination with those previously acquired in the same cell, result in malignant transformation. Malignantly transformed cells acquire enhanced abilities to reproduce, live, and resist dying. With longer human lifespans due to enhanced health care and standard of living, the time in a person's life for genetic mutations to accumulate expands, thus increasing the probability that any one person could develop cancer [172]. Rates of invasive cancer incidence are highest among the 60-69 year old age group [173]. However, there is not a strictly linear relationship between age and greater risk of developing cancer, as many variables are known to affect a person's

individual risk of developing cancer. The accumulation of genetic, epigenetic, and chromosomal abnormalities is thought to largely occur during development up to early adult years when cellular division is most rapid [172, 174–176], but with the outward culmination of clinically-evident cancer only becoming apparent in older age. This is highly relevant to this study because it suggests a window of opportunity to train the immune system to recognize and eliminate cancers before they become well-established.

The activation of oncogenes and inhibition of tumor suppressor genes can occur through multiple mechanisms, including mutations, epigenetic modifications, copy number alterations (CNAs) of chromosomal regions and gene fusions, amplifications, translocations, and deletions (via structural changes of the gene or by RNA splicing) [177–186]. Changes can also occur in regions encoding RNA, microRNAs (miRNAs), or non-coding RNAs (ncRNAs). These modifications occur in the cancer cells themselves, but through secretions and extracellular vesicles, cancer cells can also regulate the protein expression of proximal immune and stromal cells, establishing a tumor microenvironment (TME) [182, 183, 185]. Regardless of whether it is due to an inherited predisposition or is due to non-inherited factors, cancer is a process involving multiple stages of transformation. One mutation, epigenetic modification, or chromosomal abnormality will not be the sole generator of cancer. Instead, it is a gradual, step-wise progression from healthy cells to cells that are less and less normal, and, finally, to cells that have reprogrammed many of the normal physiological control systems [172, 174, 187]. These changes occur over time and accumulate. The result of the gradual, step-wise progression from benign mass to malignancy results in formation of pre-cancerous lesions along the way [107, 108, 188–193].

The progression of cells through stages of transformation before becoming malignant is evident and well-documented in many cancer types. The normal pancreas goes through 3-4 transformation stages before becoming pancreatic ductal adenocarcinoma (PDAC) [188]. Colorectal cancer follows a similarly well-defined progression [189, 190]. The lesion becomes more malignant in successive stages, going from small to large adenomas with activating and inactivating mutations. Healthy skin biopsies with no macroscopic malignancy show robust signs of UV-induced DNA damage, similar to those seen in cutaneous squamous cell carcinomas [191]. In the absence of malignancy, these biopsies contained a number of mutations enriched in oncogenes, including *NOTCH1*, *FAT1*, *KRAS*, *NRAS*, and *HRAS* [191]. Other studies have found that an abundance of normal, healthy keratinocytes harbor *TP53* mutations, are clonal, and persist after extended time of complete sun protection [192, 193]. Furthermore, autopsy reports often demonstrate evidence of pre-malignant lesions in people who had displayed no evidence of cancer [107, 108]. These data support the generality of the progressive transformation of healthy tissue into neoplastic tissue and demonstrate that many, if not all, people have pre-malignant lesions that arise in their bodies, sometimes without ever progressing to clinically-evident cancer. As described in **1.3.4**, there is evidence of immune infiltration at these stages of pre-malignancy.

1.5.2 De facto cancer

Cancer cells have a supra-physiological ability to live, replicate, survive, and thrive. They are "immortalized," meaning they can proliferate outside the bounds of normal control mechanisms. Instead of telomere shortening, which constrains a cell to a certain number of replicative life cycles before undergoing natural cell death, apoptosis, or entering into

cellular senescence, they can lengthen their telomeres [194, 195]. They can acquire additional means by which to inhibit or disrupt signaling pathways that would trigger cellular apoptosis or senescence, including disruption of the proteins Rb or p53 from their roles as tumor suppressors [196]. The normal pathways for apoptosis or senescence can be blocked even in response to outright damage to the cell or to its DNA, as with chemotherapy or irradiation [197]. In addition to being more resistant to death, cancer cells also have improved abilities for growth and survival. Proliferation is highly dependent on kinases and phosphatases, particularly cyclin-dependent kinases that regulate gene expression and transitions through the cell cycle and cell division, as well as phosphatases that remove inhibitory phosphates on cell-cycle regulators such as CDC25A and CDC25C [198–201]. Growth factor receptors and their downstream signaling molecules can become upregulated or constitutively activated, while their inhibitors can become down-regulated, essentially creating a perpetual “on” signal [202–205]. Mitogen signaling pathways contain a multitude of kinases and are highly dependent on phosphorylation to perpetuate the signaling such as through the MAPK, JNK, ERK, and mTOR pathways, to name a few [206–211]. Protein synthesis for cell growth and function is induced by mitogen signaling The initiation of protein translation is also regulated by kinases and phosphatases, with phosphorylation of initiation factors and ribosomal subunits necessary for synthesis to begin [212–214]. To satisfy their own needs, cancer cells can over-produce their own growth factors, signaling in an autocrine manner, or signal stromal cells in the tumor microenvironment to produce growth factors for paracrine signaling. Because of the persistent “on” signal, cancer cells require a great deal of nutrients. Cancer cells sequester nutrients from the tumor microenvironment: oxygen, glucose, growth factors, adenosine

triphosphate (ATP), amino acids, etc. as well as the precursors of these molecules [215–218]. Many of the pathways activated by acquisition of nutrients, energy, and growth factors involve phosphorylation. As one example, ATP-driven signaling through P2X7R, an ATP non-selective cation channel expressed in many cancers [219–223], activates PKA, PKC, MEK, ERK1/2, caspase 1/3, AKT, JNK, PIK3, and p38/MAPK pathways for growth, cytoskeletal rearrangement, proliferation, migration, invasion, survival, and inflammasome activation [[219, 222, 224] and as reviewed in [225]]. Lastly, cancer cells secrete pro-angiogenic factors to recruit formation of new vasculature to the tumor to bring oxygen and nutrients, which involves VEGFR signaling and other pathways that activate kinases [226–230]. Cancer cells have multiple mechanisms, both redundant and complementary, by which they are cellular super-survivors, and many of these processes involve phosphorylation events.

To preserve healthy tissue structure and organization, normal cells are inhibited from growing into other cells. This is called "contact inhibition" and its purpose is to maintain normal tissue function, which depends on normal tissue structure. Cancer cells overcome their contact inhibition and become able to grow on top of other cells in a disorganized manner [231, 232]. Some of this is potentiated by loss of connectivity between cell surface adhesion molecules and mitogen signaling, or by the down-regulation or loss of certain adhesion molecules like E-cadherin [231]. Other mechanisms involve loss of enforced cellular polarity [233]. This change in phenotype of cancer cells, termed "the epithelial-to-mesenchymal transition" (EMT), allows and promotes their metastasis from the primary tumor site [231, 232]. This change reflects aberrant forms of cell motility, loss of cell adhesion dependence, morphological change, and ability to degrade the extracellular

matrix (ECM) that are normal processes in wound healing and embryogenesis but not in normal, healthy tissue [234–236]. Many of the wound healing and embryogenesis signaling pathways that become activated during EMT and metastasis involve phosphorylation, including through EGFR, VEGFR, FGFR, and Fyn kinase signaling [237–240].

Tumor cells' genetic instability provides the potential to acquire malignant properties and continue adapting to their environment. The accumulation of mutations, chromosomal abnormalities, and epigenetic changes that inhibit tumor suppressor genes and activate oncogenes causes cellular malignant transformation. Additional changes can be acquired through epigenetic mechanisms including DNA methylation and histone modification [185]. Changes that result in an advantage are perpetuated in sub-clones. Due to the inherent instability of cancer cells, multiple sub-clones can arise in the primary site and may be quite different from those that seed and ultimately colonize a distant site. Metastasis in different organs and pressures from treatment force cancer cells to adapt or perish, which also enriches the variability of sub-clones [241–243]. Tumor-promoting inflammation is another enabling feature due to the pro-tumorigenic characteristics of immune infiltrates [244–248]. Immune cells produce many factors that aid the tumor development, including growth factors, survival factors, pro-angiogenic factors, enzymes to modify the ECM, and signals that facilitate EMT. Pro-tumorigenic immune cells also suppress anti-tumorigenic immune cells, protecting the cancer.

1.5.3 Challenges in treating cancer

There are numerous obstacles in treating cancer. Most of these challenges stem from the fact that cancer often goes undetected until it has reached an advanced stage, at which point it has achieved great intratumoral heterogeneity, metastasis, invasion into multiple

sensitive tissues, and additional acquired intertumoral heterogeneity between the primary and metastatic sites [241, 249, 250]. Most cancers develop invisibly and only become apparent when symptoms interfere with the person's well-being. Cancers detected in earlier stages have much higher rates of survival after treatment [251].

Over the course of treatment, some cells will arise that spontaneously are resistant to that therapy. This is due, at least in part, to their inherent instabilities supporting random alterations in the cells' phenotypes. Because of this natural selection of the most fit cells, over time, the treatment-resistant cells will survive and flourish, while the treatment-sensitive cells perish [241, 249, 250]. Via the same mechanisms of spontaneously arising variations of cellular phenotypes, some cells will be able to adapt to living in different tissues; some cells will be better survivors in some environments, while other cells will fare better in others [242]. Cells that have left the primary tumor may also hide in privileged sites like in lymph nodes or the central nervous system, where treatment is difficult or impossible because it would cause irreparable damage to the function of the tissue, as in the brain or spinal cord. The capacity of cancer cells to migrate and set up new niches elsewhere in the body improves the cancer's ability to thrive and makes it that much more challenging to completely eradicate.

Any time there is great phenotypic diversity, one treatment is not going to be able to eliminate all tumor cells because they will not all be equally susceptible. Identifying tumors and beginning treatment before they've reached an advanced stage is a strong positive prognostic factor for patient survival. On the other hand, inducing a strong immune response, including identifying tumor antigens that are highly maintained on the majority

of the cancer cells, provides a strategy that could potentially target even late-stage cancer cells with heterogeneous phenotypes.

1.6 Pre-Cancer & Factors that Influence the Development of Cancer

The influences on the development of cancer are multifactorial. An individuals' exposures – including the inheritance of specific genetic variants, lifestyle habits and environment, and infectious history – can contribute to and interact with the development of cancer in an individual. In this study, cancer-expressed phosphopeptide antigens are investigated as memory targets in individuals with no history of clinically-evident cancer. Some of the work in this study will explore possible origins of these T cells.

1.6.1 Syndromes with increased cancer susceptibility

Hereditary predisposition through inheriting gene variants known to contribute to the development of cancer is one factor that influences a person's susceptibility. Tumor suppressor genes (TSGs) such as *TP53* and *APC* control or limit cell proliferation and pro-life/anti-death signaling. In the case of genetic variants with increased predisposition for cancer, these protective control functions are lost. Some inherited TSG variants are so closely associated with increased susceptibility to cancer that there are syndromes named for them. Careful, sometimes aggressive, measures are taken to monitor these patients and/or remove certain tissues to lower or eliminate the patient's high risk of cancer. Germline mutations in the TSG, *TP53* is known as Li Fraumeni Syndrome [252]. It is associated with increased lifetime risk of adrenocortical carcinomas, pre-menopausal breast cancer, central nervous system (CNS) tumors, osteosarcomas, and soft-tissue sarcomas, as well as many other childhood and adult malignancies [252]. Children with Li Fraumeni Syndrome who survive pediatric malignancies are at increased risk of developing

additional cancers throughout their lives [252]. Patients with known *TP53* mutations undergo extensive monitoring including ultrasound, magnetic resonance imaging (MRI), colonoscopy, and mammogram and may elect to have prophylactic bilateral mastectomies to reduce their risk of developing breast cancer. Altogether, inheritance of Li Fraumeni Syndrome results in lifetime risks of developing cancer in ≥70% for men and ≥90% for women [252]. Germline mutations in any of the Mismatch Repair genes *MLH1*, *MSH2*, *MSH6*, or *PMS2* or a deletion in *EPCAM* is known as Lynch Syndrome [253]. When it results in colorectal cancer, it is known more specifically as Hereditary Non-Polyposis Colon Cancer (HNPCC) [253]. Patients with Lynch Syndrome undergo extensive monitoring and sometimes removal of tissues or whole organs, similarly to patients with Li Fraumeni Syndrome, and may elect to have prophylactic bilateral mastectomies and/or hysterectomy/oophorectomies to reduce the risk of particularly aggressive cancers. Germline mutations in the *APC* gene result in a syndrome known as Familial Adenomatous Polyposis [254]. Patients with this condition develop numerous rectal and colon adenomas, and, if left untreated, most patients will go on to develop colorectal cancer. However, because this association is well-known, most patients with Familial Adenomatous Polyposis are treated well before developing colorectal cancer. However, patients are still at increased risk of developing other cancer types, such as pancreatic mucinous adenocarcinomas, hepatoblastoma, brain tumors, and thyroid cancer [254]. Germline Phosphatase And Tensin Homolog (PTEN) mutations greatly increase a person's lifetime risk of developing cancer and of developing multiple cancer types [255]. PTEN is a phosphatase that normally suppresses signaling through the PI3K/AKT/mTOR pathway, regulating many cellular processes including those that contribute to survival and

proliferation [256, 257]. In particular, germline PTEN mutations associated with "Cowden's disease" are correlated with higher lifetime risks of thyroid, endometrial, colorectal, and kidney cancer, as well as melanoma [255]. Women are at a much higher lifetime risk of developing invasive breast cancer [255]. There are cases of pediatric and adult penetrance of cancer due to germline PTEN mutations [255]. WWP1 is an E3 ubiquitin ligase that negatively regulates PTEN [258]. Germline mutations in WWP1 have also been identified, and also increase a person's risk of PTEN-related cancer types.

In patients with genetic predispositions associated with a high risk of developing cancer, many pre-malignant lesions form in the absence of or prior to carcinogenesis, exposing the patient's immune system to some tumor-specific antigens. Multiple studies in pre-malignant colorectal adenomas from patients with Familial Adenomatous Polyposis or Lynch Syndrome have demonstrated significant somatic mutations and clonal selection, comparable to that of stage 1 colorectal cancer, suggesting that the pre-malignant lesions have already acquired a great deal of genomic variation [259–261]. These include alterations in driver genes, *APC*, *KRAS*, *FBXW7*, and *TCF7L2* [259]. Patients can develop hundreds of adenomas before carcinogenesis progresses in one or more adenomas via dysregulation of the Wnt signaling pathway [259], suggesting that patients are exposed to pre-malignant cancer cells at high frequency, all of which potentially express tumor antigens and prime the immune response. HNPCC patients have germline mutations in one of the DNA mismatch repair genes, resulting in a high frequency of frameshift mutations and microsatellite instability high (MSI-H) tumors. It has been shown that HNPCC patients without clinically-evident cancer already have effector T cells reactive to frameshift peptide neoantigens at levels higher than healthy donors and higher than colorectal cancer

patients with intact DNA mismatch repair and microsatellite stable (MSS) tumors [262]. In some patients, particularly those with Lynch Syndrome, Li Fraumeni Syndrome, and PTEN Hamartoma Tumor Syndromes, multiple tissue types are susceptible to carcinogenesis. In these cases, pre-malignant lesions can arise from multiple tissue types, likely with distinct pathway alterations, possibly exposing the patient's immune system to a broader repertoire of tumor neoantigens, some of which may include phosphopeptides. Lynch Syndrome is another predisposition resulting from a germline mutation in one of the DNA mismatch repair genes. Consequently, these patients are at higher risk of developing colorectal cancer but also breast, ovarian, cancers. In a study comparing benign adenomas and tumors from patients with Lynch Syndrome, the adenomas showed a range of mutational profiles, from low to high, and neoantigens [261]. There was also an inflammatory microenvironment in the adenomas featuring $CD4^{+}$ T cells and expression of TNFα, IL-2, LAG3, PD-L1, and CTLA-4 [261]. These studies demonstrate that the pre-malignant lesions provide opportunities for immune surveillance based on the presentation of neoantigens and the inflammatory microenvironment. Furthermore, patients who progress to malignancy respond very well to checkpoint blockade therapy [263, 264]. In patients with PTEN Hamartoma Tumor Syndromes, the phosphatase PTEN is no longer functional. Consequently, phosphorylation-dependent PI3K/AKT/mTOR signaling go unregulated, resulting in excess downstream serine and threonine phosphorylation and consequent cell proliferation and resistance to apoptosis [257, 265]. These patients often develop multiple benign tumors, and many go on to develop malignancy [258, 266]. The presence of pre-malignant lesions provide opportunities for the patient's immune system to develop pre-existing immune memory to tumor-associated antigens that are also

expressed on the pre-malignant lesions [262]. Due to the dysregulated signaling, some of the antigens expressed on pre-malignant lesions are likely to be phosphopeptides, although this remains to be determined.

1.6.2 Lifestyle and environment

Lifestyle and environment influence a person's risks of developing cancer due to increased exposure to carcinogens and heightened or prolonged inflammatory states. Known risk factors include: obesity, poor diet (particularly those high in fats and sugars), and lack of adequate exercise. The causal influences are thought to be interlinked, multifactorial and, of course, interact with the person's genetics and physiology. Chronic inflammation due to obesity or poor diet can compromise epithelial barriers through the production of free radicals during catabolism of high fat or high sugar diets. Obesity or poor diet promote persistent, local aberrant cytokine signaling, which also can compromise epithelial barriers [267, 268]. Obesity is also thought to contribute to increased risk of cancer due to high levels of hormone signaling, such as from insulin, enhanced growth factor signaling, and hyperglycemia, some of which, as already described in **1.5.2**, involves kinases. Lastly, adipocytes produce inflammatory cytokines such as leptin, TNFα, and IL-1 [269, 270], further contributing to the persistent inflammatory state associated with obesity and also involving phosphorylation.

One's eating and lifestyle habits can also drive excessive exposure to carcinogens. Carcinogens can be consumed as a result of contamination from food sources or from food additives, such as pesticides, nitrates, and nitrosamines [269]. Some cooking processes create nitrites and nitrates [269]. Lifestyle habits, such as smoking, excessive alcohol intake, and sun tanning, also increase a person's risk for developing cancer due to the high

exposure to carcinogens, namely benzopyrenediol epoxide, toluene, ethanol, and UV light [271, 272, 269]. Exposure to radiation, asbestos, mineral dust, heavy metals, and formaldehydes, to name a few, occur through workplace exposures in a number of industries [272]. Exposure to radiation occurs through medical imaging or through medical treatment. In fact, DNA damage caused by radiation or chemotherapy predisposes a cancer survivor to develop additional malignancies, often leukemias, later in life [273]. It is likely that people with enhanced susceptibility to developing cancer due to their lifestyle and/or environment have a higher rate and number of pre-malignant lesions that are surveilled by the immune system. It is conceivable, therefore, that individuals with higher risk of cancer due to their lifestyles and environments also have greater or more robust pre-existing immune memory to tumor antigens prior to developing malignant disease.

1.6.3 Chronic inflammation and cancer

Chronic infections are associated with a higher risk of developing cancer due to both persistent high levels of inflammation and the ability of many viruses that cause chronic infections to transform infected cells [25, 34]. Persistent inflammation causes an abundance of reactive oxygen and nitrogen species that can damage DNA and cells [274–276]. This damage causes a wound healing-like response, recruiting and activating lymphocytes, stromal cells, and endothelial cells [244, 275–278]. The wound healing-like response creates an inflammatory environment with signals for neovascularization and high levels of growth factors that cancer cells can use to thrive and reproduce [244, 275–278].

A number of chronic infections have been identified as causative agents of specific types of cancer [25, 34, 244, 275–278]. Infection with the bacterium *Helicobacter pylori* is associated with gastric adenocarcinoma, intestinal type mucosa-associated lymphoid

tissue (MALT) lymphoma, and non-Hodgkin's lymphoma [244, 277, 278]. Epstein-Barr virus (EBV) is associated with Burkitt's lymphoma and nasopharyngeal carcinoma [23, 24, 34, 244, 277, 278]. Human papillomavirus is associated with cervical cancer and head and neck squamous cell carcinoma [34, 244, 277, 278]. Hepatitis B and C viruses are associated with hepatocellular carcinoma [31, 33, 244, 277, 278]. It is likely that at least some patients with chronic infections have higher rates of pre-malignant lesions, which may be associated with greater pre-existing immune memory to tumor antigens prior to the development of cancer. It remains unknown whether exposure to chronic infections induces the expression of or responses to MHC-phosphopeptides.

1.6.4 Acute infection and cancer

The triad relationship between infection, the immune system and cancer is complicated and can seem paradoxical. The nature of the infection, as well as the specific infectious agent, influence the positive or negative effect on protectiveness against cancer development. While chronic infections are correlated with increased risk of developing cancer, acute infections may train the immune system in ways that are protective against the development of cancer. There is evidence that people who experienced greater childhood infections may have greater protection against the development of cancer, and antigen-specific T cell responses may play a role [279–281]. Numerous epidemiological studies and case studies have shown that higher rates of childhood infectious febrile diseases correlate with a lower lifetime risk of cancer [277, 281]. Other studies have looked at the role of acute infections in adults and again found correlations with lower risk for multiple types of cancer; however, this was more pronounced specifically for febrile infections [277]. Spontaneous regression of tumors has been attributed to concurrent infections in numerous cancer types, including

brain, lymphoma, and melanoma [282–287]. Direct evidence of infection inducing protection against cancer development is lacking. However, it's been proposed that some of the protection afforded by acute infections is due to memory induced to shared antigens between cancer cells and virally- or bacterially-infected cells. Olivera Finn has termed these "disease-associated antigens," to indicate that there may be a subset of shared antigens that are reflective not of a specific infectious or malignant cell state but of a broader signal that something has gone wrong [21, 288, 289]. This is congruent with, and builds upon, ideas first proposed by Polly Matzinger [290]. Some studies have demonstrated that socioeconomic status is correlated with the risk of cancer, which is thought to reflect the lower number and frequency of infections due to possibly cleaner conditions and better access to healthcare [277]. Therefore, a person's history of acute infections may prepare their immune systems to recognize and eliminate tumor cells. If and how MHC-phosphopeptides play a role in acute infection/cancer shared antigens remains uninvestigated.

1.7. Ovarian Cancer

1.7.1 About ovarian cancer

Epithelial ovarian cancer (EOC) is the fifth leading cause of death for women and the deadliest of gynecologic diseases with over 70% of diagnosed patients succumbing to the disease. Most cases of EOC are diagnosed at advanced stage due to the lack of available robust screening or biomarkers and general, non-specific symptoms. Patients diagnosed at early stage have a 90% chance of a 5-year survival. However, most patients are diagnosed at an advanced stage, which often corresponds with tumor metastasis throughout the abdomen and acquired resistance to chemotherapy. For these reasons, diagnosis at

advanced stage of ovarian cancer is a negative prognostic factor for response to treatment and overall patient survival [291]. Risk factors for developing EOC include: genetic mutation in the *BRCA1* or *BRCA2* genes, obesity, having a family history of ovarian, breast, or colorectal cancer, having a family cancer syndrome (HBOC or HNPCC) having genetic mutations in a number of other genes (*APC*, *BRIP1*, *RAD51C*, *RAD51D*, and *PALB2*), hormone therapy after menopause, and not having children [292]. Although the exact mechanisms of tumorigenesis remain unknown in ovarian cancer, chronic inflammation is through to contribute. Epidemiological studies have demonstrated correlations between number of ovulations, endometriosis, or pelvic inflammation with the development of EOC [291]. Common genetic alterations in EOC include: *BRCA1/2*, *TP53*, *BTAK*, *HER2*, *C-MYC*, *EGFR*, *PI3K*, *AKT2*, K*RAS* and *BRAF* [291].

1.7.2 Current treatments for ovarian cancer

Surgical debulking is the standard first step in patient treatment upon diagnosis. Adjuvant chemotherapy begins weeks after recovery from surgery, or, if deemed necessary, may be administered as neoadjuvant therapy prior to surgery to debulk. Dual chemotherapy with a platinum-based drug, such as carboplatin, and a taxane, paclitaxel, is the standard of care in ovarian cancer [293]. Paclitaxel was first introduced to patients with EOC in the 1990s, and it improved the rate of initial complete response (CR) from 31% to 51%, the time of progression-free survival from 13 to 18 months, and the overall survival from 24 months to 38 months [291]. Paclitaxel is a cell cycle specific treatment that binds beta tubulin, stabilizing the microtubules in mitotic spindles and preventing the disassembly of the spindles and centrosomes. This arrests cells in mitosis (mostly in metaphase), and is hypothesized to induce cell death by mitotic arrest, by chromosome missegregation, and/or

by aneuploidy in daughter cells resulting from multi-polarity [294]. It has been shown to have immune modulation effects through immunogenic cell death (ICD), but this is very dependent on the dose and timing [295–298]. Carboplatin is a platinum-based alkylating agent. It is not cell cycle specific. It induces tumor cell apoptosis by inhibiting DNA synthesis and crosslinking DNA [299]. This dual chemotherapy regimen is often initially successful, but recurrence is high and many patients go on to develop platinum-resistance disease, at which point alternative treatments need to be considered. One treatment is hormone therapy, in which small molecule inhibitors called aromatase inhibitors block the synthesis of estrogen. Hormone therapy is often used in patients with platinum-resistant recurrence [300]. Bevacizumab, an angiogenesis inhibitor, has also been investigated in clinical trials [301]. Other treatments for platinum-resistant disease include pegylated liposomal doxorubicin, topotecan, gemcitabine, and enrollment in clinical trials [301].

1.7.3 Role of CD8$^+$ T cells in ovarian cancer

Intratumoral CD8$^+$ tumor-infiltrating lymphocytes (TILs) independently correlate with improved disease-free and overall survival in patients with ovarian cancer [302–305]. Furthermore, CD45RO$^+$ antigen-experienced T cells are an independent prognostic factor [304]. However, other studies have found that CD8$^+$ TILs alone were not an independent prognostic factor but instead needed to be combined with an additional variable. This study found that the types of CD8$^+$ T cell subsets among TILs, the TILs' differentiation statuses, and the patient's treatments stratified CD8$^+$ TILs as being prognostic or not [306]. In support of this, in poorly infiltrated tumors, the presence of high TCR clonality correlated with improved survival, suggesting that these expanded clones were tumor antigen-specific in contrast to TILs from tumors with greater TCR clonality [307]. Another study found that

specifically $CD27^+CD8^+$ T cells, which was largely made up of $CD45RO^+CCR7^+$ central memory and $CD45RO^+CCR7^-$ effector memory T cells, was highly correlated with prognostic benefit in patients after surgery, even in patients who still had macroscopic lesions that could not be surgically removed [308]. Two recent studies may shed some light on these seemingly contradictory findings. These studies discovered that $CD8^+$ TILs in ovarian cancer are largely bystanders and not tumor-specific [307, 309]. The majority of ovarian cancers have low mutational burdens. Despite this, Bobisse *et al* recently demonstrated that even in ovarian tumors with low mutational burdens, high avidity tumor antigen-specific TCRs can be isolated from TILs, which could be utilized in ACT [151].

Overall, very few ovarian cancer-specific antigens have been identified. Mucin1 and HER2/neu were some of the first ovarian cancer associated antigens identified by their recognition by CTL lines [310, 311]. Two CTAs, NY-ESO1 and LAGE-1, were found to be often aberrantly expressed in EOC [312]. Folate receptor alpha and insulin-like growth factor binding protein 2 are MHC-II antigens in EOC [313]. Patients with p53-directed humoral responses had improved overall survival [314]. Therefore, identification of commonly expressed tumor antigens in ovarian cancer is still needed. Phosphopeptides may in part fill this need.

1.7.4 Potential of harnessing immunotherapy in patients with ovarian cancer

Analysis of TCGA data revealed an immunoreactive gene signature that correlates with improved survival [315]. In support of this, PD-1 expression on tumor cells has been shown to correlate with poor prognosis in ovarian cancer patients [305]. The accumulation of tumor-infiltrating suppressive $PD\text{-}1^+$ DCs in a murine model correlated with lower CTL infiltration and worse tumor prognosis [316]. Also, Tregs, immunosuppressive DCs, and

MDSCs create an immunosuppressive TME in ovarian cancer [303, 317]. Together with the correlation of $CD8^+$ TIL with improved survival in patients, these findings support investigation into checkpoint inhibition combined with additional T cell stimulating therapies. Clinical trials with anti-PD-1 had a response rate of 15% [318]. However, dual blockade of inhibitory receptors may be required since single blockade has been shown to induce the expression of additional inhibitory receptors as compensatory mechanisms in response to checkpoint therapy [319].

Multiple clinical trials treating patients with DC vaccines pulsed with autologous whole tumor lysate have demonstrated efficacy [320–322]. One phase I trial analyzed this type of DC vaccine with freeze-thaw tumor lysate following anti-VEGF to inhibit tumor angiogenesis and cyclophosphamide to inhibit or deplete Tregs [320]. Tumor-reactive $IFN\gamma^+$ $CD8^+$ T cell responses were detected only after vaccination but not before in the four responding patients. Tumor-specific humoral responses were also detected in the 2 patients with clinical responses. Patients who achieved stable disease but not complete remission after vaccination went on to receive adoptive therapy of vaccine-primed CD3/CD28-co-stimulated T cells. Tumor-reactive T cells were detectable in the transferred cells. Tumor-reactive T cells were detected after adoptive transfer of the co-stimulated T cells in the 2 responding patients but not in the non-responder. Therefore, activation of tumor-reactive CTLs is possible in patients with recurrent ovarian cancer, and clinical response correlated with tumor-reactive $CD8^+$ T cell IFNγ activity. Another phase I clinical trial analyzed a DC vaccine pulsed with autologous whole tumor oxidized lysate following anti-VEGF to inhibit tumor angiogenesis and cyclophosphamide to inhibit or deplete Tregs [321]. $CD4^+$ and $CD8^+$ T cell response were induced by vaccination. Again, clinical benefit in patients

correlated with tumor-reactive $CD8^+$ T cell IFNγ activity. In a third study, an oxidized whole-tumor lysate DC vaccine induced T cell responses to autologous tumor antigens in recurrent EOC patients by augmenting neoantigen-specific T cell responses and driving out new T cell clones [322]. Responsive patients had a median of 90 neoantigens [322].

The standard of care chemotherapy for patients with ovarian cancer, carboplatin and paclitaxel, may be amenable to combination with $CD8^+$ T cell directed immunotherapy. One group demonstrated that the frequency of Tregs decreased while the frequency of $CD45RO^+CD8^+$ T cells increased 2 weeks post-chemotherapy. Also, DCs loaded with tumor cell lysates isolated post-chemotherapy expressed higher levels of co-stimulatory molecules and there was a higher percentage of $IFN\gamma^+$ $CD8^+$ T cells [293]. Recently, paclitaxel (but not carboplatin) was shown to induce immunogenic cell death through TLR4 and induction of calreticulin on the cell surface [295]. The combination of carboplatin and paclitaxel did not have significant effects on T cell differentiation, activation, or proliferation, although low levels of MHC-I after chemotherapy could be a barrier to T cell directed therapy [323, 324]. Therefore, patients' immune and tumor profiles should be routinely assessed following treatment to determine their potential to respond to $CD8^+$ T cell-focused therapies. Timing and dosing would need to be investigated to determine the best way to incorporate T cell inducing therapy with the dual chemotherapy regimen.

Clinical trials assessing the efficacy of single agent PD-1 or PD-L1 antagonist antibodies have had minimal success in patients with recurrent or resistant ovarian cancer. Overall response rates were between 10.7% and 15% [325]. Dual agent checkpoint inhibition therapy is currently being assessed in clinical trials. Rational design of

combinatorial treatment strategies will be necessary to improve overall response and persistent tumor control. Identifying ovarian cancer antigens in order to generate relevant anti-tumor immune responses using vaccines or adoptive cell therapy is one important aspect of these efforts.

1.8. Rationale for Targeting Phosphopeptides as Cancer Antigens

1.8.1 Rationale for targeting antigens derived from proteins involved in malignant properties

While $CD8^+$ T cells have been shown to control and eliminate tumors [326–329], tumor cell escape is a major problem [35, 330]. Many identified tumor antigens are derived from proteins that are not required for cell survival or proliferation, and there is no selective pressure to maintain expression. Peptides that arise from proteins that contribute to the cell's malignant phenotype may provide better targeting because it is less likely that the tumors will successfully immuno-edit to evade detection. Dysregulated cell signaling pathways are a hallmark of solid and hematological malignancies. These pathways are often associated with an overabundance of phosphorylated proteins that play essential roles in promoting malignancy, including transformation, survival, growth, invasion, angiogenesis, and metastasis [211, 331–336]. As opposed to the low and transient levels of phosphorylated proteins found in healthy cells, constitutive and high abundance phosphorylated proteins found in cancer cells may cause phosphopeptides to be processed and presented on the cell surface as tumor-specific antigens. We have already demonstrated that a number of phosphopeptides are presented on melanoma, colorectal, breast, ovarian, and/or leukemia cancer cells, with many shared by multiple malignancies [1, 2, 337]. This

suggests that efforts targeting phosphopeptides may have broader therapeutic potential in multiple cancers and may also reduce the likelihood of tumor escape.

1.8.2 Kinases in cancer

Protein kinases are major players in dysregulated signaling in cancer cells [211, 331, 333, 335, 336, 338–340]. Dysregulation of kinases occurs by autocrine signaling, somatic gain-of-function mutations, gene fusions, over-expression, and/or gene copy number amplifications [338, 341]. Through autocrine signaling or scavenging of growth factors from the TME, tumor cells constitutively activate growth factor receptors, which in turn constitutively activate downstream signaling proteins, many of which are kinases [338, 341–343]. This results in a multitude of highly activated kinases and an overabundance of phosphorylated proteins, in contrast to the transient signaling that occurs under homeostatic conditions [341–343]. It is also possible that when kinases are constitutive activated, they are more likely to phosphorylate non-canonical residues, creating *de novo* phosphorylated proteins that don’t exist under normal, homeostatic conditions.

1.8.3 Phosphatases in cancer

While kinases phosphorylate serine, threonine, and tyrosine residues on proteins, phosphatases remove the phosphate, playing a major role in balancing cell signaling. Many phosphatases function as tumor suppressors due to their ability to control cell cycling or signaling that leads to proliferation and pro-survival. Phosphatases are often inhibited or their expression silenced in cancer cells.

Phosphatase and Tensin Homolog (PTEN) is a serine/threonine, tyrosine, and lipid phosphatase and one of the most frequently dysregulated tumor suppressor genes in cancer cells [256, 257, 344]. PTEN normally acts to control cell signaling by de-phosphorylating

(and therefore, regulating) the numerous kinases that contribute to cell growth and survival as part of the PI3K/AKT/mTOR signaling pathway. In the absence of PTEN regulation, these kinases become over-activated [256, 257, 344]. Protein Phosphatase 2A (PP2A) is another serine/threonine phosphatase that has been identified as a tumor suppressor that is frequently inactivated or inhibited in cancer cells. It is also involved in signaling pathways for proliferation, apoptosis, and responding to DNA damage [345]. Other phosphatases that have been identified as tumor suppressors and that are inhibited in one or more malignancies include: DEP1, SHP1, GLEPP1, PTP1B, PTPBAS, PTPD2, PTPH1, PTPp, LAR, and PTPγ [346]. Therefore, the inhibition or loss of phosphatases is a common occurrence in cancer cells, contributing to the enhanced cell signaling as well as an over-abundance of phosphorylated proteins that could undergo processing and presentation as MHC-I presented epitopes.

1.9. MHC-I presented phosphopeptides

1.9.1 Identification MHC-phosphopeptides

A large number of MHC-I and MHC-II phosphopeptides presented on patient-derived tumors, adjacent "healthy" tissue, cancer cell lines, and EBV-transformed cell lines have been identified by several groups over the past twenty years. The body of work presented here focuses on MHC-I presented phosphopeptides, but others have identified and characterized immunity to MHC-II presented phosphopeptides as well [347–349]. Previous work in our lab isolated and identified phosphopeptides expressed on MHC-I molecules on cancer cell lines, patient-derived tumors, normal splenic B and T cells, bone marrow cells, melanocytes, and colorectal and esophageal primary tissue [1, 2, 350, 351]. MHC-peptide complexes were isolated using immunoaffinity purification, and peptides were isolated by

acid extraction, enriched for phosphopeptides using Immobilized Metal Ion Affinity Chromatography (IMAC), and identified using mass spectrometry.

Phosphopeptides are naturally presented *in vivo* on MHC-I molecules in a TAP-dependent manner, demonstrating that they come from proteins that are degraded in the cytosol into phosphorylated peptides and enter the antigen-processing machinery in the endoplasmic reticulum [337, 352]. MHC-I phosphopeptides have been detected on numerous types of cancer, including melanoma, ovarian cancer, breast cancer, colorectal cancer, lung cancer, renal cell carcinoma, and multiple types of leukemias (acute myeloid leukemia/AML, chronic lymphocytic leukemia/CLL, hairy cell leukemia/HCL, acute lymphoblastic leukemia/ALL) [1, 347, 348, 350, 2, 353, 4, 45]. Normal, healthy cells also present MHC-phosphopeptides. A subset of phosphopeptides identified on primary leukemias were also seen on resting splenic T and B cells and in bone marrow [2], suggesting that a subset of phosphopeptides may be involved in normal homeostatic functioning, but that additional phosphopeptides get expressed in the context of aberrant signaling. Many phosphopeptides have been found on multiple cancer types. Therefore, vaccination with this phosphopeptide or a T cell product, a recombinant TCR or an adoptive cell therapy protocol, could be applied to more cancer types and more patients if it is targeted at a highly-conserved, shared tumor antigen.

The great majority of MHC-expressed phosphopeptides that we and others have identified to date contain phosphorylated serine or threonine residues [1–4, 45, 337, 352]. As tyrosine phosphorylation accounts for less than 1% of total cellular phosphorylation [338], this is consistent with an overall sampling of the phosphoproteome by the MHC-I processing and presentation pathway. Some phosphopeptides are derived from known

oncogenic source proteins. However, for most, the function and possible role of the identified phosphosite in oncogenesis has not been determined or even investigated [1, 2].

1.9.2 Structure of the MHC-I-phosphopeptide complex

For phosphopeptides associated with all MHC-I molecules to date, phosphorylation is most prevalent on the fourth amino acid of the peptide, which does not engage with the binding pockets of the MHC-I molecule. The biased representation of peptides phosphorylated at P4 seems to reflect binding preferences of the HLA molecule that overlap with recognition motifs of certain kinases [2, 354]. There is also a preference for a positively charged arginine or lysine at P1 of HLA-A2 presented phosphopeptides, relative to non-phosphorylated peptides, and for subdominant anchor residues (methionine, threonine, glutamine, or valine instead of leucine) at P2 [354]. Phosphorylation can result in improved, diminished, or no effect on the peptide's binding affinity for the MHC-I molecule. This depends on the MHC molecule, and is thus likely also influenced by the anchor residues [352, 354, 355]. In the case of HLA-A2-phosphopeptide complexes, many phosphopeptide binding affinities were enhanced over their non-phosphorylated counterparts. However, phosphorylation led to comparable or decreased peptide binding affinities for HLA-B7-restricted phosphopeptides versus their non-phosphorylated counterparts for reasons that remain unclear.

In crystal structures of four HLA-A2-phosphopeptide complexes, the anchor residues were positioned similarly to those in non-phosphorylated peptides [354]. However, the phosphoserine at P4 was pointed up out of the cleft, and toward the N terminus of the peptide, in contrast to much more flexible P4 residue orientations observed in non-phosphorylated peptides [354]. This enabled electrostatic interactions with side chains of

the MHC molecule, creating different peptide backbone conformations from the corresponding non-phosphorylated peptides [354, 356]. This additional stabilization by the phosphate-MHC-I side chain interactions likely explains the prevalence of subdominant residues at the P2 anchor position.

These crystal structures demonstrated that the phosphate moiety creates an electronegative charge that extends beyond the peptide cleft and is directly accessible to TCR ligation near the CDR3α region. This suggests that the phosphate plays a predominant role in TCR recognition, and points to certain features of TCR that would be required for phosphopeptide recognition [354–356]. Computational studies on non-post-translationally modified proteins have demonstrated that the amino acid residues at positions 4, 5, and 6 are most crucial to the MHC-peptide's immunogenicity, further supporting the crucial role of the phosphate at P4 in its recognition by the TCR [357]. In the HLA-A2-phosphopeptide complex, the phosphate was protected from phosphatases, supporting the persistence of the phosphorylation once in complex [356]. Altogether, these data support the MHC-I-presented phosphopeptides as unique, distinct epitopes for $CD8^+$ T cells.

1.9.3 Responses to phosphopeptides in healthy donors

In previous work, several phosphopeptides were shown to be directly immunogenic, either via immunization of naïve mice or via repeated peptide stimulation *in vitro* [1–4, 337, 352]. These responses were repeatedly demonstrated to be both sequence- and phosphate-dependent [1–4, 337, 352].

Our group has previously shown that a cohort of 10 healthy donors had robust $IFN\gamma^+$ responses in an ELISpot assay after stimulation and 7-day *in vitro* culture of PBMCs with a panel of 76 HLA-B7-restricted phosphopeptides. These peptides are expressed to

varying extents on a variety of leukemia cells, EBV-transformed cell lines, and normal immune cells [2]. Individual donors responded to 14-29% of these 76 phosphopeptides. Seven of 10 phosphopeptides derived from leukemia oncogenes were immunodominant targets, inducing responses in at least 7/10 donors. The magnitude of the responses to some phosphopeptides were at levels comparable to those to Influenza and Adenovirus [2], suggestive of an expanded population of antigen-specific memory cells.

In this previous study, $CD8^+$ T cells isolated from 2 healthy donors were sorted into naive ($CD45RA^+CD27^+$), central memory ($CD45RA^-CD27^+$), effector memory ($CD45RA^-CD27^-$) and effector ($CD45RA^+CD27^-$) T cells. Responses to 4 phosphopeptides were observed largely in the central memory compartment, with 2 of the 4 also having responses in the effector memory compartment [2]. This is consistent with the hypothesis that elevated phosphopeptide immunity is due to previous exposure to phosphopeptides expressed on transformed or infected cells. In another study from the lab, 4 healthy donors showed $CD45RO^+CD8^+$ T cell responses to the HLA-A2-restricted phosphopeptide $pCDC25B_{38\text{-}46}$ [3]. Two of the 4 donors also had responses to the HLA-A2-restricted phosphopeptide $pIRS2_{1097\text{-}1105}$. Other T cell subsets were not assessed for responses, nor were the T_{CM} and T_{EM} explicitly analyzed from the bulk $CD45RO^+$ T cells. These results demonstrate that at least some phosphopeptide responses in healthy donors are due to pre-existing immune memory generated after an immunogenic exposure, but it is unknown whether this is a general trend. It is possible that, with the isolation of $CD45RO^+$ T cells as well as the addition of cytokines to the cultures, which were lacking in the larger previous study [2], broader memory patterns in healthy donors would be observed. On the other hand, it is also possible that responses would be more limited when explicitly assessing

only memory responses. Furthermore, up to now there has been limited analysis of *ex vivo* responses in healthy donors from any T cell subset. For responses to be evident immediately *ex vivo*, there would need to be antigen-specific T cells in the blood that can immediately respond to an exposure with cytokine and be at high enough cell number to be detectable in an assay. If this was observed, it would suggest either much larger frequencies of memory T cells which would be indicative of repeated exposures in that individual. Alternatively, it would suggest that there had been a recent antigen exposure with effectors still present in the blood.

1.9.4 Responses to phosphopeptides in patients with cancer

The previous study described above also demonstrated that phosphopeptide immunity is severely compromised in patients with AML or CLL. Of the 12 AML patients analyzed, 10 patients had limited day 7 ELISpot responses to 12 AML-associated phosphopeptides, while 2 patients had responses comparable to those of healthy donors [2]. Of the 14 CLL patients analyzed, 9 patients had limited responses to 12 CLL-associated phosphopeptides, while 5 patients had responses comparable to those of healthy donors [2]. CD3 stimulation confirmed that the donors were not immune incompetent, and *in vitro* treatment with IL-2 suggested that limited responses were not due to anergic T cells as IL-2 would have been expected to revive functionality if anergy was the problem [2]. Instead, limited responses were hypothesized to be due to either cancer-induced deletion of phosphopeptide-specific cells or to a lack of prior exposure to phosphopeptides. However, the MHC-phosphopeptide repertoires on these patients' tumors were unknown, and, thus, there was insufficient data to address the reason for limited reactivity in patients.

1.10. Thesis Rationale

There is increasing evidence that healthy individuals develop "pre-existing immune memory" to cancer antigens in the absence of clinically-evident tumors. At the time this project was initiated, memory responses had been demonstrated to 2 HLA-B7-restricted phosphopeptides in 2 healthy donors and 1-2 HLA-A2-restricted phosphopeptides in 4 healthy donors, but the methodologies utilized did not analyze whether responses to most other phosphopeptides were also from memory cells. Elevated responses could have also been due to effector cells or naïve cells if there was a high enough naïve precursor frequency as has been demonstrated for MART-1 [358]. If elevated phosphopeptide responses were predominantly derived from pre-existing memory, this would indicate that healthy individuals had prior immunogenic exposures to these cancer-expressed antigens, possibly through immune surveillance. If elevated immunity was due to effector responses, it would indicate that healthy individuals had recent or ongoing immunogenic exposures to phosphopeptides. In conjunction with this, it was unknown whether exposures to any particular phosphopeptides are prevalent in the population, suggesting a common underlying mechanism of display. In association with this, these responses could also have been due to virus-infected or virally transformed cells. Given the number of phosphopeptides we had identified on Epstein-Barr virus (EBV) transformed cells, and pervasive chronic EBV infection and immunity in most human populations, we wanted to test the hypothesis that exposure to EBV provided a common source of phosphopeptide antigens that most people encounter. These issues are all addressed in **Aim 1**.

When this project was initiated, we also knew that responses to phosphopeptides that were targets of elevated responses in healthy donors were compromised or absent in

AML and CLL patients [2]. One possible explanation is that patients have not been previously exposed to phosphopeptides or that their ability to generate phosphopeptide specific responses is impaired. A second possibility is that phosphopeptide-specific immune responses are initially evident and lost over time due to tumor-intrinsic factors, including chronic antigen exposure and an immunosuppressive tumor microenvironment. However, it was unknown whether any of the analyzed phosphopeptides were expressed on any patient's tumor. Finally, the tumor microenvironments are quite distinct between solid and liquid malignancies, and it was unknown whether diminished phosphopeptide immunity would extend to patients with solid malignancies. These issues are addressed in **Aim 2**. Regardless, it was also important to determine whether phosphopeptide-specific responses could be induced or augmented in patients by vaccination. This issue is addressed in **Aim 3**.

1.11. Specific Aims

1.11.1 Aim 1: To characterize the elevated immune responses to cancer-expressed HLA-A2- and HLA-B7-restricted phosphopeptides evident in normal donors

Aim 1A. To test the hypothesis that elevated immune responses to HLA-A2 and HLA-B7-restricted phosphopeptides in healthy donors reflect pre-existing immune memory.

Aim 1B. To test the hypothesis that phosphopeptide-specific $CD8^+$ T cells are predominantly central memory cells but show discrete timepoints of effector memory and effector cells, consistent with recent or ongoing antigen exposures.

Aim 1C. To test the hypothesis that memory responses to HLA-A2 and HLA-B7-restricted phosphopeptides correlate with phosphopeptide association with EBV.

Aim 1D. Determine if there are immunodominant memory targets that are recognized by the majority of healthy donors.

1.11.2 Aim 2: To test the hypothesis that responses to phosphopeptides in patients with solid tumor malignancies are compromised.

Aim 2A: Determine if phosphopeptide responses are impaired in patients with solid tumor malignancies compared to healthy donors.

Aim 2B: Determine if patients respond to phosphopeptides expressed on their tumors.

1.11.3 Aim 3: To test the hypothesis that responses to phosphopeptides in cancer patients can be induced or augmented with vaccination.

Successful completion of this thesis work will establish the basis for responses in healthy donors to cancer-expressed phosphopeptides and the basis for compromised immunity in cancer patients. This work will suggest mechanisms by which immunity is induced in healthy donors and compromised in cancer patients. For the first time, we will assess the possibility of vaccine therapy as a method of inducing responses in patients.

Chapter 2: Materials & Methods

2.1 Reagents

2.1.1. Peptides: MHC-presented phosphopeptides previously identified on cancer cells lines and/or primary tumor cells by mass spectrometry (**Table 2.1**) were purchased from GenScript. Lyophilized peptides were stored at -80°C until reconstituted to 10mg/ml in dimethyl sulfoxide (DMSO) and stored in a desiccator at room temperature. Stock aliquots of individual peptides or combinations of peptides were diluted in Dulbecco's Phosphate Buffer Solution (DPBS) to 200μg/ml and stored at -80°C.

2.1.2. Cytokines: Human IL-2 (Cat#202-IL), GM-CSF (Cat#215-GM), IL-4 (Cat#204-IL), IL-6 (Cat#206-IL), and TNF-α (Cat#210-TA) were from R&D. IL-1β (Cat#AF-200-01B), IL-7 (Cat#200-07), and IL-15 (Cat#200-15) were from PeproTech. Prostaglandin E_2 (Cat#0409) was from Sigma.

2.2 Cell Lines

2.2.1 T2 and T2-B7 Cells: A transfectant of the antigen-processing mutant cell line, CEMx721.174.T2 (HLA-A2$^+$ T2 cell line) [359], expressing HLA-B7 (T2-B7 cell line) was kindly received from Dr. Charles Lutz (University of Kentucky). Low-passage T2 or T2-B7 cells were grown at 37°C with 5% CO_2 in RPMI-1640 (Corning), 2mM L-glutamine (Fisher), 1% Penicillin-Streptomycin (Invitrogen), and 10% fetal bovine serum (FBS) (Sigma). 150μg/ml Hygromycin (Fisher) was used as a selective reagent for surface expression of HLA-B7. Cells were regularly assessed by flow cytometry for expression of HLA-A2 (clone BB7.2, BD Cat#BDB561341) and HLA-B7 (clone BB7.1, Novus Biologicals Cat#NB10064159Q or clone REA176, Miltenyi Cat#130-118-471).

2.2.2 K562 Cells: A transfectant of the K562 cell line expressing HLA-A*02:01, CD80, CD83, and CD137L/4-1BBL was a kind gift from Dr. Naoto Hirano (University Health Network, Princess Margaret Cancer Center). K562 cells were grown at 37°C with 5% CO_2 in RPMI-1640 (Corning), 50ug/ml Gentamicin (Fisher), and 10% FBS (Sigma). K562 cells were assessed by flow cytometry for expression of HLA-A2 (clone BB7.2, BD Cat#BDB561341), CD54 (clone HA58, Biolegend), CD58 (clone TS2/9, Biolegend), CD80 (clone 2D10, Biolegend), CD83 (clone HB15e, BD Biosciences), and CD137L (clone 5F4, Biolegend). All cells were confirmed as mycoplasma negative by PCR (ATCC 30-1012K: Universal Mycoplasma Detection Kit) before freeze, after thaw, and intermittently while in culture.

2.3 Human Studies

2.3.1 Human blood and tissues: Healthy donor and patient-derived specimens were collected after informed consent following protocols (#3467 and #10598) approved by the University of Virginia Institutional Review Board for Health Sciences Research.

2.3.2 Healthy donors: Blood was collected from 15 healthy donor volunteers (**Table 2.2**). Inclusion criteria for the study were: ability and willingness to give informed consent, age 18 years and older, no current or past cancer diagnosis, and healthy/free from known illness at all times of blood collection.

2.3.3 Ovarian cancer patients: Blood and tumor specimens were collected from 48 patients at the University of Virginia from August 2017 through December 2019. Tumor specimens and immune responses were analyzed in 10 patients that met all criteria for continuation in the study (**Table 2.3**). Inclusion criteria were as follows: ability and willingness to give informed consent to data collection, blood collection, tumor collection

at time of surgery, and HLA genotyping, age 15 years and older, suspected ovarian cancer, and patient without prior surgical removal of tumor. For continuation in the study, patient had to have a confirmed pathologic diagnosis of epithelial ovarian cancer following surgery, be determined as HLA-A02:01^{+} and/or HLA-B07:02^{+} by the American Red Cross HLA genotyping, and express continued consent. Exclusion criteria were as follows: unable or unwilling to consent to any of the study criteria. Blood was collected at the time of normal clinical care phlebotomy at one or more of the following disease/treatment timepoints: prior to surgery, post-surgery, post-chemotherapy, and at disease recurrence. Ascites samples were collected for research if/when removed as part of normal clinical care. Tumor specimens were collected at time of surgery. Specimens collected for immune response analyses were frozen in vapor-phase liquid nitrogen until all samples from the patient could be analyzed in the same experiment.

2.3.4 Melanoma patients: See “2.12.1 Patients” (**Table 2.4**).

2.4 Human tissue processing

2.4.1 Isolation of cells from whole blood: Whole blood was collected in sodium heparin vacutainer tubes (BD #366480) and separated immediately using LymphoPrep SepMate (Stem Cell #07801) per the manufacturer’s instructions. Peripheral blood mononuclear cells (PBMCs) were washed in DPBS and frozen in 90% Human AB^{+} serum (Gemini #100-512)/10% DMSO in gas-phase liquid nitrogen until use. In some experiments, prior to freezing, monocytes were isolated from PBMCs by adherence to plastic or using the Human CD14 Positive Selection Kit (Miltenyi #130-050-201). The non-adherent or CD14^{neg} peripheral blood lymphocyte (PBL) fraction was kept frozen in vapor-phase liquid nitrogen until use.

2.4.2 Isolation of cells from solid tumors: Tumors were collected in RPMI-1640 (Corning) and stored at 4°C until processing. Tumor pieces were cut into 1-3mm^3 pieces, digested in tumor medium: RPMI-1640 (Corning), 2mM L-glutamine (Fisher), 1% Penicillin-Streptomycin (Invitrogen), and 2.5μg/ml Amphotericin B (Gibco #15290-018)] with 35.8U/ml Dispase (Gibco #17105-041) for 30 minutes at 37°C with 5% CO_2, swirling every 5-7 minutes as described [360, 361]. Samples were washed twice in cold tumor medium/10% FBS. Samples were incubated with DNase I (40U/ml) (Fisher #NC9709009) at 37°C for 10 minutes [362]. Cells were washed twice in cold tumor medium/10% FBS and filtered through 70 μm nylon mesh, using the end of a plunger to create a single cell suspension. Isolated cells were frozen in 90% human AB$^+$ serum/10% DMSO in vapor-phase liquid nitrogen until all patient samples were ready to be analyzed in parallel.

2.4.3 Isolation of cells from ascites: Ascites were kept on ice until processing. Cells were collected by centrifugation at 3000xg for 20 minutes at 4°C and washed in cold tumor medium. Samples were incubated with DNase I (40U/ml) at 37°C for 10 minutes and washed twice with tumor medium/10% FBS and filtered through 70 μm nylon mesh, using the end of a plunger to create a single cell suspension. Isolated cells were frozen in 90% human AB$^+$ serum/10% DMSO in vapor-phase liquid nitrogen.

2.4.4 Generation of monocyte-derived dendritic cells: Monocytes were matured into dendritic cells as described [363]. Briefly, monocytes were supported with GM-CSF (1,500U/mL) and IL-4 (2,900U/mL). Cultures were supplemented with additional GM-CSF and IL-4 on day 3 or 4. On day 6 or 7 immature dendritic cells were removed, washed and reseeded with GM-CSF (800U/mL), IL-4 (500U/mL), IL-6 (1,000U/mL), IL-1β

(10ng/mL), TNFα (10ng/mL), and PGE_2 (1μg/mL). Mature dendritic cells (mDCs) were harvested at day 10 and frozen until ready to use in culture.

2.5 Enrichment of T cells

2.5.1 Enrichment of $CD8^+$ T cells from healthy donors and from peripheral blood of patients: Previously frozen PBMCs or PBLs were thawed and rested overnight at 37°C with 5% CO_2 in culture medium: AIM-V media (Gibco #12055083), 5% human AB^+ serum (Gemini #100-512), 1% L-glutamine (Fisher), 1.5% HEPES (Gibco), 0.1% β-mercaptoethanol (Sigma M3148). Cells were supported with low dose IL-7 (1ng/ml). The following day, $CD8^+$ T cells were resuspended in MACS Buffer (PBS, 0.1% BSA, and 2mM EDTA at pH 7.2 and filtered) and isolated by negative depletion with a Human CD8 T cell isolation kit (Miltenyi #130-096-495) following the manufacturer's instructions and filtered through 70μm nylon mesh. In most experiments, antigen-experienced $CD45RO^+CD8^+$ T cells were isolated by positive selection using anti-CD45RO microbeads (Miltenyi #130-046-001). In one experiment, $CD45RA^+CD8^+$ and $CD45RA^-CD8^+$ T cells were isolated using anti-CD45RA microbeads (Miltenyi #130-094-412).

2.5.2 Enrichment of $CD8^+$ T cells from tumors: Frozen single cell suspensions of tumor specimens were thawed and treated with DNase I (40U/ml) for 10 minutes at 37°C, washed twice with tumor medium/10% FBS, and rested overnight in culture medium with low dose IL-2 (20IU/ml) [151]. $CD8^+$ T cells were resuspended in MACS Buffer and were either isolated by positive selection with a human CD8 T cell isolation kit (Dynabead #11333D) or by Fluorescence Activated Cell Sorting (FACS).

2.5.3 Fluorescence activated cell sorting (FACS): Previously frozen PBMCs or PBLs were thawed and rested overnight in culture medium with low dose IL-7 (1ng/ml).

Previously frozen tumors were thawed, treated with DNase I as described in **2.5.2**, and rested overnight in culture medium with low dose IL-2 (20IU/ml). Cells were washed, filtered through 70μm nylon mesh, and resuspended in sorting medium: RPMI-1640 lacking phenol red (Gibco), with 1% L-glutamine and 2% human AB^+ serum. Cells were incubated with Fc block (BD Pharmingen #564220) for 10 minutes at room temperature, washed, and stained for 20 minutes at 37°C followed by 10 minutes at 4°C. 7-AAD (BioLegend #420403) was added immediately before sorting on the Influx Cell Sorter (BD Model 646500). $CD8^+$ T were gated as live, singlet, lymphocyte, $CD3^+CD8^+$ cells and sorted on CCR7 and CD45RO, using "Fluorescence Minus One" (FMOs) to set gating as described [88] (**Figure 2.1**). To isolate naïve cells from memory stem cells, the $CCR7^+CD45RO^-$ subset was further gated on CD95 (**Figure 2.1**). In patients, a similar sorting strategy was used, except that cells were collected as 2 subsets instead of 4-5 subsets as done in the healthy donors (**Figure 2.2**). Fluorophore-conjugated antibodies specific for human cell-surface antibodies are as follows: CD3 (UCHT-1) and CCR7 (150503) from BD Pharmingen and CD8 (RPA-T8), CD45RO (UCHL1), and CD95 (DX2) from BioLegend. Sorted cells were collected in AIM-V media, 20% human AB^+ serum, 1% L-glutamine, 1.5% HEPES (Gibco), 0.1% β-mercaptoethanol, and 1% Pen/Strep. Flow cytometry files were analyzed using FlowJo version 10.7.1 (Treestar).

2.6 T Cell Cultures

2.6.1 Stimulation of $CD8^+$ T cells: Mature dendritic cells (mDCs) and PBMCs, PBLs, or TILs were thawed and rested overnight prior to stimulation. TILs were thawed with DNase treatment as described (**2.5.2**). On the day of stimulation, $CD8^+$ T cells or subsets thereof were isolated. Autologous mDCs were irradiated at 3000 rads, washed, and then peptide

pulsed (10μg/ml each of 1-9 peptides) with 3μg/ml beta-2-microglobulin (β_2m) (Millipore Sigma #47-582-3250) for at least 2 hours. The mDCs were washed two times before being co-cultured with CD8$^+$ T cell subsets in a 1:10 ratio (10,000 mDCs:100,000 T cells or when stimulating CD8+ T cells without further subset isolation, 30,000 mDCs:300,000 T cells) in culture medium with IL-7 (10ng/mL) at 37°C with 5% CO_2. Due to limited numbers of patient-derived mDCs, patient CD8$^+$ T cell subsets were co-cultured with autologous peptide-pulsed mDCs plus autologous peptide-pulsed CD4$^-$CD8$^-$ PBLs (irradiated at 4000 rads). Three days later, IL-7 (10ng/mL) and IL-15 (10ng/mL) were added. On day 7 and then as needed, the media was replaced with fresh media and IL-7 and IL-15. Patient TIL-derived CD8$^+$ T cells were cultured 6,000U/ml IL-2 instead of IL-7 and IL-15. As a negative control, CD8$^+$ T-cell subsets were co-cultured with mDCs pulsed with Ebola peptide, NP_{44-52} or $NP_{294-302}$, and then analyzed for responses at days 7, 10, or 14. No responses were detected in any donor to Ebola, demonstrating that the assay assessed memory responses and did not induce *de novo* responses.

2.6.2 Cell counting: The viability and total number of viable cells were measured using a hemocytometer and Trypan Blue staining or by ViaCount staining on the Guava easyCyte flow cytometer (Luminex Corporation).

2.7 T Cell Functional Assays

2.7.1 Assessment of immunocompetency: The immunocompetency of healthy donors and patients was assessed by response to PMA (3ng/ml) plus Ionomycin (3uM) stimulation and to highly conserved HLA-A2 or HLA-B7 restricted viral antigens, such as those derived from Influenza, Epstein Barr Virus (EBV), and Cytomegalovirus (CMV). We

would expect that all patients would have responses to PMA/Ionomycin stimulation and to antigens from at least two common viruses.

2.7.2 Assessment of phosphopeptide-specific responses by IFNγ ELISpot assay: Responses were assessed either directly *ex vivo* ("direct") or after one *in vitro* stimulation with peptide-pulsed antigen-presenting cells and 14-day culture with cytokines ("cultured"). T2-B7 target cells were pulsed with 3μg/ml β_2m and 10μg/ml peptide or 0.1% DMSO alone for 2-4 hours at room temperature on a rocker. IFNγ Single-Color ELISpot Plates (Cellular Technology Limited #hIFNγ-1M/2) were activated with 70% ethanol and incubated with primary antibody at 4°C overnight. Isolated subsets of $CD8^+$ T cells (25,000 cells), either direct or cultured, were plated with 75,000 target cells per well, incubated at 37°C in 5% CO_2 for 18-20 hours, and developed per the manufacturer's instructions. Spots were counted using a BioReader (BioSys Models 4000 and 7000F). Responses were determined in triplicate and quantified as the mean of peptide-pulsed over DMSO-pulsed target cells. Positive responses met 3 criteria: mean of response was at least 10 spot-forming cells (SFCs)/25,000 cells over mean of background, mean of response was at least two times the mean of the background, and the standard deviations of the response and background samples were non-overlapping. Responses meeting these 3 criteria invariably had p values of <0.05 using Unpaired Student's t test.

2.7.3 Calculation of total responses apportioned among T cell subsets: We calculated the total number of $IFN\gamma^+$ cells per 10^6 $CD8^+$ T cells for each subset, and the fraction that each subset contributed to the overall cultured and direct response. First, the total number of $IFN\gamma^+$ cells in each culture was calculated from the number of $IFN\gamma^+$ SFCs over background per 25,000 cells in the ELISpot assay (Equation 1). For cultured samples, this

number was multiplied by the total number of recovered cells in the culture (Equation 1). We normalized the number of IFNγ$^+$ cells based on the T cell subset's percentage out of CD8$^+$ in the donor's blood, determined by flow cytometry (Equation 2). The percentage contribution of each subset to the total response was calculated after summing these values for all subsets.

(1) Total # IFNγ$^+$ cells = [Mean$_{peptide}$ SFC – Mean$_{background}$ SFC]/25,000 cells X *Total # cultured cells recovered (Cultured samples only)*

(2) For each T cell subset: # IFNγ$^+$/10^6 CD8$^+$ T cells = Total # IFNγ$^+$ cells X # subset T cells in 10^6 initially sorted CD8$^+$ T cells

2.8 Kinase recognition motifs

2.8.1 Kinase recognition motifs: Peptide sequences +/- 20 amino acids were compiled using BLASTp [364]. The kinase recognition motifs (KRMs) that could generate each phosphosite were determined using: http://hprd.org/PhosphoMotif_finder, selecting for "Serine/Threonine motifs" and "Kinase Phosphatase motifs" [365]. For nested KRMs, the analysis of enrichment of memory targets was performed for each set of nested motifs, from most inclusive to most restrictive.

2.9 Isolation of HLA-associated peptides

2.9.1 Isolation of HLA-associated peptides: Class-I HLA molecules were immunoaffinity purified from samples, and phosphopeptides were isolated as previously described [366]. Cells from ascites were collected by centrifugation at 3000xg for 20 minutes at 4°C and washed in cold tumor medium. Samples were incubated with DNase I at 37°C for 10 minutes and washed twice with tumor medium/10% FBS and filtered through 70 μm nylon mesh, using the end of a plunger to create a single cell suspension.

Cells were washed twice with cold DPBS, pelleted in a 50ml conical tube, and frozen at -80°C. Tumor pieces were cut into 1-3mm^3 pieces and frozen in a 50ml conical tube at -80°C. Cells (3-4 x 10^9) or 1 gram of tissue were lysed in a solution containing 20mM Tris-HCl, pH 8.0, 150mM NaCl, 1% CHAPS, 5mM EDTA, and 1mM PMSF. Phosphatase inhibitors (Sigma-Aldrich, Cocktail II Cat #P5726, Cocktail III Cat#P0044) were used at 1:100 dilution to prevent dephosphorylation during extraction. Lysates were centrifuged for 1 hour at 100,000×g at 4°C and subsequent supernatant added to anti-HLA-A,B,C antibody (clone W6/32, Bio X Cell Cat#BE0079) bound to NHS-activated Sepharose 4 Fast-Flow (GE Cat#17-0906-01) beads. After rotation overnight at 4°C, beads were transferred to poly-prep columns (Bio-Rad Cat#731-1550) and serially washed once with Lysis buffer, twice with 20mM Tris-HCL, 150mM NaCl, pH 8.0, twice with 20mM Tris-HCL, 1M NaCl, pH 8.0, and 3 times with 20mM Tris-HCL, pH 8.0. Contents were transferred to Amicon Ultra-4 centrifugal filters (Millipore #UFC801024) and centrifuged at 5,000×g at 4°C for 30-60 minutes until all fluid was removed from beads. Samples were then subjected to Liquid Chromatography/MS and phosphopeptide enrichment as described [366].

2.10 Multimers and Flow Cytometry

2.10.1 Multimers to identify antigen-specific CD8$^+$ T cells: Phycoerythrin- (PE) or Allophycocyanin (APC)-conjugated tetramers for viral antigens were used in preliminary experiments (MBL International Corporation). MHC-I monomers conjugated with phosphopeptides or viral control peptides were obtained from the NIH Tetramer Core Facility at Emory and conjugated per the manufacturer's protocol into dextramers using PE- or APC-conjugated "Klickmers" (Immudex).

2.10.2 Multimer staining: The protocol was first optimized using viral control peptides, such as Influenza M1, CMV pp65, and EBV BMLF1. Peptide-MHC I multimer specific for the Ebola NP peptide was used as a negative control to set the background of non-specific labeling of cells by the multimer. Antigen-specific $CD8^+$ T cells were enriched from previously frozen PBMCs, rested overnight in low dose IL-7 (1ng/ml), or from $CD8^+$ T cells isolated the next day. Cells were incubated in complete RPMI (RPMI, 5% human AB+ serum, 1% L-glutamine, 1% Pen/Strep, and 1.5% HEPES) plus 50nM of the protein kinase inhibitor dasatinib at 37°C for 45 minutes, then washed in MACS Buffer (PBS, 1% L-glutamine, 1% sodium pyruvate, 1% non-essential amino acids, 2% essential amino acids, dextrose, 1% bovine serum albumin, 0.5mM EDTA, and 0.2% sodium azide, pH 7.4 and 0.2µm filtered) or FACS Buffer depending on whether the samples were going to be sorted using magnetic columns or if the samples were going to be analyzed by flow cytometry. Cells were incubated with Fc block (BD Pharmingen #564220) for 10 minutes at room temperature, washed, and stained with multimers. (In most cases, phosphopeptide-specific cells were labeled on both PE and APC to enhance confidence of true antigen-specific T cells, which would be double positive.) Multimer staining was performed using one of three different methodologies: 2 hours at room temperature, 10 minutes at room temperature then 20 minutes at 37°C, or 30 minutes at 37°C. For samples to be analyzed by flow cytometry, samples were washed, and then incubated with Zombie Aqua (Life Technologies Cat# L34957) for 15 minutes at 4°C for discrimination of dead cells. Cells were fixed in 2% para-formaldehyde at room temperature for 10 minutes. Fluorophore-conjugated antibodies specific for human cell-surface antibodies are as follows: CD3

(clone SK7), CD8 (RPA-T8), CD4 (clone RPA-T4), CD14 (clone M5E2), CD16 (clone 3G8), and CD19 (clone HIB19) from BD Biosciences.

2.10.3 Flow cytometry: Antigen-specific $CD8^+$ T cells were identified by gating on singlet live lymphocytes that were Dump Gate $(CD4/CD14/CD16/CD19)^{neg}$, $CD3^+$, $CD8^+$, and $multimer^+$. Gates were set based on FMOs. Samples were analyzed on the BD Fortessa, Cytek Aurora, or BD Cytoflex.

2.10.4 Multimer-enrichment: After multimer staining, samples were resuspended in 2ml of MACS Buffer. Ten percent of the sample, 200µl, were saved as the “pre-enriched” fraction. The remaining 90% of the sample was washed and resuspended in 200µl MACS Buffer. Both anti-PE microbeads (Miltenyi #130-048-801) and anti-APC microbeads (Miltenyi #130-090-855 Lot#) were added simultaneously because two antigen specificities were co-staining. Samples were incubated with anti-PE and anti-APC microbeads in the dark for 20 minutes at 4°C, washed with MACS Buffer, resuspended in 2ml AutoMACS running buffer, and sorted on the AutoMACS (Miltenyi, AutoMACS Pro 590) “Positive Selection – Sensitive” program. If enriched cells were going to be analyzed by flow cytometry, Fc block, antibody staining, and viability dye staining would occur after collection from the AutoMACS as described (**2.10.2**). Cells were fixed in 2% para-formaldehyde at room temperature for 10 minutes. To calculate the precursor frequency of antigen-specific T cells:

$$= [\text{Number of Multimer}^+ \text{ cells in enriched sample}] / [\ \#\ \text{CD8}^+ \text{ T cells in pre-enrichment sample} * 10]$$

2.11 Analyses

2.11.1 Statistical analyses: Statistical analyses were performed in GraphPad Prism version 9.0.0. A p value < 0.05 was considered significant for Unpaired Student's t test. To evaluate the levels of viral responses in patients to those in healthy donors, Welch's t test was performed. Correlations for donor age and percentage of phosphopeptides recognized were analyzed by calculating a Pearson R value. Ninety-five percent confidence intervals (95% CI) for proportions were calculated using the Wilson-Brown Method.

2.12 Clinical trial

2.12.1 Patients: Patients with resected AJCC stage IIA–IV (V.7) melanoma [367] were eligible, as well as patients with disease who failed other approved therapies. However, all who enrolled were clinically free of disease. Inclusion criteria included: HLA-A2 expression, ages 18 years and above, Eastern Cooperative Oncology Group performance status 0–1, adequate liver and renal function, and ability to give informed consent. Exclusion criteria included: pregnancy; cytotoxic chemotherapy, IFN-therapy, immune-modulatory therapies, or radiation within the preceding month; known or suspected allergies to vaccine components; multiple brain metastases; use of steroids; Class III–IV heart disease; systemic autoimmune disease with visceral involvement or uncontrolled diabetes (hemoglobin A1C ≥7%) (**Table 2.4**).

2.12.2 Vaccine preparation and administration: $pBCAR3_{126-134}$ (IMDR(pT)PEKL) and $pIRS2_{1097-1105}$ (RVA(pS)PTSGV) were synthesized and purified (>95%) by PolyPeptide Laboratories (San Diego, California, USA), solubilized in aqueous solution, sterile-filtered, vialed in sterile single-use vials, lyophilized and stored at −80°C. The tetanus helper peptide AQYIKANSKFIGITEL (P2830-844, modified by adding alanine to the N-terminus) [142, 368], was prepared, sterile filtered, vialed and lyophilized under Good

Manufacturing Practice conditions as described [142, 369]. Vials were submitted for quality-assurance studies including sterility, identity, purity, concentration, general safety, pyrogenicity and stability in accordance with Code of Federal Regulations guidelines and BB-IND#15 134. Polyinosinic-polycytidylic acid stabilized with poly-L-lysine and carboxymethylcellulose (poly-ICLC, Hiltonol; Oncovir, Washington, D.C., USA) was provided by the Ludwig Institute (New York, New York, USA) as a clinical grade reagent. Each vaccination consisted of 100µg of either or both phosphopeptides and 200µg of the tetanus peptide in a water-in-oil emulsion with an equal volume of an incomplete Freund's adjuvant (IFA) (Montanide ISA-51 VG adjuvant, Seppic, Paris, France). Vaccines were administered subcutaneously (0.5 mL) and intradermally (0.5 mL) at one skin puncture site. One mg of poly-ICLC was administered immediately thereafter by a separate 0.5 mL injection (0.25 mL subcutaneously and 0.25 mL intradermally) into the precise sites where the peptide emulsion was given. Vaccine sites were rotated to different skin regions on each vaccination date, using a minimum of two separate extremities. Vaccines were administered in two treatment cycles (**Figure 2.3**).

2.12.3 Clinical trial design: This was an open-label, pilot, proof-of-concept study to assess phosphopeptide vaccine safety and immunogenicity. It was designed with three arms to assess each phosphopeptide individually before assessing the combination: Arm A, $pBCAR3_{126\text{-}134}$ only; Arm B, $pIRS2_{1097\text{-}1105}$ only; and Arm C, $pBCAR3_{126\text{-}134}$ + $pIRS2_{1097\text{-}1105}$. Arms A and B were restricted to patients with stage IIIB–IV melanoma (at original presentation or at recurrence, with or without measurable disease). Once safety was evident for each peptide in these higher-risk patients, Arm C was open for stage IIIB–IV patients and also for patients with lower risk (stage IIA–IIIA) melanoma rendered clinically free of

disease by surgery, other therapy or spontaneous remission. Maximum target accrual was set at 21 eligible participants who received a vaccination. Provided safety rules were met, immunogenicity would be assessed for each phosphopeptide in up to 12 evaluable total participants.

2.12.4 Toxicity assessment: The study was monitored continuously for treatment-related adverse events. Adverse events were described and coded based on NCI CTCAE v4.03. A dose-limiting toxicity (DLT) was defined as any unexpected adverse event that is possibly, probably or definitely related and: (1) ocular ≥grade 1, (2) non-hematologic/ non-metabolic ≥Grade 3, or (3) hematologic/metabolic ≥Grade 3. Toxicities with known or likely autoimmune features and affecting vital organs, including colitis, hepatitis, hypophysitis, uveitis and adrenal insufficiency, were recorded and assessed with special interest. These were considered DLTs if ≥grade 2 and probably or definitely related. Grade 2 autoimmune toxicities involving these organs/tissues were not considered DLTs if only possibly related. Vitiligo was not considered a DLT, but was recorded. Hyperthyroidism or hypothyroidism was considered a DLT if ≥grade 3. All vaccinated participants were followed for a minimum of 4 weeks for assessment of DLTs.

2.12.5 Safety assessment: The number of vaccinated participants who experienced DLTs guided decisions about safety. In the initial safety phase for arm A or Arm B, one participant was accrued. If that subject did not experience a DLT within 4 weeks, 2 additional participants were accrued. If 0/3 DLTs were observed, then the initial safety criteria for the arm were satisfied; if ≥2/3 participants experienced a DLT, then accrual to that arm would be halted permanently; otherwise three additional participants would be accrued to the arm to assess safety. Accrual to arm C occurred only after the initial safety

criteria for arms A and B were satisfied. A maximum of nine participants were accrued in sequential cohorts of 3. Accrual continued as long as no more than 33% of participants experienced a DLT.

2.12.6 ELISpot analysis of T-cell response to phosphopeptides: Blood (100–140 mL) was drawn on days indicated in (**Figure 2.3**). PBMCs were isolated from 80 mL (except 120 mL week 0) using Ficoll gradient centrifugation and cryopreserved in 10% dimethyl sulfoxide/90% serum. Thawed PBMCs were incubated 2 hours at 37°C with 40µg/mL each of pBCAR3126-134, pIRS21097-1105 and pS33-βcat30-39 (control), washed to remove unbound peptide, and resuspended in medium containing 10ng/mL recombinant human IL-7 (Peprotech, Rocky Hill, New Jersey, USA) for 3 days. New medium containing recombinant human IL-7 plus 10ng/mL IL-15 (Peprotech) was added on days 3, 7 and 10. Cells were assayed on day 14 for IFNγ production by ELISpot assay using methods and criteria described previously [370, 371]. Briefly, T2-B7 cells were pulsed with each of the three phosphopeptides for each assay date. Negative controls included no peptide, irrelevant peptide (HIV gag peptide restricted by HLA-A2; Atlantic Peptides, LLC, Lewisburg, Pennsylvania, USA), and positive controls included PMA-ionomycin, PHA. Responses were corrected for the fraction of $CD8^+$ T cells in PBMC as determined by flow cytometry of individual samples at culture initiation. T-cell responses were calculated using the following definitions: Nvax=number T-cells responding to vaccine peptide; Nneg=number T-cells responding to maximum negative control; Rvax=Nvax/ Nneg. A patient was considered to have a T-cell response to vaccination (binary yes/no), only if all of the following criteria were met: (1) Nvax exceeded Nneg by at least 100 SFCs/100,000 $CD8^+$ T cells, (2) (Nvax – 1 SD) ≥ (Nneg +1 SD) and (3) Rvax after vaccination ≥2 x Rvax

pre-vaccine. Pre-vaccine Rvax values less than one (e.g., control counts exceed number of responding T-cells) were set equal to one to indicate no response and to prevent overinflating adjusted fold-increases due to pre-vaccine ratios less than one or division by zero. Inter-assay coefficients of variation (CVs) were calculated for the response of 2 normal donors to a mixture of viral peptides (CEF peptide pool, Proimmune, Sarasota, Florida, USA): for the high responder, mean number of spots per 100,000 cells was 309, and CV was 29%.

2.12.7 Statistical assessment of immune response: Since this was a first-in-humans clinical trial with phosphopeptide antigens for cancer, a first goal was to assess safety with a small study. The biological primary endpoint was evidence of immunogenicity. The prespecified level of interest was two or more patients with $CD8^+$ T cell responses. Beyond that, CI for the observed proportions would determine how much the data supported immunogenicity. Assuming that the response to one phosphopeptide is not affected by the presence of the other for those in arm C, the immunogenicity of each phosphopeptide was based on the total number of participants vaccinated with the specific phosphopeptide, alone or in combination with the other (i.e., Arm A+Arm C for $pBCAR3_{126-134}$; Arm B+Arm C for $pIRS2_{1097-1105}$). Based on the upper limit of a one-sided 90% CI, if the upper limit of the observed bound was >35% to any single phosphopeptide, we would conclude that the phosphopeptides was immunogenic and worthy of further study. To define the $CD8^+$ T cell response rate with higher precision would require a much larger sample size, which was not indicated in this first-in-humans trial.

2.12.8 Analysis of exploratory endpoint: All participants were clinically free of disease at enrollment. Disease-free survival (DFS) is defined as the time from on-study until the

earliest documentation of a new metastasis or death from any cause, or censored at the date of last follow-up for those without a documented event. Overall survival is defined as the time from on-study to death from any cause, or censored at the date of last follow-up for those still alive.

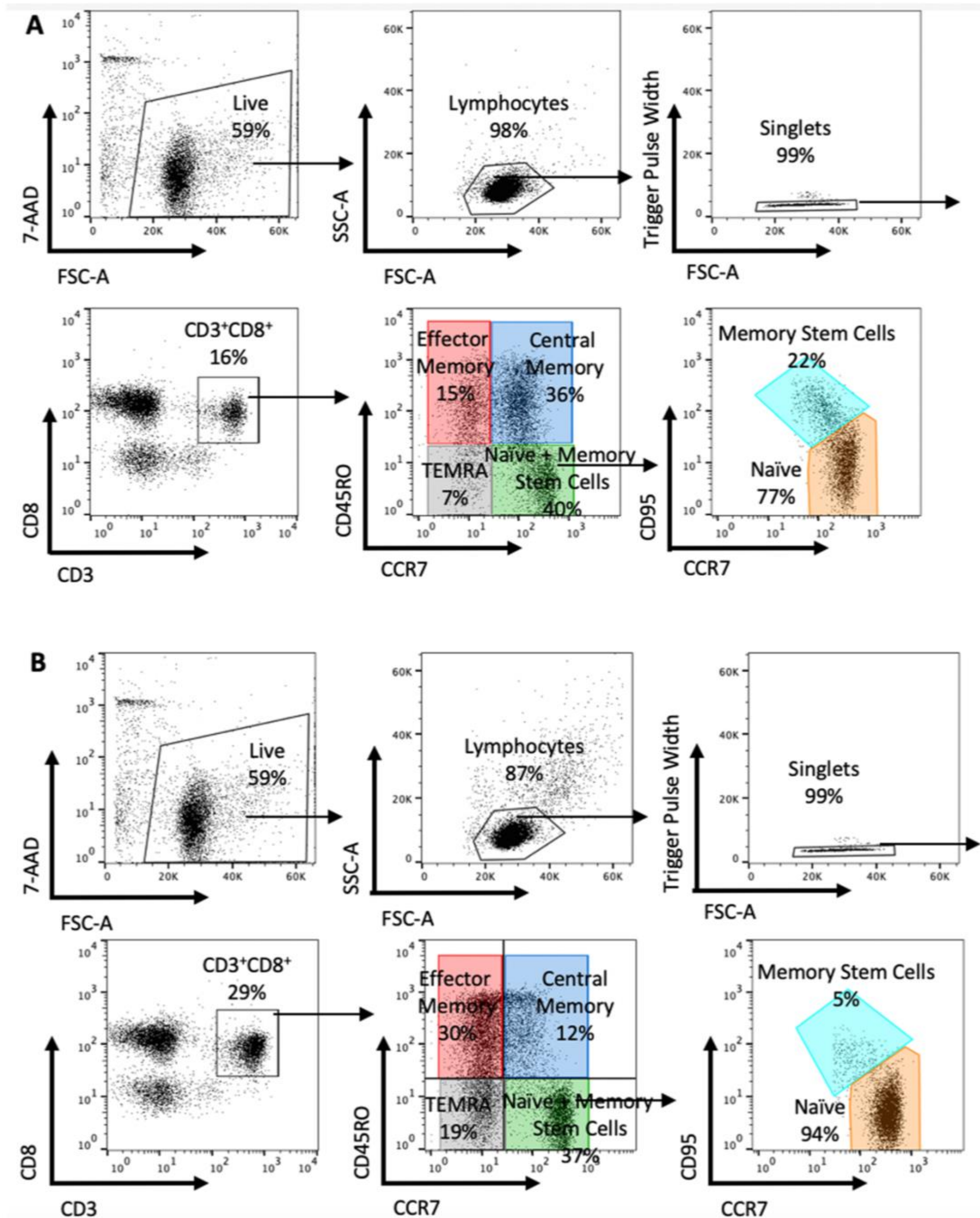


Figure 2.1. FACS sorting strategy to isolate subsets of CD8^{+} T cells in healthy donors. Representative FACS sorting strategy of PBMCs from **A,** HD43 and **B,** HD44.

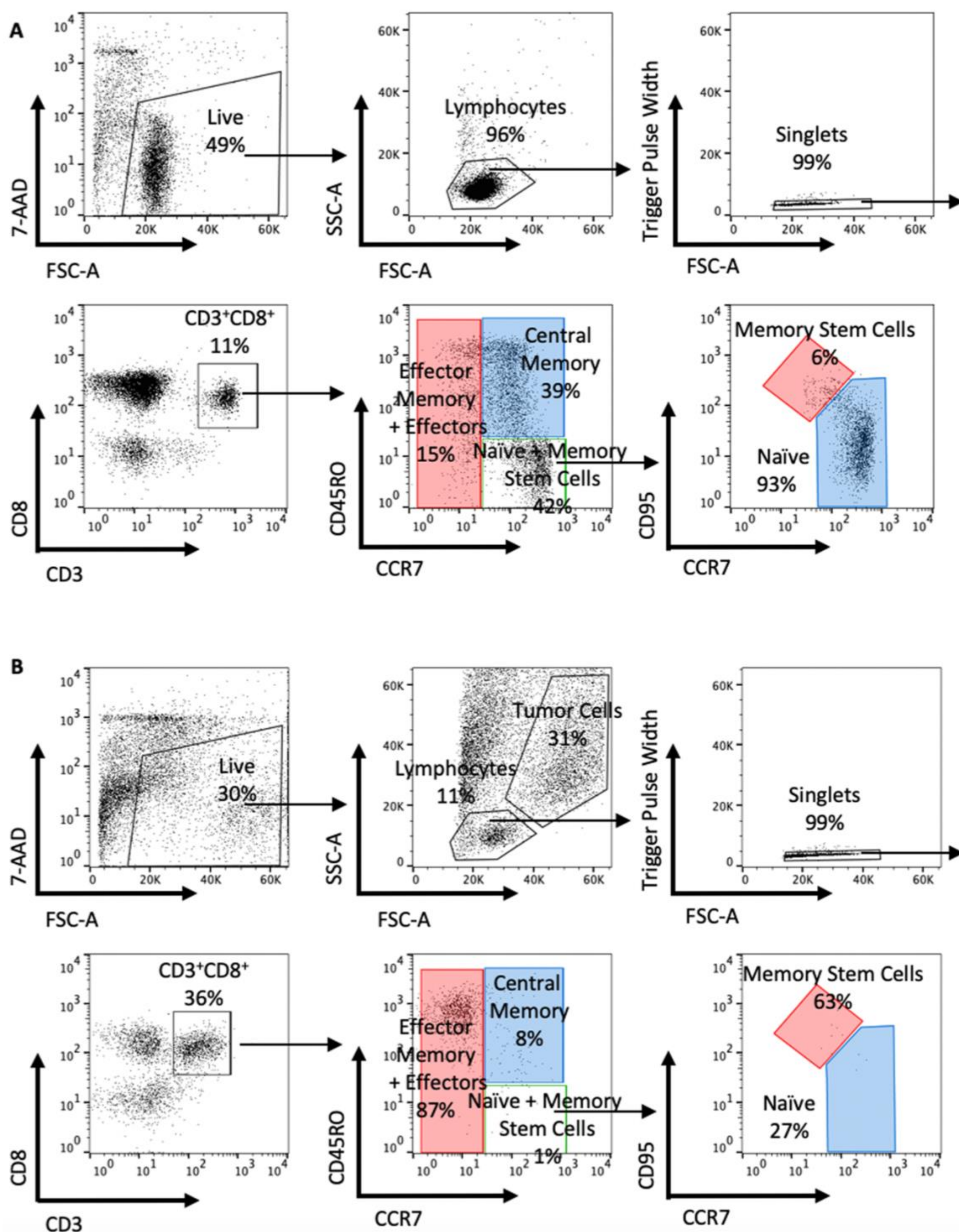


Figure 2.2. FACS sorting strategy to isolate subsets of CD8⁺ T cells in patients. Representative FACS sorting strategy from one patient's **A,** PBMCs and **B,** TILs.

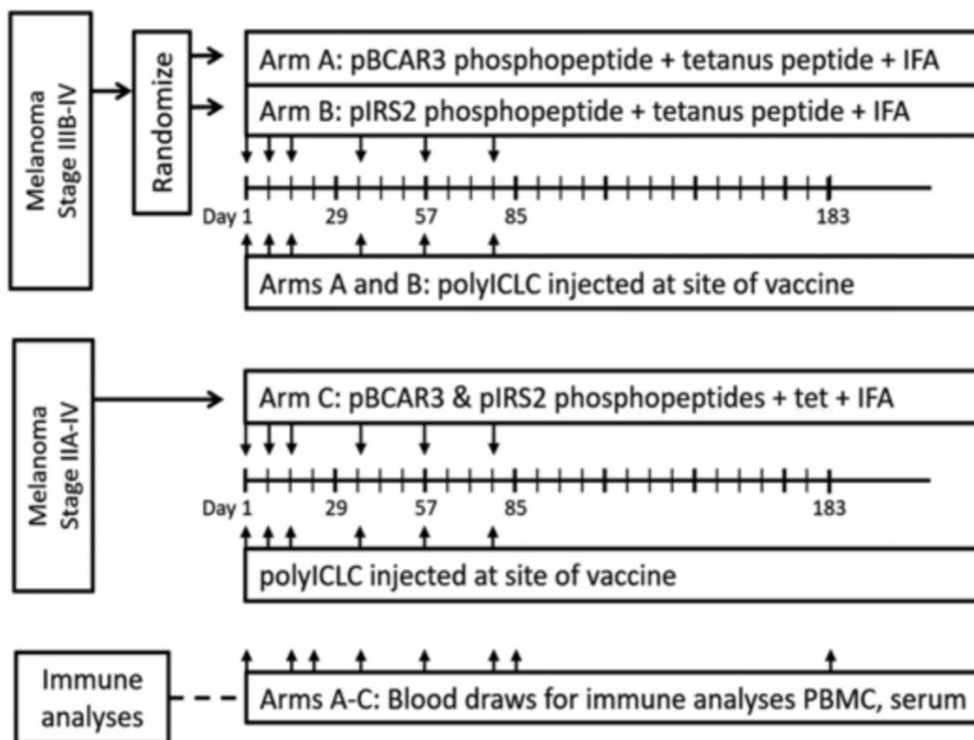


Figure 2.3. Proof of Concept Clinical Trial. Evaluation of the Safety and Immunogenicity of Phosphopeptide Vaccines plus Poly-ICLC in Participants with High Risk and Advanced Malignancies. Patients: Histologically or cytologically proven high-risk or advanced solid malignancies including melanoma, colorectal cancer, ovarian cancer, breast cancer, or non small-cell lung cancer (NSCLC).

Table 2.1. List of All Analyzed Phosphopeptides.				
Sequence[a]	**UniProt #**	**Gene (Uniprot)**	**Protein (Uniprot)**	**Reference**
HLA-A02:01 restricted phosphopeptides				
AVIHQsLGL	Q9BV87	CNPPD1	Protein CNPPD1	[372]
AIsDLQQL	O76094	SRP72	Signal recognition particle subunit SRP72	[350]
ALDsGASLLHL	P57078	RIPK4	Receptor-interacting serine/threonine-protein kinase 4	[350]
AMAAsPHAV	Q13151	HNRNPA0	Heterogeneous nuclear ribonucleoprotein A0	[1]
FLDtPIAKV	Q969G9	NKD1	Protein naked cuticle homolog 1	[350]
GLLGsPVRA	P30305	CDC25B	M-phase inducer phosphatase 2	[1]
ILDsGIYRI	Q9UPZ3	HPS5	Hermansky-Pudlak syndrome 5 protein	[372]
ILKsPEIQRA	P36578	RPL4	60S ribosomal protein L4	[1]
IMDRtPEKL	O75815	BCAR3	Breast cancer anti-estrogen resistance protein 3	[1]
KAFsPVRSV	Q02363	ID2	DNA-binding protein inhibitor ID-2	[45]
KLAsPELERL	P05412	JUN	Transcription factor AP-1	[1]
KLFPDtPLAL	Q12906	ILF3	Interleukin enhancer-binding factor 3	[1]
KLIDIVsSQKV	O14757	CHEK1[b]	Serine/threonine-protein kinase Chk1	[1]
KLIDRTEsL	P33241	LSP1	Lymphocyte-specific protein 1	[1]
KLLDFGSLsNLQV	P08708	RPS17	40S ribosomal protein S17	[1]
KLLsPSNEKL	Q14694	USP10	Ubiquitin carboxyl-terminal hydrolase 10	[1]
KLMsPKADVKL	Q86T90	KIAA1328	Protein hinderin	[1]
LLLsEEVEL	Q8IY92	SLX4	Structure-specific endonuclease subunit SLX4	[350]
LMFsPVTSL	Q9C0A6	SETD5	Histone-lysine N-methyltransferase SETD5	[1]
RIsHELDS	P10451	SPP1	Osteopontin	[350]
RLAsLNAEAL	Q8TBE0	BAHD1	Bromo adjacent homology domain-containing 1 protein	[45]
RLAsYLDRV	P05783	KRT18	Keratin type I cytoskeletal 18	[350]
RLDsYVR	Q9Y5R8	TRAPPC1	Trafficking protein particle complex subunit 1	[350]
RLDsYVRSL	Q9Y5R8	TRAPPC1	Trafficking protein particle complex subunit 1	[337]
RLFsKELRC	Q15543	TAF13	Transcription initiation factor TFIID subunit 13	[1]
RLLsPLSSA	E9PAU2	RAVER1	Ribonucleoprotein PTB-binding 1	[1]
RLQsTSERL	Q96TA2	YME1L1	ATP-dependent zinc metalloprotease YME1L1	[1]
RLSsPLHFV	Q8NC44	RETREG2	Reticulophagy regulator 2	[1]
RQAsIELPSM	P33241	LSP1	Lymphocyte-specific protein 1	[2]
RQAsIELPSMAV	P33241	LSP1	Lymphocyte-specific protein 1	[1]
RQDStPGKVFL	P13056	NR2C1	Nuclear receptor subfamily 2 group C member 1	[45]
RQDsTPGKVFL	P13056	NR2C1	Nuclear receptor subfamily 2 group C member 1	[1]
RQIsQDVKL	Q01433	AMPD2	AMP deaminase 2	[1]
RQLsSGVSEI	P04792	HSPB1	Heat shock protein beta-1	[1]
RRSsLDAEIDSL	Q93052	LPP	Lipoma-preferred partner	[2]
RTFsPTYGL	O15061	SYNM	Synemin	[1]
RTLsHISEA	Q6ZS17	RIPOR1	Rho family-interacting cell polarization regulator 1	[1]

RTLsPEIITV	Q9H4A3	WNK1[c]	Serine/threonine-protein kinase WNK1	[350]
RVAsPTSGV	Q9Y4H2	IRS2	Insulin receptor substrate 2	[337]
RVLHsPPAV	Q9Y4B5	MTCL1	Microtubule cross-linking factor 1	[45]
SLLTsPPKA	Q14669	TRIP12	E3 ubiquitin-protein ligase TRIP12	[1]
SLQPRSHsV	Q9Y2H5	PLEKHA6	Pleckstrin homology domain-containing family A member 6	[1]
SMtRSPPRV	Q9BRL6	SRSF8	Serine/arginine-rich splicing factor 8	[1]
SMTRsPPRV	Q9BRL6	SRSF8	Serine/arginine-rich splicing factor 8	[2]
TLAsPSVFKST	Q6PGQ7	BORA	Protein aurora borealis	[1]
VLLsPVPEL	Q9H1A4	ANAPC1	Anaphase-promoting complex subunit 1	[1]
VMFRtPLASV	Q9UKT4	FBXO5	F-box only protein 5	[1]
VMIGsPKKV	Q68CZ2	TNS3	Tensin-3	[1]
YLDsGIHSGA	P35222	CTNNB1	Catenin beta-1	[1]
YLDsGIHSGV[d]	P35222	CTNNB1	Catenin beta-1	[372]
YQRsFDEVEGVF	Q6Y7W6	GIGYF2	GRB10-interacting GYF protein 2	[2]
HLA-B07:02 restricted phosphopeptides				
APDsPRAFL	Unknown		Unknown	[350]
APRDRRAVsF	Q5TAA0	TTC22	Tetratricopeptide repeat protein 22	[350]
APRKGsFSAL	Q13619	CUL4A	Cullin-4A	[45]
APRKGsFSALM	Q13619	CUL4A	Cullin-4A variant	[373]
APRRYsSSL	Q68EM7	ARHGAP17	Rho GTPase-activating protein 17	[2]
APSVRsLSL	Q9Y446	PKP3	Plakophilin-3	[351]
AVRPTRLsL	Q9Y4H2	IRS2	Insulin receptor substrate 2	[350]
EPKRRsARL	P05114	HMGN1	Non-histone chromosomal protein HMG-14	[2]
EPRsPSHSM	Q03164	KMT2A	Histone-lysine N-methyltransferase 2A	[2]
FPHsLLSVI	Q9H9Y6	POLR1B	DNA-directed RNA polymerase I subunit RPA2 isoform 1	[351]
GAQPGRHsV	Q96IF1	AJUBA	LIM domain-containing protein ajuba	[45]
GPRPGsPSAL	Q9UJJ7	RPUSD1	RNA pseudouridylate synthase domain-containing protein 1	[45]
GPRsAsLLSL	Q9Y4H4	GPSM3	G-protein-signaling modulator 3	[373]
GPRSAsLLSL	Q9Y4H4	GPSM3	G-protein-signaling modulator 3	[45]
GPRSASLLsL	Q9Y4H4	GPSM3	G-protein-signaling modulator 3	[337]
GPRSAsLLsL	Q9Y4H4	GPSM3	G-protein-signaling modulator 3	[2]
GPRsPKAPP	Q6PJ34	ARHGAP4	ARHGAP4 protein	[45]
GPRsPPVTL	Q15735	INPP5J	Phosphatidylinositol 4,5-bisphosphate 5-phosphatase A	[351]
HPKRSVsL	O60238	BNIP3L	BCL2/adenovirus E1B 19 kDa protein-interacting protein 3-like	[351]
HPRsPTPTL	Q96HE9	PRR11	Proline-rich protein 11	[45]
HPRSPtPTL	Q96HE9	PRR11	Proline-rich protein 11	[45]
KARsPGRAL	Q14767	LTBP2	Latent-transforming growth factor beta-binding protein 2	[351]
KPAsPARRL	P78559	MAP1A	Microtubule-associated protein 1A	[2]
KPAsPKFIVTL	Q6PJT7	ZC3H14	Zinc finger CCCH domain-containing protein 14	[2]
KPEsRRSSLL	Q6WKZ4	RAB11FIP1	Rab11 family-interacting protein 1	[350]

KPLIRSQsL	Q9H6H4	REEP4	Receptor expression-enhancing protein 4	[45]
KPPHsPLVL	P01106	MYC	Myc proto-oncogene protein	[2]
KPPsPEHQSL	Q9Y6X9	MORC2	ATPase MORC2	[2]
KPPsPSPIEM	Q9H165	BCL11A	B-cell lymphoma/leukemia 11A	[2]
KPPtPGASF	Q96T58	SPEN	Msx2-interacting protein	[2]
KPPYRSHsL	Q96GE4	CEP95	Centrosomal protein of 95 kDa	[2]
KPQTRGKtF	Q8IV04	TBC1D10C	Carabin	[2]
KPRPLsMDL	Q9BY89	KIAA1671	Uncharacterized protein KIAA1671	[350]
KPRPPPLsP	Q15642	TRIP10	Cdc42-interacting protein 4	[45]
KPRRFsRsL	Q7L4I2	RSRC2	Arginine/serine-rich coiled-coil protein 2	[350]
KPRsPDHVL	Q9UPN3	MACF1	Microtubule-actin cross-linking factor 1 isoforms 1/2/3/5	[2]
KPRsPFSKI	Q9BXF6	RAB11FIP5	Rab11 family-interacting protein 5	[45]
KPRsPPRAL	Q86TG7	PEG10	Retrotransposon-derived protein PEG10	[45]
KPRsPPRALV	Q86TG7	PEG10	Retrotransposon-derived protein PEG10	[373]
KPRsPPRALVL	Q86TG7	PEG10	Retrotransposon-derived protein PEG10	[45]
KPRsPVVEL	P25098	GRK2	Beta-adrenergic receptor kinase 1	[337]
KPYsPLASL	Q13469	NFATC2	Nuclear factor of activated T-cells cytoplasmic 2	[2]
KVQsLRRAL	Q969G5	CAVIN3	Caveolae-associated protein 3	[350]
LPAsPRARL	Q3KQU3	MAP7D1	MAP7 domain-containing protein 1	[2]
LPIFSRLsI	P47974	ZFP36L2	mRNA decay activator protein ZFP36L2	[45]
LPKsPPYTAF	P23588	EIF4B	Eukaryotic translation initiation factor 4B	[45]
LPRGSsPSVL	Q9GZN2	TGIF2	Homeobox protein TGIF2	[45]
LPRtPRPEL	Q8N1W2	ZNF710	Zinc finger protein 710	[350]
LPVsPRLQL	P13688	CEACAM1	Carcinoembryonic antigen-related cell adhesion molecule 1	[350]
MPRQPsATRL	Q6NZ67	MZT2B	Mitotic-spindle organizing protein 2B	[45]
QPQRRsLRL	Q9ULW0	TPX2	Targeting protein for Xklp2	[350]
QPRsPGPDYSL	Q99684	GFI1	Zinc finger protein Gfi-1	[2]
QPRtPSPLVL	P33241	LSP1	Lymphocyte-specific protein 1	[2]
QPRtPsPLVL	P33241	LSP1	Lymphocyte-specific protein 1	[2]
RAHSsPASL	P46937	YAP1	Transcriptional coactivator YAP1	[350]
RAPsPSSRM	Q9UQ35	SRRM2	Serine/arginine repetitive matrix protein 2	[2]
RARGIsPIVF	Q96MU7	YTHDC1	YTH domain-containing protein 1	[350]
RPAKsMDSL	Q7Z6I6	ARHGAP30	Rho GTPase-activating protein 30	[337]
RPAsAGAML	Q14814	MEF2D	Myocyte-specific enhancer factor 2D	[337]
RPAsARAQPGL	Q9NPB0	SAYSD1	SAYSvFN domain-containing protein 1	[45]
RPAsPAAKL	Q9P2N6	KANSL3	KAT8 regulatory NSL complex subunit 3	[2]
RPAsPGPSL	Q8IY33	MICALL2	MICAL-like protein 2	[45]
RPAsPQRAQL	Unknown		Unknown	[2]
RPAsPSLQL	Q8WUF5	PPP1R13L	RelA-associated inhibitor	[45]
RPAsRFEVL	Q8IZ52	CHPF	Chondroitin sulfate synthase 2	[351]
RPAtGGPGVA	Q86TW6	N/A	Submitted name: Full-length cDNA clone CS0DI075YC18 of Placenta of Homo sapiens (human)	[2]

RPAtPTSQF	Q13115	DUSP4	Dual specificity protein phosphatase 4	[373]
RPDsAHKML	Q8WX93	PALLD	Palladin	[350]
RPDsPTRPTL	Q7RTP6	MICAL3	[F-actin]-monooxygenase MICAL3	[45]
RPDsRLGKTEL	Q9BYW2	SETD2	Histone-lysine N-methyltransferase SETD2	[2]
RPDVAKRLsL	O75815	BCAR3	Breast cancer anti-estrogen resistance protein 3	[45]
RPFHGISTVsL	Q5VZ89	DENND4C	DENN domain-containing protein 4C	[45]
RPFsPREAL	Q86V48	LUZP1	Leucine zipper protein 1	[2]
RPGsRQAGL	Q96JY6	PDLIM2	PDZ and LIM domain protein 2	[350]
RPHsPEKAF	Q53F19	NCBP3	Nuclear cap-binding protein subunit 3	[2]
RPIsPGLSY	Q16204	CCDC6	Coiled-coil domain-containing protein 6	[45]
RPIsPRIGAL	Q9Y6I3	EPN1	Epsin-1 isoform 2	[350]
RPItPPRNSA	P62136	PPP1CA	Serine/threonine-protein phosphatase PP1-alpha catalytic subunit	[2]
RPKLsSPAL	Q09472	EP300	Histone acetyltransferase p300	[2]
RPKPSSsPV	Q15366	PCBP2	Poly(rC)-binding protein 2	[2]
RPKsPLSKM	Q9HCD6	TANC2	Protein TANC2	[45]
RPKsVDFDSL	Q9Y5K6	CD2AP	CD2-associated protein	[350]
RPNsPSPTAL	Q9UKI8	TLK1	Serine/threonine-protein kinase tousled-like 1	[2]
RPPPPPDtPP	Q9Y5W3	KLF2	Krueppel-like factor 2	[2]
RPPsPGPVL	Q12770	SCAP	Sterol regulatory element-binding protein cleavage-activating protein	[2]
RPPsSEFLDL	Q9P2R6	RERE	Arginine-glutamic acid dipeptide repeats protein	[2]
RPQRAtSNVF	P24844	MYL9	Myosin regulatory light polypeptide 9	[2]
RPQRATsNVF	P24844	MYL9	Myosin regulatory light polypeptide 9	[2]
RPRAAtVV	P10644	PRKAR1A	cAMP-dependent protein kinase type I-alpha regulatory subunit	[350]
RPRAAtVVA	P10644	PRKAR1A	cAMP-dependent protein kinase type I-alpha regulatory subunit	[350]
RPRANsGGVDL	Q92766	RREB1	Ras-responsive element-binding protein 1	[2]
RPRARsVDAL	Q86X29	LSR	Lipolysis-stimulated lipoprotein receptor	[45]
RPRGsQSLL	P21860	ERBB3	Receptor tyrosine-protein kinase erbB-3	[45]
RPRPHsAPSL	Q5JXC2	MIIP	Migration and invasion-inhibitory protein	[2]
RPRPVsPSSL	P57059	SIK1	Serine/threonine-protein kinase SIK1	[2]
RPRRsSTQL	P28908	TNFRSF8	Tumor necrosis factor receptor superfamily member 8	[372]
RPRsAVEQL	Q9HAU0	PLEKHA5	Pleckstrin homology domain-containing family A member 5	[45]
RPRsAVLL	Q12802	AKAP13	A-kinase anchor protein 13	[2]
RPRsLEVTI	O15553	MEFV	Pyrin	[2]
RPRSLsSPTVTL	Q96PU5	NEDD4L	E3 ubiquitin-protein ligase NEDD4-like	[45]
RPRsMTVSA	O43312	MTSS1	Protein MTSS 1	[45]
RPRsPAARL	Q9P2Y4	ZNF219	Zinc finger protein 219	[2]
RPRsPGSNSKV	P78347	GTF2I	General transcription factor II-I	[2]
RPRsPNMQDL	Q6T310	RASL11A	Ras-like protein family member 11A	[350]

RPRsPPGGP	Q86UZ6	ZBTB46	Zinc finger and BTB domain-containing protein 46	[350]
RPRsPPPRAP	O43900	PRICKLE3	Prickle planar cell polarity protein 3	[45]
RPRsPRENSI	Q99700	ATXN2	Ataxin-2 variant	[351]
RPRsPRQNSI	Q99700	ATXN2	Ataxin-2	[2]
RPRsPSPIS	P41594	GRM5	Metabotropic glutamate receptor 5	[2]
RPRsPTGP	Q96I25	RBM17	Splicing factor 45	[2]
RPRsPTGPsNSF	Q96I25	RBM17	Splicing factor 45	[45]
RPSRSsPGL	Q8N3V7	SYNPO	Synaptopodin	[2]
RPSsLPDL	Q8NFD5	ARID1B	AT-rich interactive domain-containing protein 1B	[2]
RPsSPALYF	Q9Y3Q8	TSC22D4	TSC22 domain family protein 4	[2]
RPTKIGRRsL	Q96HN2	AHCYL2	Adenosylhomocysteinase 3	[350]
RPTsRLNRL	Q15788	NCOA1	Nuclear receptor coactivator 1	[2]
RPVsPFQEL	Unknown		Unknown	[2]
RPVsPGKDI	P31629	HIVEP2	Transcription factor HIVEP2	[337]
RPVtPVSDL	Q13118	KLF10	Krueppel-like factor 10	[2]
RPWsPAVSA	P12755	SKI	Ski oncogene	[2]
RPYsPPFFSL	Q9NYF3	FAM53C	Protein FAM53C	[2]
RSHSsPASL	Q9GZV5	WWTR1	WW domain-containing transcription regulator protein 1	[350]
RSHsSPASL	Q9GZV5	WWTR1	WW domain-containing transcription regulator protein 1	[45]
RSLsPGGAA	Q96T37	RBM15	RNA-binding protein 15	[2]
RTRsPSPTL	Q86UU1	PHLDB1	Pleckstrin homology-like domain family B member 1	[373]
RVRsPTRSP	Q03164	KMT2A	Histone-lysine N-methyltransferase 2A	[2]
SPAsPKISL	Q8WWM7	ATXN2L	Ataxin-2-like protein	[2]
SPEKAGRRsSL	A6NC98	CCDC88B	Coiled-coil domain-containing protein 88B	[2]
SPFKRQLsL	P49757	NUMB	Protein numb homolog	[45]
SPGLARKRsL	Q9H2Y7	ZNF106	Zinc finger protein 106	[2]
SPKsPGLKA	Q6JBY9	RCSD1	CapZ-interacting protein	[2]
SPKsPTAAL	Q53EZ4	CEP55	Centrosomal protein of 55 kDa	[45]
SPRAPVsPLKF	Q9UBS0	RPS6KB2	Ribosomal protein S6 kinase beta-2	[45]
SPRRsRSISL	Q16629	SRSF7	Serine/arginine-rich splicing factor 7	[2]
SPRsITSTP	Q9P0K7	RAI14	Ankycorbin	[45]
SPRsPDRTL	Q9UKN1	MUC12	Mucin-12	[350]
SPRsPGKPM	Unknown		Unknown	[2]
SPRSPsTTYL	Q13111	CHAF1A	Chromatin assembly factor 1 subunit A	[350]
SPRsPSTTYL	Q13111	CHAF1A	Chromatin assembly factor 1 subunit A	[2]
SPRTPVsPVKF	P23443	RPS6KB1	Ribosomal protein S6 kinase beta-1	[45]
SPSsPSVRRQL	O75179	ANKRD17	Ankyrin repeat domain-containing protein 17	[2]
SPSTSRSGGsSRL	Q9BUV0	RSRP1	Arginine/serine-rich protein 1	[2]
SVKPRRTsL	P15822	HIVEP1	Zinc finger protein 40	[350]
TPAQPQRRsL	Q9ULW0	TPX2	Targeting protein for Xklp2	[2]

TPIsPGRASGM	Q01196	RUNX1	Runt-related transcription factor 1	[2]
TPRsPPLGL	Q16584	MAP3K11	Mitogen-activated protein kinase kinase kinase 11	[2]
TPRsPPLGLI	Q16584	MAP3K11	Mitogen-activated protein kinase kinase kinase 11	[2]
VLKGsRSSEL	Q96B45	BORCS7	BLOC-1-related complex subunit 7	[372]
VPKsPAFAL	Q9ULW0	TPX2	Targeting protein for Xklp2	[45]
VPRPERRsSL	Q6UWJ1	TMCO3	Transmembrane and coiled-coil domain-containing protein 3	[350]
VPRsPKHAHSSSL	O95425	SVIL	Supervillin	[2]
YPGGRRsSL	P22897	MRC1	Macrophage mannose receptor 1	[350]
YPSFRRsSL	O95071	UBR5	E3 ubiquitin-protein ligase UBR5	[351]
YPSsPRKAL	O43166	SIPA1L1	Signal-induced proliferation-associated 1-like protein 1	[351]

[a] Lower case letter indicates phosphorylated residue.

[b] Natural variant (VAR_024571) I471V.[374]

[c] Natural variant (VAR_041317) M1808I.[333]

[d] A10V – Altered peptide ligand created to enhance binding.

Table 2.2. Characteristics of Healthy Donors.

Healthy Donor ID	Age at start of study	HLA Type			HLA Typing Methodology	Personal History of Cancer
		A locus	B locus	C locus		
HD29	49	24:02, 68:01	07:02, 27:05	07:02, 07:04	ARC*	No
HD43	64	02:01	07:02, 44:02	05:01, 07:02	ARC*	No
HD44	43	02:01	07:02, 44:02	07:02, 05:01	ARC*	No
HD54	30	02:01, 29:01	15:17, 44:37	07:69, 16:01	Low res*	No
HD64	64	02:01, 25:01	37, 40	03, 06	Low res*	No
HD66	48	01:01, 02:01	14:02, 18:01	08:02, 05:01	ARC*	No
HD67	54	01:01, 02:01	07:02, 15:01:01G	07:02, 05:01	ARC*	No
HD70	48	02:01, 24:02	35:02, 55:01	01:02, 04:01	ARC*	No
HD73	32	03:01, 11:01	07:02, 35:01	04:01, 07:02	ARC*	No
HD75	25	02:01, 24:02	40:01, 55:01	03:03, 03:04	ARC*	No
HD77	56	03:01	07:02	07:02	ARC*	No
HD78	31	01:01, 02:01	15:17, 35:01	07:01, 04:01	ARC*	No
HD80	50	03:01, 24:02	07:02, 15:01	07:01, 03:13	Low res*	No
HD89	28	01:01, 02:01	07:02	07:02	ARC*	No
HD90	28	03:01, 30:01	07:02, 13:02:01G	07:02, 06:02	ARC*	No

ARC = HLA genotyping was performed by the American Red Cross (ARC). From ARC: Allelic HLA typing was performed by PCR-SSOP, Sanger SBT, NGS. Low-Intermediate resolution HLA typing was performed by PCR-SSOP.
Low res = In house HLA genotyping was performed by PCR-SSP, Invitrogn/One Lambda AllSet+ Gold SSP Low-Resolution. Low resolution HLA typing was performed by PCR-SSP.
* = HLA genotyping for A02:01 and B7:02 were confirmed by antibody staining.

Table 2.3. Characteristics of ovarian cancer patients and tumor specimens analyzed by mass spectrometry.

Patient	Age At Start of Study	Ovarian Cancer Type	Stage	Tumor Mutation Status	Chemotherapies	Tumor Specimens used for the Identification of Phosphopeptides by Mass Spectrometry			
						Ascites	Ovary	Omentum	Pelvic Mass
VTB239	40	Serous carcinoma	Stage III high grade	BRCA 1	Neoadjuvant: Carboplatin + paclitaxel; Carboplatin + taxotere. Adjuvant: 3 cycles q3 of carboplatin/taxotere; Carboplatin/Doxil for recurrence.	X		X*	
VTB241	43	Endometrioid adenocarcinoma	FIGO grade 1; pT1c, pN0, pM: N/A because cannot be determined from specimens submitted	VUS in NF1	None		X		
VTB246	54	Serous carcinoma	pT1a, pN0, pM: N/A because cannot be determined based on the submitted specimens	Negative panel testing	Carboplatin/Taxol	X	X		
VTB247	56	High grade serous carcinoma	FIGO IIIC; pT3c, pN1a	Deleterious mutation in CDKN2A, c.97dupG. MS Stable, Tumor Mutational Burden Low (5mut/Mb)	GOG 3015 Carboplatin/Taxol with Bevacizumab/ Atezolizumab vs placebo (blinded)			X	

VTB269	Unknown	High grade serous carcinoma	high grade; pT3c, pNX	VUS CHEK2	Neoadjuvant: Carboplatin/Taxol. Adjuvant: Carboplatin/Taxol.		X*		
VTB279	68	Serous carcinoma	pT3c, pN0	VUS in STK11 (c1180G>A(p.Gly 3945er)	Neoadjuvant: Carboplatin/Taxol. Adjuvant: Carboplatin/Taxol.			X	
VTB280	61	Serous carcinoma	Unknown	Unknown	Unknown		X*		
VTB285	48	Endometrioid adenocarcinoma	FIGO IIA grade 3; pT1c2, pNX	BRCA 1/2 negative	Carboplatin/Taxol				X
VTB288	59	High grade serous carcinoma	FIGO IIIC; pT3c, pNX	Negative panel testing	Carboplatin/Taxol; Carboplatin/Abraxane; Carboplatin/Docetaxel		X		
VTB291	48	Granulosa cell tumor, adult type	FIGO IIA; pT2a, pNX	Unknown	Carboplatin/Taxol				X

FIGO = International Federation of Gynecology and Obstetrics
VUS = variant of unknown specificity
* = neoadjuvant chemotherapy

Table 2.4. Melanoma patient demographics by treatment group.

Peptide		Arm A pBCAR3	Arm B pIRS2	Arm C Both	Total
N		3	3	9	15
Gender (female)		1	1	4	6 (40%)
Caucasian, non-Hispanic		2	3	9	11 (92%)
Hispanic		1	0	0	1 (7%)
Age—median (range)		64 (40–67)	63 (50–85)	52 (30–75)	56 (30–85)
Performance status 0		2	3	8	13 (87%)
Stage at registration	II–IIIA	0	0	4	4 (27%)
	IIIB, IIIC	1	1	5	7 (47%)
	IV	2	2	0	4 (27%)
Distant metastatic sites	None	1	1	9	11
	Nodes	1	0	0	1
	Soft tissue	0	2	0	2
	Lung	0	1	0	1
	Brain	1	0	0	1
	Other visceral	0	1	0	1
Clinically NED at study entry		3	3	9	15 (100%)

NED, no evidence of disease; pBCAR3, phosphopeptide from breast cancer antiestrogen resistance 3; pIRS2, phosphopeptide from insulin receptor substrate 2.

Chapter 3: The Development of Culture and Analysis Conditions to Characterize T Cell Responses to Phosphopeptides in Healthy Donors and Cancer Patients

Experimental contributions: Cummings KL contributed to experiments testing the use of IL-2 or IL-7/15, 10- or 14-day cultures, and fresh or frozen mDC stimulation. Mir RM contributed to the phenotyping of mDCs and K562 cells.

3.1 Highlights

- $CD45RO^+$ enrichment of antigen-experience $CD8^+$ T cells improves the resolution of identifying antigen-specific memory T cells.
- IL-7/15 is superior to IL-2 for the expansion and support of most antigen-specific $CD45RO^+CD8^+$ T cells.
- Previously frozen mDCs are equally effective as freshly isolated mDCs in stimulating $CD45RO^+CD8^+$ T cells as freshly isolated mDCs.
- Stimulation of $CD45RO^+CD8^+$ T cells with greater number of peptides results in loss of detection of lower frequency antigen-specific cell populations.
- Phosphopeptide-specific monomer technology needs to be optimized for effective use in identifying antigen-specific T cells.

3.2 Abstract

The detection of low frequency T cells from human specimens can be challenging. Limitations in access to human specimens make resources particularly precious and emphasize the need to have an optimized assay protocol to maximize data gained from each analysis. T cell isolation, antigen presenting cells, cytokine support, and culture and assay conditions must be optimized for low background, high response, and efficiency of methodology. We have developed an *in vitro* T cell enrichment, priming, and expansion methodology to optimally induce and detect robust recall responses from both healthy donors and patients with cancer. The findings of these studies not only provide a methodology for detecting memory T cell responses from humans but also illuminate avenues for future investigation into memory T cells' requirements for optimal recall responses.

3.3 Introduction

Circulating memory T cells specific for non-viral epitopes are often low frequency. Therefore, *in vitro* expansion is required to increase the representation of low-frequency antigen-specific T cells over an assay's limit of detection. Appropriate culturing conditions should preferentially increase the antigen-specific T cells so that they represent a higher frequency of the total T cell population, while minimizing the cytokine production of the non-specific T cells.

The *in vitro* conditions supporting activated antigen-specific T cells need to promote their survival, expansion, and gain/retention of effector activity, while minimizing the background effector activity of bystander cells. HLA-matched antigen presenting cells (APCs) expressing co-stimulatory molecules must be used to stimulate T cells with peptide. Adequate MHC-cognate antigen expression, co-stimulatory molecules, and possibly adhesion molecules influence the strength of T cell re-activation, which could impact the T cell's ability to survive, expand, and/or produce effector functions. HLA negative cell lines can be transduced to express the HLA allele of interest and then utilized as artificial APCs to stimulate T cells *in vitro* [375, 376]. Cytokines must be added to cultures to support T cell activation, proliferation, and survival after engagement with their cognate antigen. IL-2 is often used in culture as a T cell survival and growth factor [132, 377]. IL-7 and IL-15, on the other hand, are homeostatic cytokines for memory T cell survival and proliferation [378, 379]. *In vitro*, IL-7 and IL-15 can induce homeostatic and activation-driven proliferation [79–81, 380]. Lastly, the amount of time T cells spend in culture influences their cell numbers, viability, and effector function. Terminal effectors have a window of time in which they can survive and still be capable of producing IFNγ upon

recognition of their cognate antigen. However, if the T cells are at low frequency, they need to be expanded to a high enough abundance to be above the threshold of detection in the ELISpot assay. Due to the immunosuppression of T cells in cancer patients, alternative methodology may be required from that of healthy donors to optimally detect responses.

We previously found that healthy donors had robust $CD8^+$ T cell responses to a subset of HLA-A2- and HLA-B7-restricted phosphopeptides [2, 3]. Here we established optimal culture and analysis conditions to more comprehensively and explicitly interrogate phosphopeptide immunity in healthy donors and in cancer patients. We identified parameters of T cell enrichment, antigen presenting cells, cytokine support, and length of culture that promoted robust detection of responses. We also identified HLA-phosphopeptide multimer technology as an area of research requiring additional technological development.

3.4 Results

3.4.1 Culture conditions for optimal survival, expansion and frequency of antigen-specific T cells

We wanted to ensure that our *in vitro* culture conditions enabled us to detect low numbers of antigen-specific T cells in the IFNγ ELISpot assay, while avoiding culture periods that were too long, resulting in T cell death or loss of function. Therefore, we assessed magnitude of responses to viral and phosphopeptide antigens at 10 or 14 days to determine an optimal culture window for response detection. Responses in healthy donor (HD) 44 to EBV BMLF1 and CMV pp65 and in HD43 to Influenza M1, EBV BMLF1, and pIRS2 were higher in 14-day cultures than in 10-day cultures (**Figure 3.1.A, Figure 3.1.B**). The responses in HD44 to Influenza M1 and pIRS2 were higher in 10-day cultures than in 14-day cultures. We also compared the effects of IL-2 and a combination of IL-7 and IL-15, after stimulation and 14 days in culture. Responses in HD44 to Influenza M1, CMV pp65, and pIRS2 were higher when cultured with IL-7 and IL-15 than when cultured with IL-2, although the response to EBV BMLF1 was higher when cultured with IL-2 (**Figure 3.2.A**). Responses in HD43 to Influenza M1, EBV BMLF1, and pIRS2 were higher when cultured with IL-7 and IL-15 than when cultured with IL-2 (**Figure 3.2.B**). In addition to assessment of responses by ELISpot assay, we utilized multimer staining to assess the antigen-specific and homeostatic expansion/survival of antigen specific cells at day 16 from cultures supported with either IL-7/15 or IL-2. We analyzed the percentage of T cells in HD43 specific to Influenza M1$_{58\text{-}66}$ and to EBV BMLF1$_{300\text{-}308}$ from cultures that were either stimulated with antigen-pulsed mDC and cytokines (stimulated) or cytokines only (unstimulated), IL-7 and IL-15 better supported the maintenance or survival of Influenza

M1- and EBV BMLF1-specific T cells in unstimulated cultures, and expanded the frequency of M1- and BMLF1-specific T cells in stimulated cultures twice as much as IL-2 (**Figure 3.2.C**). Therefore, both the frequency of antigen-specific T cells under homeostatic expansion and expansion in response to peptide stimulation were better supported by IL-7 and IL-15 than by IL-2.

We asked if IL-7 and IL-15 were also better than IL-2 for supporting and expanding T cells from melanoma patients' PBMCs after peptide stimulation. We interrogated total cell numbers, viability, and strength of responses after stimulation and culturing either with IL-2 or a combination of IL-7 and IL-15. Melanoma patient PBMCs were frozen without isolation of monocytes for preparation into DCs. Therefore, whole PBMC cultures were stimulated with peptides, relying on the endogenous monocytes, DCs, and B cells to present peptide to the T cells. Since patients are in an immunocompromised state due to cancer, we wanted to know whether a T cell survival cytokine, IL-2, would promote better T cell expansion and survival, reverse anergy if required, and result in higher frequencies of responsive antigen-specific T cells. Therefore, we stimulated PBMCs from two patients, VMM728 and VMM934, at two timepoints. The first timepoint was prior to vaccination. The second timepoint was 7 (VMM728) or 6 (VMM934) weeks after their first vaccination in a clinical trial. PBMCs were pulsed with peptides and cultured for 14 days with IL-7 and IL-15 or IL-2. Responses in both patients from both timepoints were the same or higher when cultured with IL-7 and IL-15 than with IL-2 (**Figure 3.3.A, Figure 3.3.B**). From PBMCs at both timepoints, VMM728's responses to Influenza M1 were higher when cultured with IL-7 and IL-15 compared to IL-2 (**Figure 3.3.A**). The response at Week 0 to pIRS2 and the response at Week 7 to the common melanoma antigen $gp100_{209\text{-}217}$

(IMDQVPFSV) were also higher when cultured with IL-7 and IL-15. No responses to pCHEK1 were detected from either timepoint under either condition. From PBMCs at both timepoints, VMM934's responses to Influenza M1 were higher when cultured with IL-7 and IL-15 compared to IL-2 (**Figure 3.3.B**). The response at Week 6 to $gp100_{209-217}$ was also higher when cultured with IL-7 and IL-15. There were no responses in VMM934 to pIRS2 or pCHEK1 regardless of the timepoint or cytokine conditions. In a similar experiment with stimulated melanoma patients' PBMC, when the resulting cell numbers for all cultures at day 14 were analyzed, the percent viability of the cells was 156% higher when cultures were supported with IL-7 and IL-15 over cultures supported with IL-2 (**Figure 3.3.C**). The total number of viable cells was 9.6 times higher when cultures were supported with IL-7 and IL-15 than with IL-2 (**Figure 3.3.C**). Therefore, culturing stimulated cells with IL-7 and IL-15 cytokine support promotes better expansion, survival, and frequency of antigen-specific T cells than IL-2 support when analyzing responses from PBMCs in melanoma patients.

3.4.2 Peptide stimulation conditions

Because we intended to analyze approximately 200 phosphopeptides in at least 10 healthy donors and a subset of these in 10 cancer patients, we needed to make the response analysis more high-throughput. To this end, we asked whether we could stimulate one culture with multiple phosphopeptides and viral control peptides. We initially analyzed memory responses in healthy donors by stimulating each culture with 1 phosphopeptide. After 14 days *in vitro* culture, the expanded cells were interrogated for responses against T2-B7 target cells pulsed individually with each peptide. However, stimulation of each culture with only 1 phosphopeptide required an immense number of cells to start. Therefore, we

moved to stimulating each culture with 3 phosphopeptides. The CD45RO$^+$CD8$^+$ T cells in one culture, therefore, were stimulated by mDCs that were pulsed (simultaneously) with all three phosphopeptides. A recent study demonstrated that a single culture could be stimulated with up to 300 irrelevant peptides and not inhibit the detection of expected responses to known relevant peptides [381]. We therefore tested whether the number of phosphopeptides stimulated per culture could be increased to improve the throughput of responses analyzed. We analyzed responses from CD45RA$^-$CD8$^+$ T cells or CD45RA$^+$CD8$^+$ T cells stimulated with Ebola NP (our negative viral control) plus 3 or 21 phosphopeptides. Based on previous data, expected phosphopeptide responses could be detected in the cultures stimulated with 3 or with 21 phosphopeptides, but were diminished when stimulated with 21 phosphopeptides (**Figure 3.4.A**). Based on our results, we chose to remain with a microculture system in which mDCs are pulsed with 3 phosphopeptides so that we would not lose detection of low-level responses. While requiring more PBMCs, this microculture system lowered the possibility of false-negative results that were present but below the limit of detection in the ELISpot assay.

We also tested whether the addition of a viral antigen to the culture stimulation would change the detection or strength of responses to phosphopeptides from that culture. T cell cultures from HD43 were stimulated with 3 phosphopeptides or 3 phosphopeptides plus Influenza M1, EBV BMLF1, EBV LMP2A, or CMV pp65. Responses to pCDC25B were highest when stimulated only with phosphopeptides (**Figure 3.4.B**). The inclusion of any viral peptides lowered the response to pCDC25B. Responses to pIRS2 were highest when stimulated only with phosphopeptides or with phosphopeptides and Influenza M1 or EBV LMP2A. The inclusion of the strong viral peptides, EBV BMLF1 or CMV pp65,

lowered the response to pIRS2. Therefore, we stimulated cultures with phosphopeptides only and with viral control peptides in discrete cultures. We were curious why the inclusion of additional peptides in the stimulation diminished responses to immunogenic peptides. To test the hypothesis that additional immunogenic peptides in the culture competed for MHC binding, we assessed responses to Influenza M1 in HD44 after stimulation with different peptide combinations: Influenza M1 only or in combination with 5 or 9 immunogenic phosphopeptides or 9 non-immunogenic phosphopeptides. The stimulation with M1 peptide plus 5 or 9 immunogenic phosphopeptides did not diminish the response compared to stimulation with M1 alone (**Figure 3.4.C**). The only condition that resulted in diminished response to Influenza M1 was in the presence of 9 non-immunogenic phosphopeptides (**Figure 3.4.C**). These results were surprising because if peptides were competing for MHC binding, we would have expected to see diminished responses driven out of conditions with 9 phosphopeptides, regardless of whether they were immunogenic or not. However, there was no discernible difference in the Influenza response when the culture was stimulated with 9 immunogenic phosphopeptides. This suggests that there is peptide competition for MHC binding, but the activation of additional T cells in the culture with 9 immunogenic phosphopeptides adds additional IL-2 which overcomes re-activation shortcomings.

Overall, our established system yielded low background, improving the ability to detect low level responses. We made a few additional modifications to the culture and response analysis conditions to maximize the antigen-specific responses and minimize the background. It has been demonstrated that higher responses can be detected when frozen mDCs and PBLs are thawed and rested 1-2 days in advance of antigen stimulation;

therefore, we rest thawed cells overnight in AIM-V+5% AB$^+$ serum media prior to stimulation [381, 382]. We rested PBLs in low dose IL-7 and mDCs in GM-CSF plus IL-4. We also changed the target cell in the ELISpot assay to T2-B7 cells rather than T2s, which in experiment-to-experiment comparisons, had lower background (data not shown because these conditions were never compared head-to-head in an assay). We hypothesized that the additional MHC expression on the cell surface (due to dual expression of HLA-A2 and HLA-B7), in addition to the stabilization of the MHC-peptide complex on the cell surface with the addition of β_2m, resulted in the T2-B7 target cells' lower background. However, we did not explicitly assess these hypotheses.

3.4.3 Enrichment of antigen-experienced T cells

Our goal was to assess CD8$^+$ T cells in healthy donors for recall responses to phosphopeptides. CD45RO is a cell surface marker that is expressed only after a T cell has encountered its cognate antigen and undergone activation [383, 384]. We hypothesized that enrichment of CD45RO$^+$CD8$^+$ T cells would enable better detection of memory responses than interrogating responses from unenriched CD8$^+$ T cells. We compared IFNγ responses after stimulation with mDC plus 10μm peptide and 14 days culture with IL-7 and IL-15 from either unenriched or CD45RO$^+$ enriched CD8$^+$ T cells, obtained from normal donor peripheral blood mononuclear cells (PBMC). For cultures containing CD45RO$^+$ enriched CD8$^+$ T cells, one-third the numbers of T cells and DCs were added to account for memory cells making up about one-third of the CD8$^+$ T cell compartment. Despite decreasing the number of CD8$^+$ T cells stimulated, responses to Influenza M1, EBV BMLF1 and pCHEK1 in two healthy donors, HD43 and HD75, were higher when starting with CD45RO$^+$CD8$^+$ T cells rather than CD8$^+$ T cells (**Figure 3.5A, Figure 3.5.B**). These results show that

enriching CD45RO$^+$CD8$^+$ T cells improves the numbers of antigen-specific memory cells in a one-time stimulated *in vitro* assay.

3.4.4 Antigen presenting cells for T cell stimulation

Autologous mDCs are a very good APC in terms of being HLA-matched and expressing some co-stimulatory molecules, but the processing and maturation of monocytes from PBMCs into mDCs takes 10-12 days and yields a limited number of APCs. Having a cell line that performs as an "artificial" APC would remove this 10-12 day mDC maturation protocol. It would also remove variability in the DC maturation and yield among donors and especially among cancer patients who may be leukopenic and unable to provide enough CD14$^+$ monocytes to mature into DCs. K562, a poorly differentiated cell line derived from a patient with Chronic Myeloid Leukemia (CML) in the 1970s, is immunologically inert, as it does not express a number of relevant molecules, including MHC-I, MHC-II, ICOS-L, PD-L1, CD40, CD40L, CD27, and CD70, and does not secrete IL-2, IL-4, IL-7, IL-10, IL-15, IL-21, IFNγ, or GM-CSF [385]. It does, however, express the adhesion molecules CD54/ICAM-1 and CD58/LFA-3, which promote T cell attachment [385]. [386, 387]. Here we compared responses after stimulation with one of two types of APCs, autologous monocyte-derived dendritic cells (mDCs) and K562 cells transduced to express HLA-A*02:01 and the co-stimulatory molecules CD80, CD83, and CD137L/4-1BBL [363, 376, 385]. Responses to Influenza M1 and the phosphopeptide pCHEK1) were substantially higher in cultures stimulated by autologous mDCs, although the response to another phosphopeptide, pSRP72, was higher in cultures stimulated by K562 (**Figure 3.6.A, B**). Although the results are limited and somewhat contradictory, they suggest that in some cases autologous mDCs provide better stimulation of memory T cells for recall responses.

We compared the expression and expression levels of MHC and co-stimulatory molecules on autologous mDCs and K562 differed. All K562 cells and mDCs expressed HLA-A2, CD54, and CD58, but the MFIs for these three molecules were much higher on mDCs (**Figure 3.6.C, D**). However, K562 expressed CD137L but mDCs did not (**Figure 3.6.E**). A higher percentage of K562 cells expressed CD80 and CD83 and the MFIs of both molecules were also substantially higher (**Figure 3.6.F**). We did not assess the production of cytokines by mDCs and it is possible that they produce IL-12 or IL-15. However, cytokine secretion by K562 has been shown to be negligible [385]. It is possible that the higher MFI of HLA-A2 on mDCs promoted better T cell stimulation; this could be followed up with future studies.

To gain flexibility for our response analysis, we compared the effectiveness of unfrozen mDCs used immediately after the 10-12 day *in vitro* mDC maturation process, and mDCs that were frozen after maturation and thawed at a later time. Both DC populations were pulsed with peptide and cultured with $CD45RO^{+}CD8^{+}$ T cells for 10 days with IL-7 and IL-15. Responses in HD43 to Influenza M1, EBV LMP2A, pCDC25B, pIRS2, or pCTNNB1 were comparable whether stimulated with fresh or frozen DCs (**Figure 3.7.A, B**). The response in HD54 to Influenza M1 was comparable or possibly higher when stimulated with frozen DCs (**Figure 3.7.A, B**). However, the responses in HD54 to pCDC25B and pIRS2 were higher when stimulated with fresh DCs (**Figure 3.7.B**). The absence of a response elicited by frozen DCs to EBV BMLF1 suggests there may have been something inherently faulty about this aliquot of HD54's frozen DCs. Responses in both HD43 and HD44 to EBV BMLF1 were higher when stimulated with fresh mDCs over

frozen mDCs. Overall, frozen and thawed mDCs were comparably effective at stimulating most antigen-specific $CD45RO^+CD8^+$ T cells.

3.4.5 Identification of phosphopeptide-specific $CD8^+$ T cells by multimer staining and enrichment

We wanted to be able to identify and isolate phosphopeptide-specific T cells. Multimer staining detects all antigen-specific cells, regardless of their functionality. Because of this feature, multimer staining can be utilized to detect exhausted or dysfunctional antigen-specific T cells, particularly in cancer patients, where suppressed T cells are more likely. Furthermore, identification of antigen-specific cells allows for multi-dimensional characterization by flow cytometry and can be used to isolate T cells for downstream analyses such as TCR sequencing [388–390]. We worked to optimize multimer staining of phosphopeptide-specific T cells for all three of these purposes. For the detection of low frequency T cells, enrichment on magnetic columns using magnetic bead-conjugated multimers is one way to increase the frequency of antigen-specific T cells in the sample, as described in [391]. In preliminary experiments, we used magnetic enrichment to identify T cells specific to CMV pp65 and EBV BMLF1, two strong viral epitopes against which HD44 historically has robust responses (**Figure 3.8.A**). There was an approximately 10-fold increase in the frequency of multimer-stained T cells after magnetic enrichment. However, when we applied the same strategy to phosphopeptide-specific T cells after peptide stimulation by autologous mDCs and 14-day culture with IL-7 and IL-15, the staining did not correspond to the IFNγ ELISpot assay results (**Figure 3.8.B, Figure 3.8.C**). To gain confidence that we were detecting phosphopeptide-specific T cells, we conjugated each antigen-specific monomer to both APC and PE, such that truly specific cells would

be double positive. In HD43, IFNγ responses were evident against pCHEK1 and Influenza M1, but multimer staining was only evident against Influenza M1 (**Figure 3.8.B**). In HD44, responses were evident against pCHEK1, pSRP72, and Influenza M1, but multimer staining was only evident against Influenza M1 and, seemingly, pIRS2 (**Figure 3.8.B**). The pIRS2 double positive staining looked clean and discrete, but had no correlate to the ELISpot response, and could not be reproduced in future experiments.

Because we experienced issues with reproducibility from assay to assay as well as non-discrete single positive staining, we performed an experiment to assess different multimer staining protocols. We stimulated and expanded Influenza- and phosphopeptide-specific T cells in culture with IL-7 and IL-15 after peptide stimulation by autologous mDCs. At day 14 we compared three multimer staining techniques. We found that staining with the multimers at room temperature for 10 minutes, followed by the addition of other surface marker antibodies and additional incubation at 37°C for 20 minutes resulted in this highest percentage of Influenza M1-specific T cells (**Figure 3.9.A**). However, there were no double positive cells observed under any staining condition for pCDC25B (**Figure 3.9.B**) or pSRP72 (**Figure 3.9.C**) in HD44. Although there were insufficient cells to perform an ELISpot assay in this experiment to compare the frequencies of responsive cells by ELISpot to the frequencies of antigen-specific cells by FACS, HD44 historically had robust day 14 responses to both epitopes. Therefore, we were confident there should have been some antigen-specific cells. When we applied this staining strategy to pIRS2-specific T cells in HD54 *ex vivo*, we again saw indiscriminate staining (**Figure 3.9.D**). Therefore, we were unable to proceed with analyses using multimer technology due to limited reproducibility and discordance with ELISpot responses. This work identifies a crucial

technological barrier that requires further investigation in order to identify and isolate phosphopeptide-specific T cells for additional downstream analyses.

3.5 Discussion

In this work, we parsed variables during the peptide stimulation, cell culture, and response analysis to determine their influence on the detection or robustness of memory T cell recall responses. Although useful tools, the use of artificial APCs in this study were less ideal than autologous mDCs for stimulating responses. It is possible that lower expression of HLA-A2, CD54, and CD58 on K562 limited their ability to productively present antigen to T cells in this culture system, despite their higher and more uniform expression of several costimulatory ligands. Increased MHC expression would likely improve the responses generated after stimulation with peptide-pulsed K562 cells. Fourteen days culture with IL-7 and IL-15 after peptide stimulation with autologous mDCs promoted the best detection of robust responses. Peptide stimulation with more peptides limited the robustness of responses. Lastly, multimers are useful tools for the identification and isolation of antigen-specific T cells. While the ELISpot assay depends on T cell functionality, specifically the production of one cytokine, multimer detection identifies all antigen-specific T cells, regardless of whether the cells are functional and, if so, which cytokine(s) they can produce. As demonstrated, additional technological advances are required to apply multimer detection to phosphopeptide-specific T cells.

One caveat of the use of IL-7/15 rather than IL-2 to support one-time stimulated T cells is that IL-7/15 favors the survival and expansion of T_{CM} and T_{SCM} over T_{EM}, T_{EMRA}, or T_{eff}. Therefore, the culture system itself was somewhat biased in that it better supported some cell subsets over others. Therefore, it is possible that antigen-specific T_{EM} cells were present but were less likely to have been detected in this culture/assay system. Furthermore, the use of cytokines influences T cell metabolism and, therefore, their function, which is

relevant since the readout of antigen-specific cells is a functional assay. Activated T cells undergo a shift from catabolic to anabolic metabolism, aerobic glycolysis [392, 393]. This occurs downstream of TCR signaling through the PI3K, mTOR, and Myc pathways with the purpose of generating amino acid and fatty acid building blocks that are needed for protein translation for growth, proliferation, and effector activity [394, 395]. IL-2 strongly promotes glycolysis by activating the PI3K-AKT/mTOR pathway, which in turn also activates Myc signaling [394]. IL-7 signaling is also known to activate these same pathways, but seemingly to a lesser extent [394]. Therefore, it is likely that the metabolic reprogramming of memory T cells upon re-activation with cognate antigen differs depending on whether their expansion is supported by IL-2 or IL-7. It is also likely that this metabolic reprogramming has durable effects that impact the T cells' functionality 14 days following re-activation.

Future work should interrogate the mechanism by which additional either non-immunogenic or strong viral peptides during stimulation inhibit the detection of responses from antigen-specific T cells. One possible explanation for decreased strength of responses is that the increased number of peptides are competing for binding to HLA molecules on the surface of the mDCs, resulting in lower avidity for antigen-specific T cells to bind their cognate HLA-peptide complexes. This may result in no or sub-optimal T cell priming. Another possible explanation is that the peptide pool concentration needs to be increased. We followed the authors' suggestions of concentrations of 0.1uM per peptide which is a 100-fold decrease in peptide concentration than we have previously used [381]. We have not yet tested responses from microcultures stimulated with the combination of 21 phosphopeptides at 10uM concentration. If the number of HLA molecules available for

binding is saturated, the diminished peptide concentration will be a non-issue, but this remains to be determined. Further work is required to determine the underlying mechanism(s) limiting responses in order to streamline high-throughput analysis without sacrificing detection of low-level positive responses. Similarly, the addition of strong viral peptides, such as Influenza M1, EBV BMLF1, or CMV pp65, lowered the strength of phosphopeptide responses. One hypothesis is that the abundant number of T cells specific to any of these viral peptides compete for resources, such as cytokines or nutrients, inhibiting the optimal expansion of phosphopeptide-specific T cells. An alternative hypothesis is that highly abundant viral-specific T cells compete for interaction with MHC-peptide complexes, limiting the ability of T cells of lower abundance to surveil the MHC-peptide complexes or physically engage with cognate antigens as long or with high avidity. One other hypothesis is that there is some degree of Fas-dependent T cell activation-induced cell death of recently re-activated T cells [396–398] and that the T cells of higher abundance are more likely to survive. The data on Influenza M1 responses from cultures stimulated under different conditions suggest there may be alternative mechanisms in play when the response being analyzed is against a strong viral antigen. In this case, more immunogenic peptides in the stimulation did not diminish responses to Influenza, but the addition of non-immunogenic peptides did. This suggests that access to MHC-peptide was not an issue for T cells specific to Influenza M1, or it is possible that cultures stimulated with immunogenic phosphopeptides were supported by the production of IL-2 from the additional activated T cells, which could have overcome culture limitations caused by the addition of peptides. This suggests that competition for MHC binding is not the limiting factor, since T cells specific for the immunogenic phosphopeptides would likely interact

longer with their cognate MHC-antigen but the response to Influenza was not diminished in this condition. It also suggests that competition for cytokines is not likely the reason for diminished responses in the presence of additional peptides because the T cells stimulated with immunogenic phosphopeptides would use the cytokines more quickly and robustly than would non-stimulated T cells. However, there is a possibility that the additional activated T cells produce greater levels of IL-2 in the culture, overcoming any other limitation brought on by the inclusion of additional peptides in the culture, such as competition for MHC-peptide and reduced TCR avidity during reactivation. These surprising results requiring additional investigation to understand the mechanisms underlying the effects of non-immunogenic peptides and strong viral peptides on detection of responses to Influenza and phosphopeptides, respectively. Future work investigating the mechanisms of memory T cells' requirements for optimal recall responses will illuminate important molecular interactions that would likely be relevant to physiological memory T cell re-activation, as well as establish nuances for interpretation of the detection of *in vitro* stimulated responses.

3.6 Figures and Tables

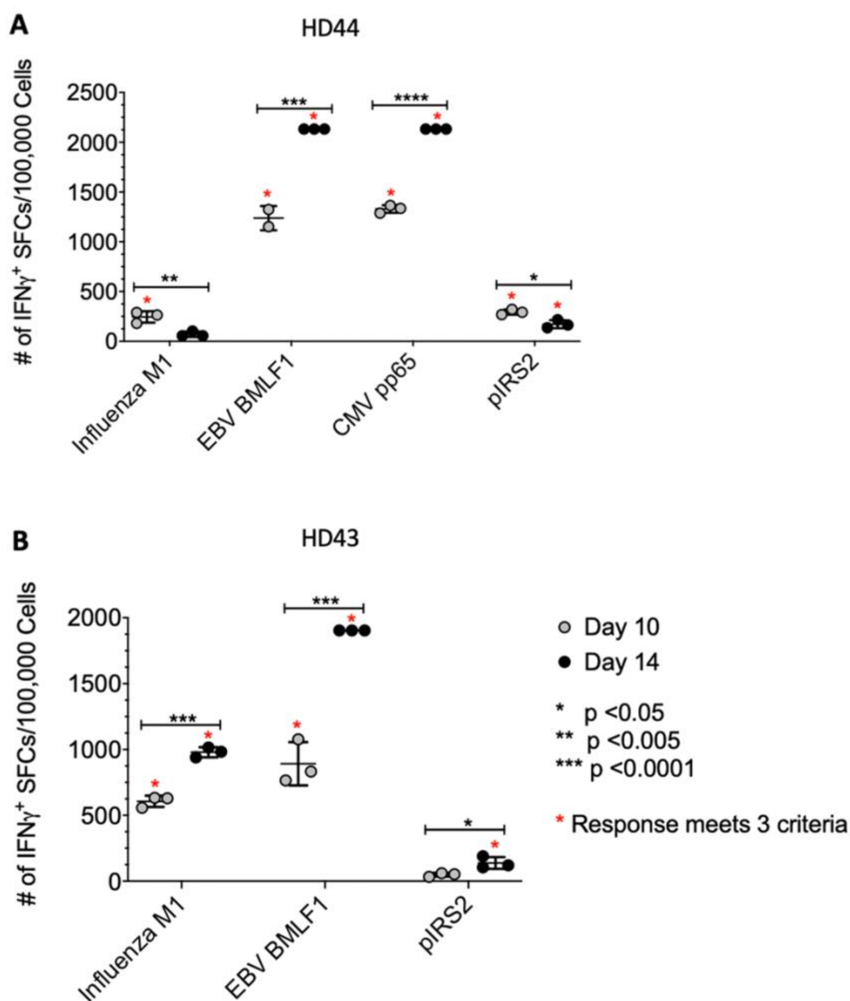


Figure 3.1. Fourteen day expansion of stimulated CD45RO⁺CD8⁺ T cells often results in higher responses than 10 day expansion. Isolated CD8⁺CD45RO⁺ T cells were stimulated one-time *in vitro* stimulation with peptide-pulsed autologous dendritic cells ("Stimulated") or cultured alone with no DCs or peptide ("Unstimulated"). Responses from CD8⁺CD45RO⁺ T cells 10 or 14 days in culture with IL-7 and IL-15, as measured in triplicate wells in an IFNγ ELISpot assay, reported as SFCs/25,000 cells over background (target T2 cells plus DMSO). **A,** Responses in HD44 and **B,** HD43 to HLA-A2 restricted phosphopeptides and viral control peptides. Data reflect one experiment. Differences in responses under different conditions were analyzed by unpaired t tests. SFCs = spot-forming cells. HD = Healthy Donor.

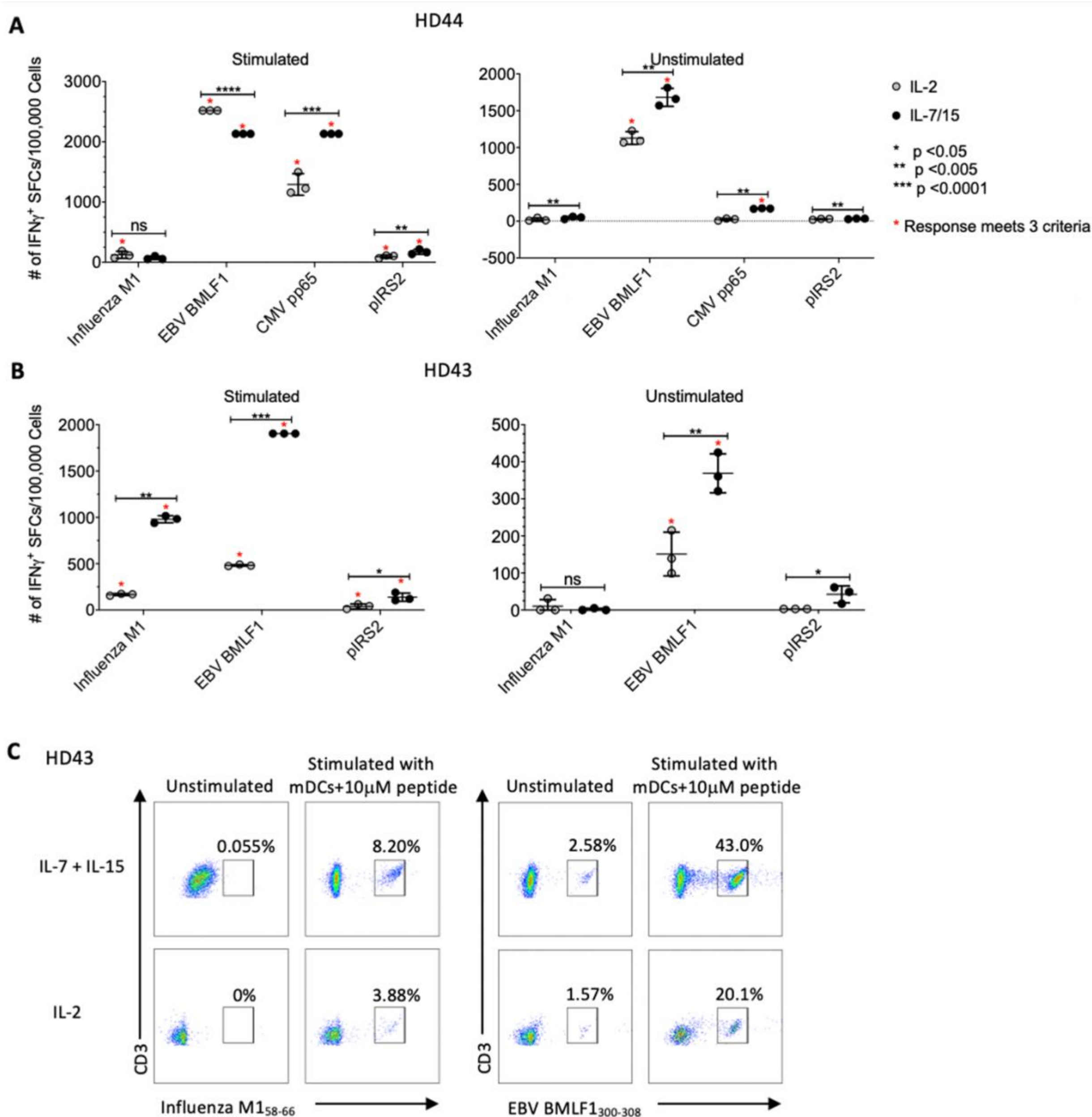


Figure 3.2. Culture of stimulated CD45RO⁺CD8⁺ T cells from healthy donors with IL-7 and IL-15 promotes higher recall responses than culture with IL-2. Responses from CD8⁺CD45RO⁺ T cells after a one-time *in vitro* stimulation with peptide-pulsed autologous dendritic cells ("Stimulated") or co-cultured without peptide-pulsed mDCs ("Unstimulated") and 14 days in culture with IL-7 and IL-15 or with IL-2, as measured in triplicate wells in an IFNγ ELISpot assay, reported as SFCs/100,000 cells over background (target T2-B7 cells plus DMSO). **A,** Responses in HD44 and **B,** HD43 to HLA-A2 restricted phosphopeptides and viral control peptides. C, Flow cytometry staining of viral-specific T cells after a one-time *in vitro* stimulation of CD8⁺CD45RO⁺ T cells with peptide-pulsed autologous dendritic cells ("Stimulated") or co-cultured without peptide-pulsed

mDCs ("Unstimulated") and 16 days in culture with IL-7 and IL-15 or with IL-2. Data reflect one experiment. Differences in responses under different conditions were analyzed by unpaired t tests. SFCs = spot-forming cells. HD = Healthy Donor.

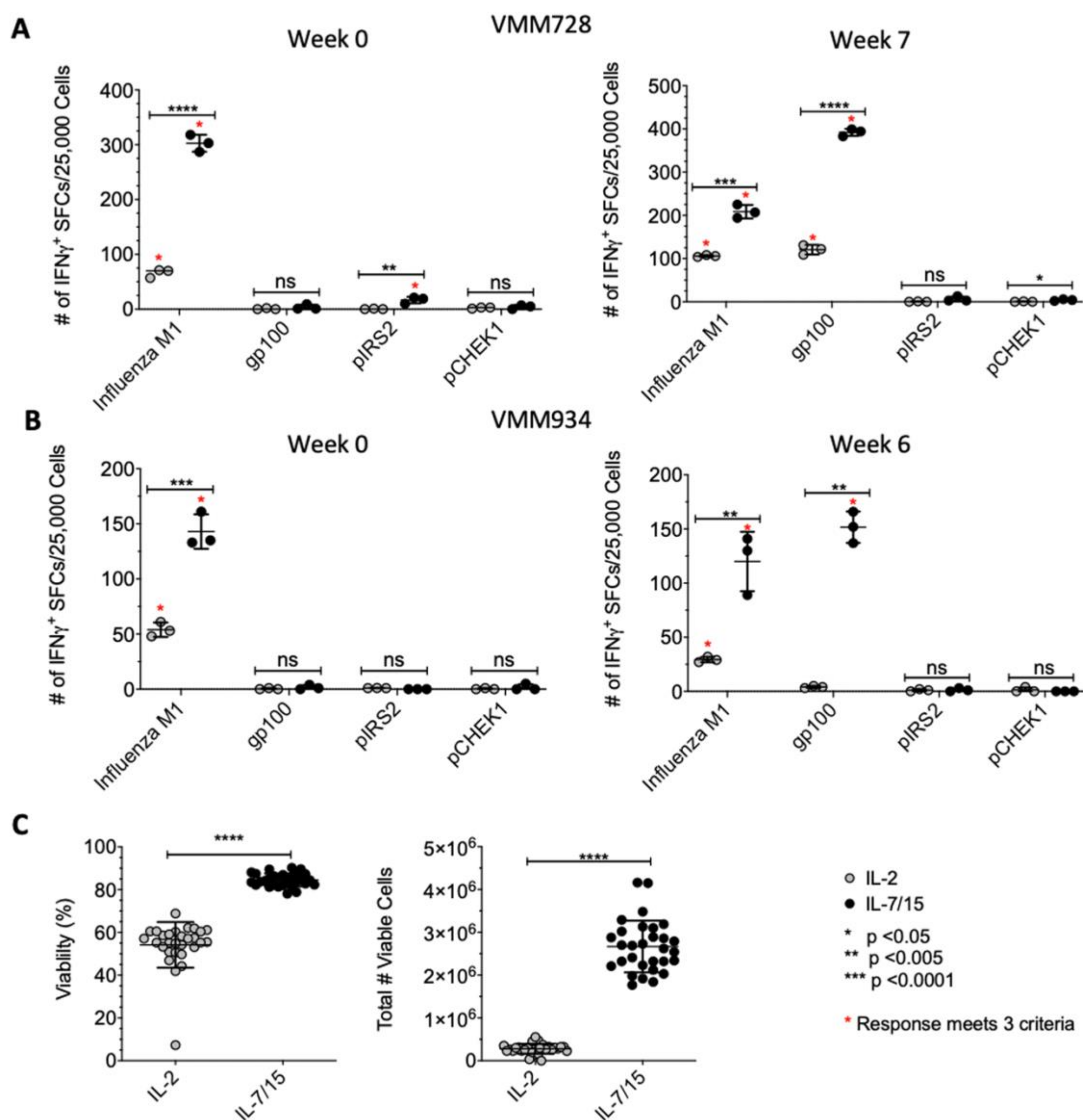
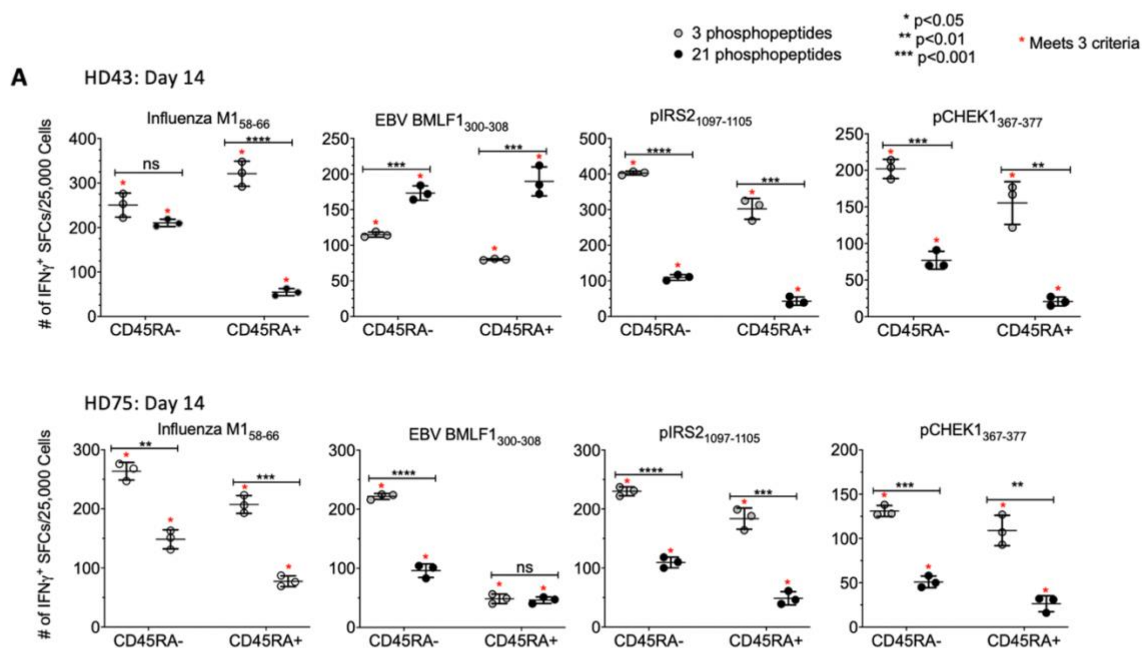
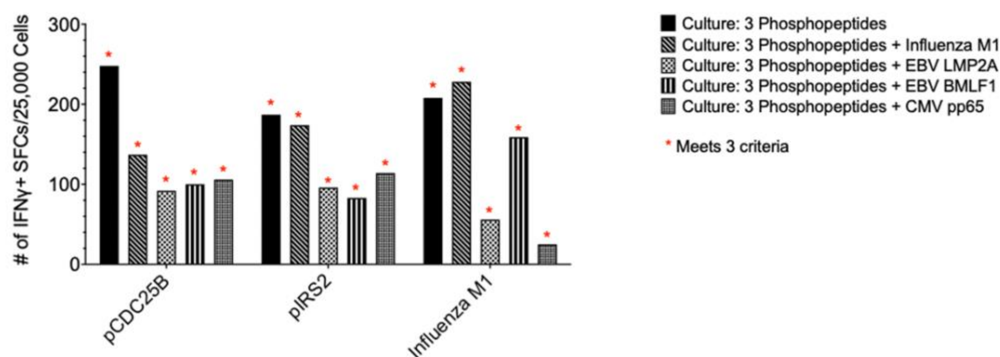


Figure 3.3. Culture of stimulated PBMCs from melanoma patients with IL-7 and IL-15 promotes higher recall responses and better cell numbers and viability than culture with IL-2. Responses from melanoma patients' PBMCs after a one-time *in vitro* stimulation and 14 days in culture with IL-7 and IL-15 or with IL-2, as measured in triplicate wells in an IFNγ ELISpot assay, reported as SFCs/25,000 cells over background (target T2-B7 cells plus DMSO). **A,** Responses in VMM728 and **B,** VMM934 to HLA-A2 restricted phosphopeptides and viral control peptides. **C,** The viability and total number of viable cells at day 14 as measured by ViaCount staining on the Guava. Data are representative of 2 experiments. Differences in responses under different conditions were analyzed by unpaired t tests. SFCs = spot-forming cells.


3 phosphopeptides
21 phosphopeptides
* p<0.05
** p<0.01
*** p<0.001
* Meets 3 criteria
A
HD43: Day 14
Influenza M1 58-66
EBV BMLF1 300-308
pIRS2 1097-1105
pCHEK1 367-377
# of IFNγ+ SFCs/25,000 Cells
CD45RA-
CD45RA+
HD75: Day 14


B
HD43: Day 10
# of IFNγ+ SFCs/25,000 Cells
pCDC25B
pIRS2
Influenza M1
Culture: 3 Phosphopeptides
Culture: 3 Phosphopeptides + Influenza M1
Culture: 3 Phosphopeptides + EBV LMP2A
Culture: 3 Phosphopeptides + EBV BMLF1
Culture: 3 Phosphopeptides + CMV pp65
* Meets 3 criteria

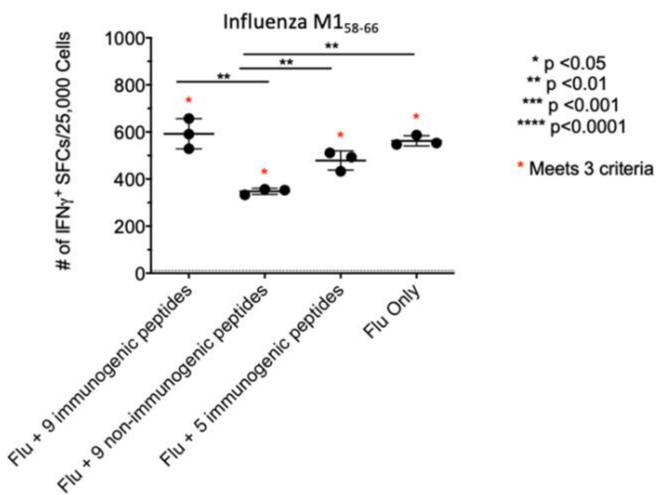

C
HD44: Day 14
Influenza M1 58-66
# of IFNγ+ SFCs/25,000 Cells
Flu + 9 immunogenic peptides
Flu + 9 non-immunogenic peptides
Flu + 5 immunogenic peptides
Flu Only
* p <0.05
** p <0.01
*** p <0.001
**** p<0.0001
* Meets 3 criteria

Figure 3.4. Lower recall responses detected when T cells are stimulated with more peptides per culture. Responses from $CD8^+CD45RO^+$ T cells after a one-time *in vitro* stimulation with peptide-pulsed autologous dendritic cells and 10 days in culture with IL-7 and IL-15, as measured in triplicate wells in an IFNγ ELISpot assay, reported as SFCs/25,000 cells over background (target T2-B7 cells plus DMSO). **A,** Responses in HD43 to HLA-A2 restricted phosphopeptides and viral control peptides when stimulated with 3 phosphopeptides or 3 phosphopeptides plus 1 viral control peptide. **B,** Responses in HD43 and HD75 from $CD8^+CD45RA^-$ or $CD8^+CD45RA^+$ T cells after 14 days in culture with IL-7 and IL-15 to HLA-A2 restricted phosphopeptides and viral control peptides when stimulated with 3 or 21 phosphopeptides. **C,** Responses in HD44 from $CD8^+CD45RO^+$ T cells after 14 days in culture with IL-7 and IL-15 to HLA-A2 restricted Influenza M1 when stimulated with Flu only or Flu plus a number of immunogenic or non-immunogenic phosphopeptides (based on previous experiments in this donor). Data reflect one experiment. Differences in responses under different conditions were analyzed by unpaired t tests. SFCs = spot-forming cells. HD = Healthy Donor.

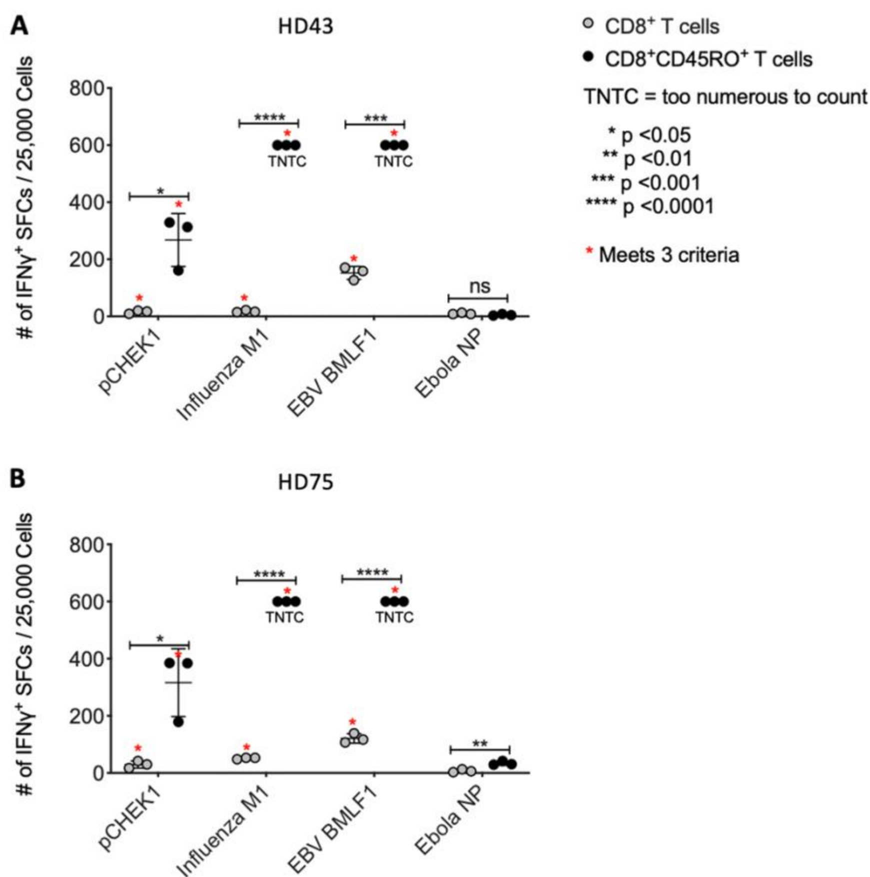


Figure 3.5. Higher memory T cell responses are detected from enriched CD45RO$^+$CD8$^+$ T cells than whole CD8$^+$ T cell population. Responses from CD8$^+$CD45RO$^+$ T cells or CD8$^+$ T cells after a one-time *in vitro* stimulation with peptide-pulsed autologous dendritic cells and 14 days in culture with IL-7 and IL-15, as measured in triplicate wells in an IFNγ ELISpot assay, reported as SFCs/25,000 cells over background (target T2-B7 cells plus DMSO). Cultures began with 1e5 CD8$^+$CD45RO$^+$ T cells plus 2e4 peptide-pulsed mDCs or 3e5 CD8$^+$ T cells plus 6e4 peptide-pulsed mDCs (to account CD45RO$^+$ making up approximately one-third of CD8$^+$ T cells. Data reflect 1 experiment. **A,** Responses in HD43 and **B,** HD75 to HLA-A2 restricted phosphopeptides and viral control peptides. Differences in responses under different conditions were analyzed by unpaired t tests. SFCs = spot-forming cells. HD = Healthy Donor.

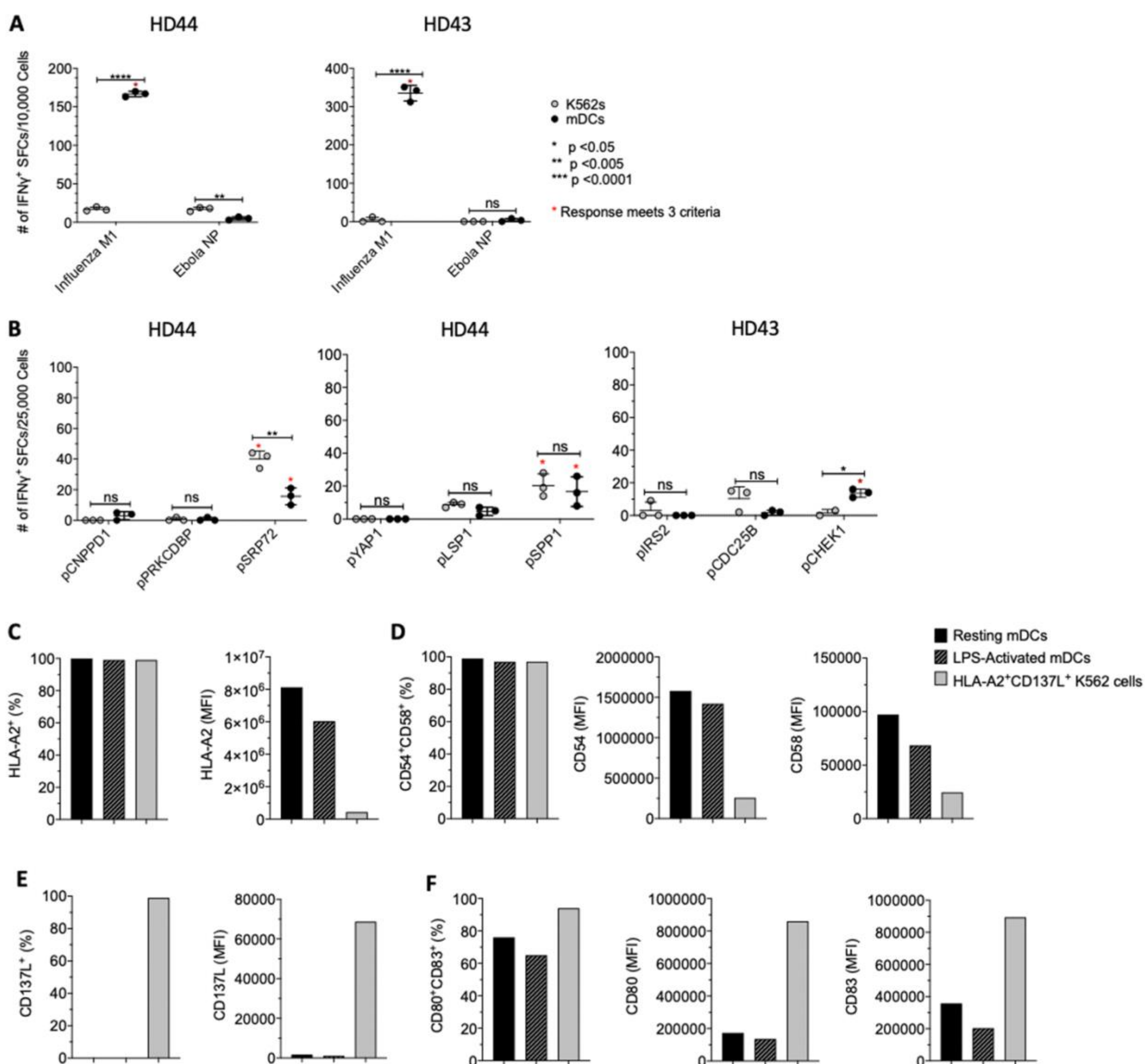


Figure 3.6. Stimulation with peptide-pulsed autologous monocyte-derived dendritic cells results in higher recall responses than stimulation with peptide-pulsed K562 articial APCs. Responses from $CD8^{+}CD45RO^{+}$ T cells after a one-time *in vitro* stimulation with either peptide-pulsed autologous monocyte-derived dendritic cells or peptide-pulsed K562 cells and 14 days in culture with IL-7 and IL-15, as measured in triplicate wells in an IFNγ ELISpot assay, reported as SFCs/25,000 cells over background (target T2-B7 cells plus DMSO). **A,** Responses in HD43 and HD44 to HLA-A2 restricted viral control peptides (representative of 2 independent experiments). **B,** Responses in HD43 and HD44 to HLA-A2 restricted phosphopeptides. Data reflect 1 experiment. **C-F,** Flow cytometry analysis of cell surface marker expression on mDCs or K562s. Data reflect 1 experiment. Differences in responses under different conditions were analyzed by unpaired t tests. SFCs = spot-forming cells. HD = Healthy Donor.

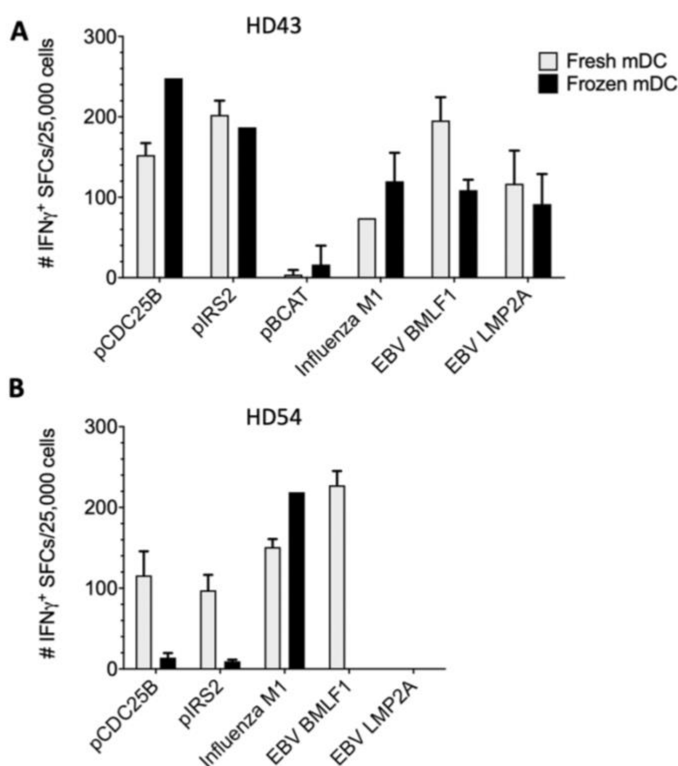


Figure 3.7. CD8⁺CD45RO⁺ T cell responses are comparable when stimulated with freshly isolated or previously frozen autologous monocyte-derived dendritic cells. Responses from CD8⁺CD45RO⁺ T cells after a one-time *in vitro* stimulation with peptide-pulsed autologous dendritic cells and 10 days in culture with IL-7 and IL-15, as measured in duplicate wells in an IFNγ ELISpot assay, reported as SFCs/25,000 cells over background (target T2 cells plus DMSO). **A,** Responses in HD43 and **B,** HD54 to HLA-A2 restricted phosphopeptides and viral control peptides. Differences in responses under different conditions could not be statistically analyzed because wells were in duplicate. SFCs = spot-forming cells. HD = Healthy Donor.

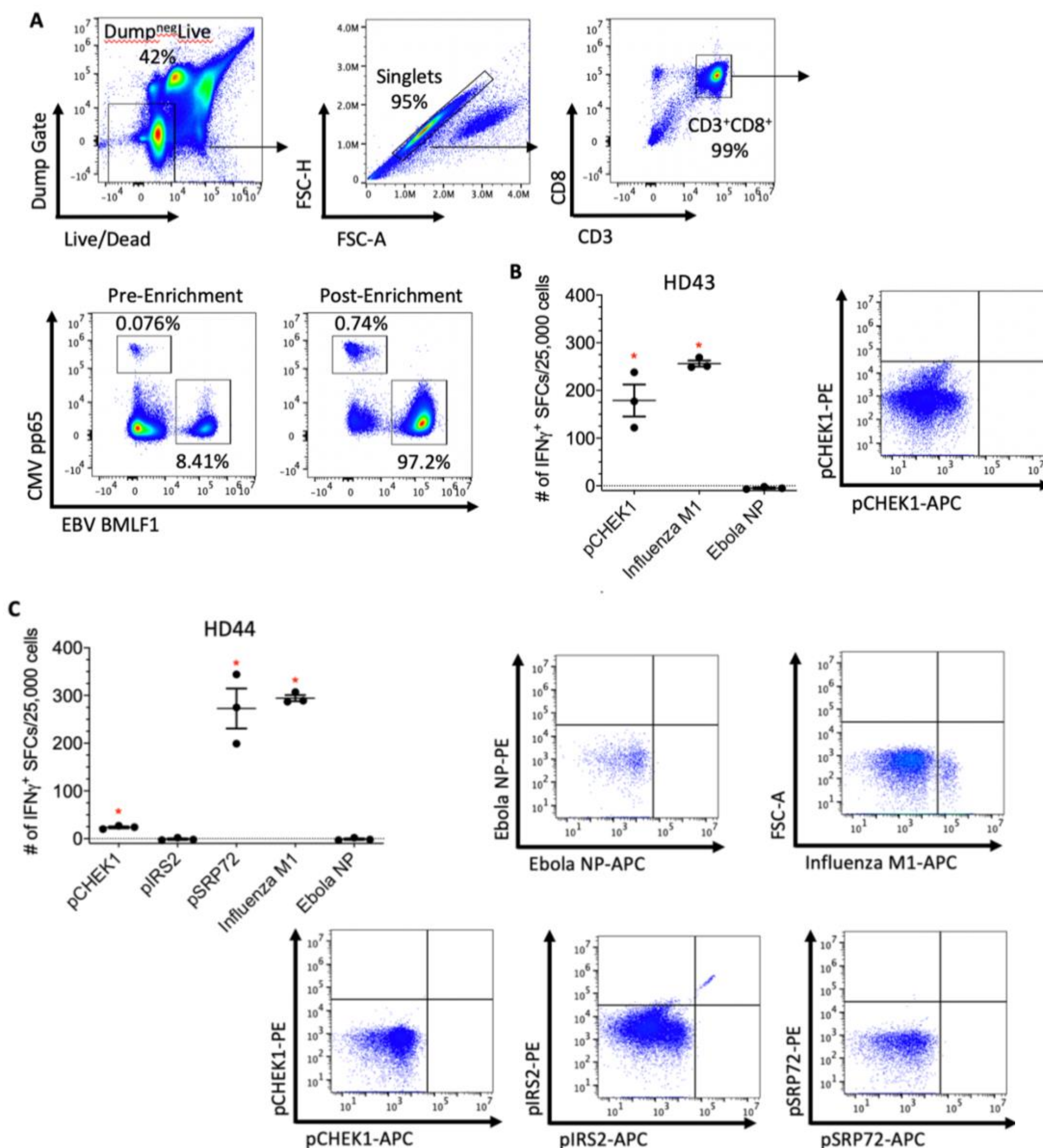


Figure 3.8. Multimer staining to detect antigen-specific T cells. Multimer detection by flow cytometry of viral and phosphopeptide-specific T cells. **A,** *Ex vivo* detection of viral-specific T cells in HD44 before and after magnetic enrichment. **B,** Flow cytometry staining and responses in HD43 and **C,** HD44 to HLA-A2 restricted phosphopeptides and viral control peptides from CD8^{+}CD45RO^{+} T cells after a one-time *in vitro* stimulation with peptide-pulsed autologous dendritic cells and 14 days in culture with IL-7 and IL-15. Responses are measured in triplicate wells in an IFNγ ELISpot assay, reported as SFCs/25,000 cells over background (target T2-B7 cells plus DMSO). Cells are gated on Singlet, Live, CD3+CD8+ T cells for flow cytometry staining. SFCs = spot-forming cells. HD = Healthy Donor.

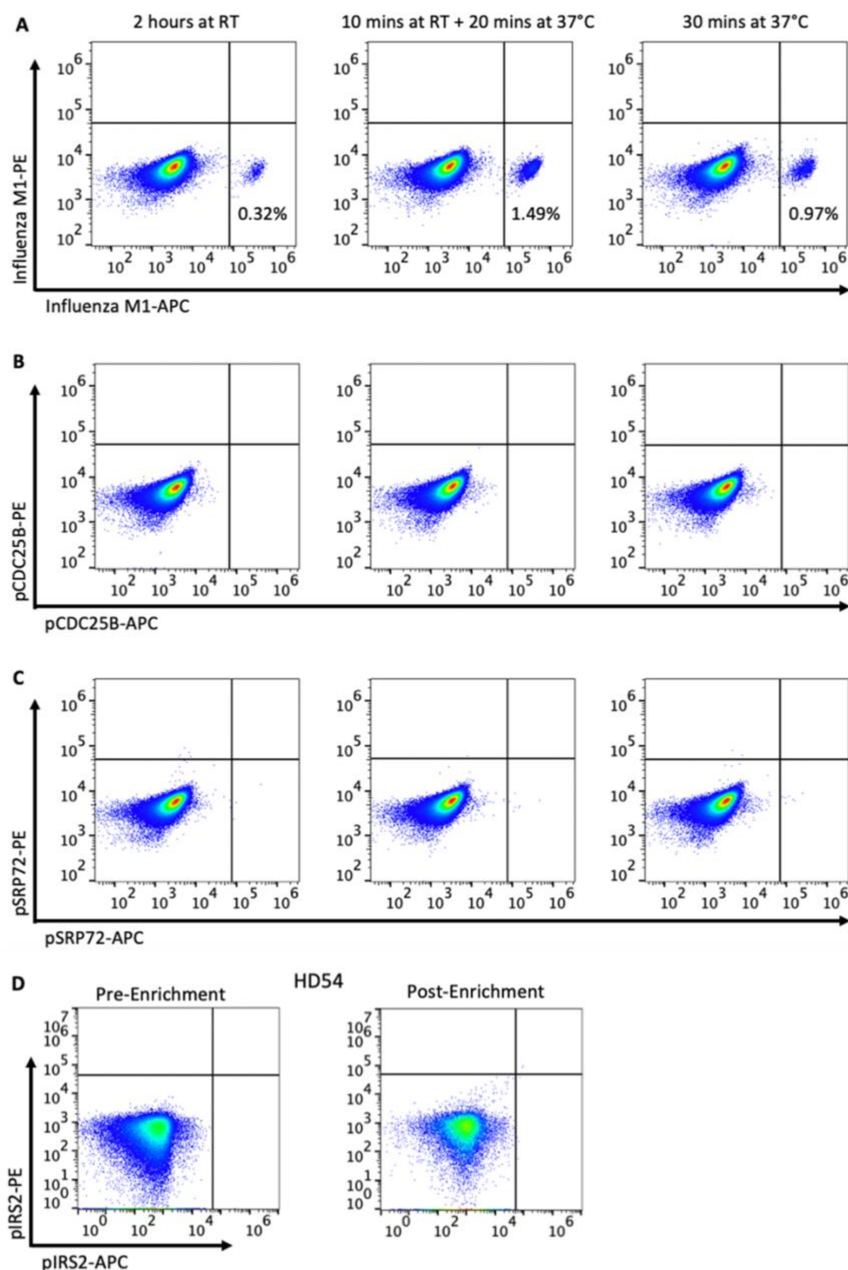


Figure 3.9. Assessment of multimer staining protocols. A-C, Viral or phosphopeptide-specific T cells in HD44 detected by multimers and flow cytometry after peptide stimulation and 14 days culture. Three multimer staining protocols were utilized to optimize multimer staining. **A,** Influenza M1-specific, **B,** pCDC25B-specific, and **C,** pSRP72-specific T cells in HD44 as detected by multimer staining using 3 different staining protocols. **D,** Phosphopeptide-specific T cells in HD54 detected *ex vivo* by multimers and flow cytometry after magnetic enrichment. Staining protocol used was 10 minutes at room temperature followed by 20 minutes at 37°C, as determined in panel A. HD = healthy donor. RT = room temperature.

Chapter 4: Immune memory and effector activity to cancer-expressed MHC-I phosphopeptides in healthy donors and cancer patients

This chapter contains a modified version of the article: Lulu AM, Cummings KL, Jeffery ED, Myers PT, Underwood D, Lacy RM, Chianese-Bullock KA, Slingluff CL, Modesitt SC, and Engelhard VH. "Immune memory and effector activity to cancer-expressed MHC-I phosphopeptides in healthy donors and cancer patients." Cancer Immunology Research. (2021, in press)

Experimental Contributions: Cummings KL (together with AML), conducted CD45RO^{+} healthy donor experiments and kinase recognition motif analysis.

Authors' contributions:

A. Lulu: Conceptualization, methodology, data curation, formal analysis, visualization, supervision, writing – original draft, writing – review and editing. **K. Cummings:** Conceptualization, methodology, data curation, formal analysis, visualization, supervision, writing – original draft, writing – review and editing. **E. Jeffery:** Methodology, data curation, writing – review and editing. **P. Myers:** Methodology, data curation, writing – review and editing. **D. Underwood:** Data curation, writing – review and editing. **R. Lacey:** Data curation, project administration, writing – review and editing. **K. Chianese-Bullock:** Resources, data curation, writing – review and editing. **C. Slingluff:** Resources, data curation, writing – review and editing. **S. Modesitt:** Patient recruitment, data curation, supervision, project administration, writing – review and editing. **V. Engelhard:**

Conceptualization, resources, supervision, funding acquisition, project administration, writing – original draft, writing – review and editing.

This chapter contains a subset of data from the previously published article: Engelhard VH, Obeng RC, Cummings KL, Petroni GR, Ambakhutwala AL, Chianese-Bullock KA, Smith KT, Lulu AM, Varhegyi N, Smolkin ME, Myers P, Mahoney KE, Shabanowitz J, Buettner N, Hall EG, Haden K, Cobbold M, Hunt DF, Weiss G, Gaughan E, and Slingluff CL. "MHC-restricted phosphopeptide antigens: preclinical validation and first-in-humans clinical trial in participants with high-risk melanoma." Journal for Immunotherapy of Cancer. 8.e000262 (2020), doi, 10.1136/jitc-2019-000262.

Experimental and Analysis Contributions: Smith K (ELISpot analyses of Mel59 clinical trial patient responses), Cummings KL (together with AML, optimization of patient culture conditions, ELISpot analyses of responses in non-clinical trial melanoma patients), Petroni GR (Statistical analyses of responses, Survival analyses).

4.1 Highlights

- Most healthy donors have pre-existing immune memory to cancer-expressed phosphopeptides.
- There is high donor-to-donor variability in pre-existing phosphopeptide-specific immune memory.
- A few phosphopeptides are immunodominant memory targets against which most healthy donors are exposed and develop immunity.
- Phosphopeptide immunity in ovarian cancer patients is negligible to tumor-expressed phosphopeptides and to immunodominant memory targets.
- Phosphopeptide immunity in melanoma patients is negligible, but responses can be induced by vaccination.

4.2 Abstract

Elevated immunity to cancer-expressed antigens has been demonstrated in people with no history of cancer and may contribute to cancer prevention. We previously established MHC-restricted phosphopeptides are cancer-expressed antigens and targets of immune recognition. However, the extent to which immunity reflects prior or ongoing exposures was not investigated. Here we demonstrate that pre-existing immune memory to cancer-expressed phosphopeptides is evident in most healthy donors but the breadth among donors is highly variable. Three phosphopeptides were recognized by most donors, suggesting exposures to common microbial/infectious agents. However, most of 205 tested phosphopeptides were not recognized by PBMCs from any donor and the remainder were recognized by only 1-3 donors. In longitudinal analyses of 2 donors, effector immune response profiles suggested active re-exposures to a subset of phosphopeptides. These findings suggest that the immunogens generating most phosphopeptide-specific immune memory are rare infectious agents or incipient cancer cells with distinct phosphoproteome dysregulations, but that repetitive immunogenic exposures occur in individual donors. Lastly, phosphopeptide immunity in PBMCs and TILs from ovarian cancer patients was limited, regardless of whether the phosphopeptide was expressed on their tumor. However, 4/10 patients responded to 1-2 immunodominant phosphopeptides, and one showed an elevated effector response to a tumor-expressed phosphopeptide. As the tumors from these patients displayed many phosphopeptides, these data are most consistent with lack of prior exposure or impaired ability to respond to some phosphopeptides, and suggest that enhancing phosphopeptide-specific T-cell responses could be a useful approach to improve tumor immunotherapy.

4.3 Introduction

Dysregulated signaling is a hallmark of cancer cells with over-activated kinases or inhibited phosphatases driving many of the aberrant pathways that contribute to malignant transformation [338, 399]. This dysregulated signaling results in over-expressed phosphorylated proteins or phosphorylation at noncanonical sites. Phosphorylated proteins undergo proteasomal degradation, and some of the resulting phosphorylated peptides can be presented by MHC molecules on cancer and Epstein-Barr Virus (EBV)-transformed cells [1–5, 45, 347, 348, 352, 353, 356]. MHC-presented phosphopeptides are recognized by T cells in a phosphate- and sequence-dependent manner [1–4, 337, 349, 352, 354–356]. Therefore, MHC-presented phosphopeptides are a new class of tumor antigens.

Phosphopeptide-specific T cells can recognize cancer cell lines [2–4, 347], and many tumor types are known to express phosphopeptides [1–4, 45, 347, 348, 353]; thus, phosphopeptide-specific T-cell responses may play a role in tumor control. Phosphopeptide-specific cytotoxic T cells can be generated *in vivo* by immunization [1, 3–5, 337] or *in vitro* by repeated peptide stimulation [352]. Adoptive transfer of phosphopeptide-specific T cells slows tumor growth in a humanized melanoma model [3, 5], and phosphopeptide immunization delays tumor growth in a humanized lung cancer model [4]. We recently demonstrated the safety and immunogenicity of a vaccine in inducing phosphopeptide-specific immune responses in melanoma patients [5], establishing the potential therapeutic application of phosphopeptide neoantigens. Altogether, these data support further investigation into the role of phosphopeptide-specific T cells in tumor control.

There is increasing evidence that the immune systems in otherwise healthy individuals perform surveillance and destruction of incipient cancer cells, developing "pre-existing immune memory" to cancer antigens in the absence of clinically evident tumors. Tumor antigen-specific antibodies and T cells have been found in liver, colon, and pancreatic pre-malignant lesions prior to progression to clinically-detectable cancer [110–112]. $CD4^+$ and $CD8^+$ T-cell responses to myeloma-associated cancer testis antigens are evident in patients with Monoclonal Gammopathy of Undetermined Significance prior to multiple myeloma [113]. Evidence of immunosurveillance in healthy individuals with no known pre-malignant lesions includes effector T cells and antibodies to cyclin B1, which is overexpressed in lung, colorectal, cervical, and head and neck cancers [118], and to hypoglycosylated MUC1, which is overexpressed in most human adenocarcinomas [119]. The immunogenic exposures that generate pre-existing immune memory to tumor antigens are unknown, but are thought to be incipient cancer cells or viral infections [124, 279, 280, 289]. Some viruses dysregulate many of the same kinases and/or phosphatases that are dysregulated in cancer [400–402]. Regardless of how it is generated, pre-existing immune memory against tumor antigens may protect against future development of cancer.

We previously found that healthy donors with no evident prior cancer had robust $CD8^+$ T-cell responses to two HLA-A2-restricted melanoma-expressed phosphopeptides and to a cohort of HLA-B7-restricted leukemia-expressed phosphopeptides [2, 3]. Some of these responses arose from $CD8^+CD45RO^+$ antigen-experienced T cells, providing evidence of phosphopeptide-specific memory T cells in healthy individuals in the absence of clinically-evident malignancy [2, 3]. Here we explicitly determined the pervasiveness of pre-existing immune memory to over 200 phosphopeptides in 15 healthy donors. We

also determined the existence of immunodominant phosphopeptides that were recognized by the majority of donors. By characterizing memory and effector subsets, we tested the hypothesis that some immunogenic exposures to phosphopeptides were current or reoccurred over time. Finally, we evaluated memory and effector activity in ovarian cancer patients to test the hypothesis that their phosphopeptide-specific responses were limited compared to healthy donors and that this limitation was confined to the phosphopeptides expressed on their tumors.

4.4 Results

4.4.1 CD8 T cell memory to cancer-expressed phosphopeptides is highly variable among healthy donors.

To determine the extent to which healthy donors show pre-existing immune memory to phosphopeptides, we purified $CD8^+CD45RO^+$ T cells from 15 healthy donors, including 6 HLA-$A2^+$, 5 HLA-$B7^+$, and 4 HLA-$A2^+$/-$B7^+$ donors. This T cell population consists of central and effector memory cells [403]. We evaluated responses after *in vitro* stimulation with peptide-pulsed mDCs and culture with cytokines for 14 days ("cultured"). We assessed responses to 205 HLA-A2- or HLA-B7-restricted phosphopeptides that were previously shown to be presented on: 1) one or more patient-derived tumors (leukemias, colorectal cancer, ovarian cancer) and/or 2) cancer cell lines (melanoma, ovarian cancer, breast cancer), and, in some cases, also 3) EBV-transformed B-cell lines (**Table 2.1**). There were no responses detected to Ebola peptides in any donor, consistent with assay conditions testing memory and not naive immune responses (**Fig. 4.1A, 4.1B**). Responses were frequently detected to peptides from Influenza, CMV, and EBV. All ten HLA-$A2^+$ donors showed pre-existing immune memory to at least one of 51 HLA-A2-restricted phosphopeptides (**Fig. 4.1A**), while 7/9 HLA-$B7^+$ donors showed pre-existing immune memory to at least one of 154 HLA-B7-restricted phosphopeptides (**Fig. 4.1B**). Individual donors responded to between 1 and 14 (mean=7, median=5.5) HLA-A2-restricted and between 0 and 20 (mean=6, median=3) HLA-B7-restricted phosphopeptides. However, in donors analyzed later, we examined responses only to the subset of phosphopeptides recognized by at least one earlier donor (**Tables 4.1, 4.2**). No responses were detected to a subset of unphosphorylated peptides, which is consistent with the generation of

phosphopeptides—but not the unphosphorylated version of the peptide—under immunogenic exposures and the absence of tolerance in that donor against the recognized phosphopeptide (**Fig. 4.2A, 4.2B**). Thus, most healthy donors exhibited pre-existing immune memory to some MHC-I-restricted phosphopeptides, although the breadth varied.

We asked whether multiple donors recognized the same phosphopeptides, which would suggest common immunogens. Of the 26 HLA-A2-restricted phosphopeptides that were targets of pre-existing immune memory (hereafter referred to as "memory targets"), 17 were recognized by 2 or more donors (0.65, 95% CI, 0.46, 0.81) (**Fig. 4.1A**). However, only 3 of these (pIRS2$_{1097\text{-}1105}$, pCDC25B$_{38\text{-}46}$, and pCHEK1$_{367\text{-}377}$) were "immunodominant", in that they were memory targets in more than half of donors. In contrast, of the 32 HLA-B7-restricted memory targets, only 12 were recognized by 2 or more individuals (0.38, 95% CI, 0.23, 0.55) (**Fig. 4.1B**). While two donors (HD43 and HD44) recognized a large number of HLA-B7-restricted memory targets in common, the remaining 5 donors recognized mostly non-overlapping memory targets, and none met the criterion for immunodominance.

We asked whether any kinase recognition motifs (KRMs) were enriched among memory targets, which could suggest that a subset of kinases is differentially responsible for generating immunogenic phosphopeptide exposures in healthy donors. However, there was no statistically significant enrichment of any KRMs among memory targets relative to the phosphopeptides not recognized by any donor (**Fig. 4.3A, Table 4.3**). Although pathogen exposures and transformation events are expected to increase with age, the percentage of phosphopeptides recognized did not correlate with donor age (**Fig. 4.3B**).

Finally, although chronic EBV infection is prevalent in humans, there was no difference in the proportion of memory targets between those expressed (0.24, 95% CI, 0.17, 0.33) and not expressed (0.33, 95% CI, 0.25, 0.44) on EBV-transformed cells. These results suggest that pre-existing immune memory to most phosphopeptides reflects exposures that are not common among donors.

4.4.2 Immunogenic re-exposures to a subset of phosphopeptides occurred in two healthy donors.

To test the hypothesis that immunogenic exposures to phosphopeptides occurred recently or were ongoing, phosphopeptide responses in subsets of memory and effector cells were quantitated in HD43 and HD44. These donors were chosen because of the breadth of memory targets they recognized, many of which were shared, and their expression of both HLA-A2 and HLA-B7. First, their response patterns indicative of recent or ongoing exposure to well-characterized viral antigens were established. Using CD45RO and CCR7 expression, $CD8^+$ T cells were sorted into central memory (T_{CM}), effector memory (T_{EM}), TEMRA/effector (T_{EMRA}), and naïve plus memory stem cells (T_N+T_{SCM}) (**Fig. 2.1**). In some experiments the latter population was separated using CD95. Cultured responses to viral peptides or phosphopeptides from each subset were quantified, and the contribution of each subset to the overall $CD8^+$ PBMC response was determined by taking into account the increase in number of T cells during the culture period and the fractional representation of each subset in the original PBMC isolate (see Methods). A similar method was used to evaluate direct effector activity in freshly isolated PBMC after 18-20 hours of incubation with the same peptides.

Under resting conditions, T cells specific for antigens that arise transiently or are expressed at low level are typically found mostly as T_{CM} [98, 101]. Consistent with this, cultured Influenza $M1_{58-66}$-specific effectors in both donors were derived predominantly (57-83%) from T_{CM}, while T_{EM} and T_N+T_{SCM} subsets contributed to a lesser extent, and contribution from T_{EMRA} was negligible (**Fig. 4.4A, 4.4B**). Direct effectors to $M1_{58-66}$ were low-level (300 and 100-300 IFNγ^+ cells/10^6 CD8$^+$ T cells in HD44 and HD43, respectively) and also derived predominantly from T_{CM}. Cultured responses to $LMP2A_{307-315}$, a latent phase epitope expressed at low levels during chronic EBV infection, were also derived predominantly (71-98%) from T_{CM}. Direct responses for $LMP2A_{307-315}$ were low level (40-200 and 15-40 IFNγ^+ cells/10^6 CD8$^+$ T cells in HD44 and HD43, respectively) and derived from T_{EM} in HD44 and T_{CM} in HD43. The contributions of the T_N+T_{SCM} subset to the cultured responses in HD44 and HD43 were largely due to T_{SCM}, although a small contribution from the T_N subset was also evident in the HD44 response to $M1_{58-66}$ (**Fig. 4.4C, 2D**). In PBMCs collected from HD44 and HD43 16-17 days after their annual Influenza vaccinations, T_{CM} cells were no longer the predominant contributors (less than 35%) to the cultured response; instead, T_{CM}, T_{EM}, and T_N+T_{SCM} contributed similarly and there was also a low but significant contribution from T_{EMRA}, consistent with an ongoing response. (**Fig. 4.4A, 4.4B**). Direct responses from PBMCs harvested 7-8 days after vaccination shifted entirely to T_{EM} in HD44 and modestly in HD43, again consistent with an ongoing response, although the number of direct effectors (300-500 IFNγ^+ cells/10^6 CD8$^+$ T cells in both donors HD43 and HD44) was only modestly increased relative to the pre-vaccine condition.

Responses to peptides from proteins expressed at high levels during the lytic phase of chronic viral infections are typically skewed toward T_{EM} and T_{EMRA} [94, 98, 101]. In HD44, all four cell subsets contributed substantially to cultured responses to EBV $BMLF1_{300-308}$ and $BBLF2/3_{624-632}$ and to CMV $pp65_{417-426}$ and $pp65_{495-503}$, with effectors derived from T_{CM} contributing only 21-37% and effectors derived from T_{EMRA} contributing 18-35% of the total response (**Fig. 4.4A**). The contribution of the T_N+T_{SCM} subset was largely due to T_{SCM} although a small contribution from the T_N subset was also evident for $BMLF1_{300-308}$ (**Fig. 4.4C**). Strong direct responses (2,500-150,000 IFNγ^+ cells/10^6 $CD8^+$ T cells) were also evident, predominantly from the T_{EM} and T_{EMRA} subsets. The cultured response of HD43 to $BMLF1_{300-308}$ differed from that of HD44, in that T_{CM} were the predominant contributor, with only minor contributions from T_{EM} and T_{EMRA} (**Fig. 4.4B**). The contribution of the T_N+T_{SCM} subset was similar to that of HD44 and largely due to T_{SCM} (**Fig. 4.4D**). However, a strong direct response (14,000 IFNγ^+ cells/10^6 $CD8^+$ T cells) was also evident, with elevated contributions from T_{CM} and T_N+T_{SCM} compared to HD44. The elevated representation of T_{SCM} in HD43's direct response to $BMLF1_{300-308}$ likely reflects the elevated representation of T_{SCM} (10%) in circulating $CD8^+$ T cells in this donor versus 2% in HD44 (**Fig. 2.1**) and 1-3% in humans overall [82], but the elevated representation of T_{CM} is surprising. Nonetheless, these results establish two distinct response patterns: Response Pattern 1, in which T_{CM} are the predominant contributors (greater than 55% of the total response) to cultured responses and direct effector activity is low, associated with non-recent or low-level antigen exposure; and Response Pattern 2, in which the contribution of T_{EM}, T_{EMRA}, and/or T_{SCM} to cultured responses is enhanced at the

expense of T_{CM} (making up less than 50% of the total response) and direct effector activity is high, associated with recent or ongoing high-level antigen exposure.

Using these patterns as a guide, we evaluated responses to a cohort of phosphopeptides recognized by these two donors in blood draws taken over 13-16 months. However, because of sample size limitations, we were often unable to compare cultured and direct responses in the same blood draw. Consequently, we categorized response patterns based on T cell subset distribution in cultured responses. In the initial blood draws, both response patterns were observed to different phosphopeptides. Response Pattern 1 was evident in HD44 cultured responses to $pLSP1_{249-258}$, $pWWTR1_{86-94}$, $pSRP72_{466-473}$, $pCHEK1_{461-471}$, and $pPEG10_{248-259}$, (**Fig. 4.5A**), and in HD43 cultured responses to $pCHEK1_{461-471}$ and $pSRP72_{466-473}$ (**Fig. 4.6A**). In these instances, responses in the T_N+T_{SCM} subset were exclusively from T_{SCM} (**Fig. 4.5B, 4.6B**). Response Pattern 2 was evident in initial HD44 cultured responses to $pPEG10_{248-258}$ and $pCDC25B_{38-46}$ (**Fig. 4.5A**), and in HD43 responses to $pPEG10_{248-259}$ and $pCDC25B_{38-46}$ (**Fig. 4.6A**). Interestingly, these response patterns changed in subsequent blood draws. In HD44, the response to $pLSP1_{249-258}$ shifted from Pattern 1 to 2 at +10 months. The response to $pPEG10_{248-258}$ shifted from Pattern 2 to 1 at all other timepoints, and the response to $pCDC25B_{38-46}$ remained Pattern 2 until +13 months. In HD43, Pattern 1 to 2 shifts were evident in responses to $pCHEK1_{461-471}$ at +4 months and $pSRP72_{466-473}$ at +3 and +13 months, and Pattern 2 to 1 shifts were evident in responses to $pPEG10_{248-259}$ at +13 months and to $pCDC25B_{38-46}$ at +3, +13, and +16 months. Interestingly, the timepoints at which Pattern 2 responses for different peptides were evident were largely distinct for both donors (**Fig. 4.5C, 4.6C**), indicating that the processes resulting in their immunogenic expression were independent. These

results suggest that, while phosphopeptides are infrequently expressed in the population, they are repeatedly re-expressed in an individual. We evaluated direct responses to the same phosphopeptides in both donors to determine whether *ex vivo* effector activity was consistent with the exposure status suggested by the cultured response. Direct responses in both donors at most timepoints were predominantly or exclusively derived from T_{CM} or T_{EM} and low-level (5-472 and 0-183 SFCs/10^6 $CD8^+$ T cells in HD44 and HD43, respectively) (**Fig. 4.7A, 4.7B**). These responses are consistent with Pattern 1 cultured responses to the same peptides observed at many of the same time points (**Fig. 4.5A, 4.6A**), and with direct responses to $M1_{58\text{-}66}$ and $LMP2A_{307\text{-}315}$ (**Fig. 4.4A, 4.4B**). The HD44 cultured responses to $pCDC25B_{38\text{-}46}$ at +3 and +4 months and HD43 cultured response to $pPEG10_{248\text{-}259}$ and $pCDC25B_{38\text{-}46}$ at +6 months were all Pattern 2 (**Fig. 4.5A, 4.6A**). The HD43 direct response to $pCDC25B_{38\text{-}46}$ was not evident, but the HD44 direct response to $pCDC25B_{38\text{-}46}$ at +3 months was predominantly T_{EM}, consistent with viral Pattern 2 direct responses (**Fig. 4.7A, 4.7B**). However, the direct responses of HD44 to $pCDC25B_{38\text{-}46}$ at +4 months and of HD43 to $pPEG10_{248\text{-}259}$ at +6 months were almost exclusively T_{CM} (**Fig. 4.7A, 4.7B**). This is inconsistent with viral Pattern 2 direct responses in which T_{EM} and T_{EMRA} are predominant (**Fig. 4.4A, 4.4B**). These direct responses mediated by T_{CM} suggest that these cells are not resting, but may represent alternatively differentiated effectors, which may suggest distinct immunogenic exposures to phosphopeptides.

4.4.3 Immunity to ovarian cancer patients

We next evaluated phosphopeptide-specific responses of 8 HLA-$A2^+$ and 2 HLA-$A2^+$/$B7^+$ ovarian cancer patients in relation to the phosphopeptides expressed on their tumors, which were identified by mass spectrometry (**Table 4.4**). From 12 tumor specimens (1 tumor from

8 patients and 2 tumor specimens from 2 patients), we identified 242 total and 180 unique phosphopeptides. Individual tumors expressed between 2 and 67 (mean=20) phosphopeptides. For analysis of patient immune responses, phosphopeptides were chosen that were expressed on at least 1 tumor and/or were common memory targets in healthy donors, because these responses demonstrated the peptides' immunogenicity and likely absence of self-tolerance.

Because cell numbers in patient PBL and tumor were low, $CD8^+$ T cells were sorted into only 2 subsets, (T_{CM}+T_N) and (T_{SCM}+T_{EM}+T_{EMRA}) (**Fig. 2.2A, 2.2B**), which still enabled discrimination between Pattern 1 and Pattern 2 responses. Fourteen-day cultured responses to $M1_{58-66}$ and $BMLF1_{300-308}$ viral peptides were evident in 9/9 and 8/9 assessed patients, respectively) (**Fig. 4.8, 4.9**). Responses to the immunodominant $pIRS2_{1097-1105}$ were also evident in 4/10 patients (0.4, 95% CI, 0.17, 0.69), which was not significantly different from healthy donors (0.6, 95% CI, 0.31, 0.83) (**Fig. 4.8**). This included VTB239, whose tumor expressed $pIRS2_{1097-1105}$, and 2 patients who also responded to the immunodominant $pCHEK1_{367-377}$. However, the fractions of patients responding to $pCHEK1_{367-377}$ (0.2, 95% CI, 0.04, 0.51) and the other immunodominant memory target, $pCDC25B_{38-46}$ (0.0, 95% CI, 0.00, 0.30), were significantly lower than those of healthy donors ([1.0, 95% CI, 0.72, 1.0] and [0.7, 95% CI, 0.40, 0.89], respectively). TILs from two of these patients also showed responses to the same phosphopeptides. There were no responses to any other phosphopeptide in these 4 patients, including 12 that were expressed on their tumors. The remaining 6 patients demonstrated no responses to any evaluated phosphopeptide, including 6 that were expressed on their tumors (**Fig. 4.9**). Overall, the fractions of phosphopeptides expressed (0.06, 95% CI, 0.00, 0.28) or not expressed (0.07,

95% CI, 0.03, 0.15) on the patient's tumor were recognized at similar rates. These data suggest a general impairment in phosphopeptide immunity in ovarian cancer patients compared to healthy donors.

To determine whether expression of $pIRS2_{1097-1105}$ on VTB239's tumor could have influenced the response in this patient, we compared the response pattern of VTB239 to that of VTB241, whose tumor did not express $pIRS2_{1097-1105}$ or $pCHEK1_{367-377}$. Cultured PBL responses in VTB241 to $pIRS2_{1097-1105}$ and $pCHEK1_{367-377}$ were predominantly derived from T_{CM} with low cell numbers (200-600 $IFN\gamma^+$ cells/10^6 $CD8^+$ T cells) (**Fig. 4.10A**), consistent with Response Pattern 1 and a non-recent antigen exposure. Responses to Influenza M1 and EBV BMLF1 peptides in this patient were consistent with Pattern 2, demonstrating active immunity. On the other hand, cultured responses of PBLs and TILs to $pIRS2_{1097-1105}$ in VTB239 were predominantly derived from the T_{EM}+T_{EMRA}+T_{SCM} subset with high cell numbers (1,200-2,700 $IFN\gamma^+$ cells/10^6 $CD8^+$ T cells) (**Fig. 4.10B**), consistent with Response Pattern 2. The representation of the T_{EM}+T_{EMRA}+T_{SCM} subset in the $pIRS2_{1097-1105}$ response was substantially higher than in responses to viral peptides. This suggests that the expression of $pIRS2_{1097-1105}$ on VTB239's tumor generated an active immune response.

4.4.4 Responses in melanoma patients to HLA-A2-restricted phosphopeptides

We tested the hypothesis that phosphopeptide immunity was also impaired in melanoma patients. We assessed responses to the two immunodominant memory targets, $pIRS2_{1097-1105}$ and $pCHEK1_{367-377}$ in five melanoma patients as well as an additional 19 phosphopeptides, including the immunodominant $pCDC25B_{38-46}$, in 3 of the 5 patients. PBMCs from four of the five patients responded to Influenza M1, and all responded to

PMA/Ionomycin, demonstrating baseline immunocompetency (**Figure 4.11**). Furthermore, 2 of the 2 assessed patients responded to the melanoma antigen gp100$_{209-217}$ ("IMD"), (**Figure 4.11**). One patient responded to pIRS2 (**Figure 4.11**). There were no responses from the other four patients to pIRS2 and no responses from any donor to pCHEK1 or pCDC25B. There were no responses in the 3 patients to the additional 18 phosphopeptides. In two patients, 2 timepoints were analyzed, and responses were analyzed after culturing with IL-2 or with IL-7 and IL-15. (**Figure 4.11**). The one response to pIRS2 was observed in one of these patients at Week 0, after stimulation and culturing with IL-7 and IL-15 but not with IL-2. As culture with IL-2 would have supported anergic T cell expansion if the T cells were present, the lack of responses in these two donors after peptide stimulation and culture with IL-2 demonstrates the absence of T cells. Rather, the data suggest the absence of phosphopeptide-specific T cells in PBMCs of these patients. Since patient tumor specimens were unavailable for mass spectrometry analysis, it is unknown if any of the analyzed phosphopeptides were expressed on the patients' tumors, and, therefore could have been lost due to tumor-induced suppression, deletion, or tolerance. However, we suggest that it is more likely that phosphopeptide immunity in melanoma patients is generally impaired rather than being tumor-specific, similar to what was observed in patients with ovarian cancer.

4.4.5 Phosphopeptide-specific responses can be induced in melanoma patients by vaccination

4.4.5.1 Clinical presentation of patients vaccinated with phosphopeptides

Having identified pIRS2$_{1097-1105}$ and pBCAR3$_{126-134}$ phosphopeptides as attractive targets for cancer immunotherapy, we designed a clinical trial to test their safety and

immunogenicity in cancer patients (**Fig. 2.3**). Vaccines were formulated as phosphopeptides together with a tetanus helper peptide emulsified in IFA (Montanide ISA-51) and co-administered with poly-ICLC. While awaiting FDA approval to accrue to $pIRS2_{1097-1105}$ peptide (Arm B), the first participant was accrued to $pBCAR3_{126-134}$ (Arm A), after which accrual was randomly assigned 1:1 to arm A and Arm B until cohort conditions were satisfied. Sequential accrual to Arm C occurred after the initial safety criteria for Arms A and B were satisfied and no additional safety bound was crossed. Fifteen eligible patients were enrolled, each of whom had undergone surgical resection of stage II, III or IV melanoma, with three patients each in Arms A and B, and nine in Arm C. Patient demographics and clinical features are summarized in (**Table 2.4**).

4.4.5.2 Clinical toxicities

Treatment-related adverse events are detailed for all 15 patients in (**Table 4.4**). There were no treatment-related grade 3–4 toxicities, no deaths on study and no dose-limiting toxicities (DLTs). All patients had grade 1 and 2 adverse events, usually limited to 24–48 hours after each vaccine. All patients developed grade 2 vaccine injection site reactions, which were more persistent and included induration in 13 patients and skin ulceration in 2 patients. Other common treatment-related grade 1 and 2 toxicities were fatigue, chills, headache, myalgias, arthralgias, autoimmune disorders, fever, nausea and diarrhea. Meaningful differences in adverse events among study arms were not evident. Autoimmune toxicities were observed in three patients: two in arm A and one in arm B, and all were grade 1. These were all treatment-associated asymptomatic elevations of serum antinuclear antibody (ANA), and were identified in patients 1A (day 183), 3B (day 85) and 4A (day 85). Three

patients had pre-treatment elevations of rheumatoid factor (10C) or ANA (7C, 15C). No patients developed treatment-related vitiligo.

4.4.5.3 Immune responses to phosphopeptides in vaccinated patients

All 15 eligible patients were evaluable for immune responses in PBMC. Initial *ex vivo* ELISpot assays to detect IFNγ production in response to peptide antigen, performed on four patients representing arms A (2A), B (3B) and C (7C, 8C), were negative. Thus, subsequent ELISpot assays were performed after one *in vitro* sensitization for all patients. Two patients had evidence of pre-existing immune responses to phosphopeptides based on a greater than twofold increase, and increase of at least 100 spots/100,000 $CD8^+$ T cells, compared with the maximum negative control at baseline. Specifically, the ratios and spot counts per 100,000 $CD8^+$ T cells at baseline were as follows: patient 7, for pIRS2 (2.8x and 373 SFCs difference); patient 8, for pBCAR3 (2.8x and 137 SFCs difference) and for $pCTNNB1_{30-39}$ (which was not in the vaccine, 2.9x and 139 SFCs difference) (**Table 4.5, Figure 4.12, Figure 4.13**). T-cell responses to vaccine peptides were identified against $pBCAR3_{126-134}$ in 2/12 patients (17%, Arms A+C) and against $pIRS2_{1097-1105}$ in 5/12 patients (42%, Arms B+C) (**Table 4.5 and Figure 4.12**). Overall 6 of 15 patients (40%) had a T-cell response to either or both of these peptides. The majority of immune responses were detected at a single time point, typically at weeks 3, 5 or 8. One patient (#11) also showed an additional immune response at week 26. No responses against the negative control phosphopeptide, pS33-$βcat_{30-39}$, were observed at any time. In addition, responses in arm A and B patients were specific for the vaccinating peptide only. Also, there were indications of subthreshold responses in several patients at other time points that were specific for the same peptide (4A, 8C, 15C). Associations of immune response with

autoimmune toxicities were not evident (**Table 4.5**). These results give confidence that the significant responses observed are a consequence of vaccination, despite their transience. The study design set a goal for immunogenicity based on an upper limit of a 90% CI for the observed immune response rate of 35%. The values calculated for pBCAR3$_{126-134}$ and pIRS2$_{1097-1105}$ were 44% and 68%, respectively, for each peptide (**Table 4.5**). These both exceeded the protocol specified upper limit immunogenicity criteria target of >35% supporting further study and development. The prespecified target for immune response rate coincided with 2 or more responses in 12 evaluable patients for each peptide, with greater interest in further development with higher immune response rates.

4.4.5.4 Clinical outcomes

Only three patients have died from melanoma with a median follow-up of 4.9 years for those still alive. Estimated survival and disease-free survival curves are displayed in (**Figure 4.14**). Four-year survival is estimated at 80%. New melanoma metastases were identified in 10 patients, with a median disease-free survival of just over 1.0 year and 4 years disease-free survival of 33% (95% CI 15% to 53%) (**Figure 4.14**). The early phase study was not powered to assess association of immune response with clinical outcome with reliable inference, as is reflected by the width of the confidence bounds. Participant specific immune responses and outcomes are noted in (**Table 4.5**) with no clear associations noted in this small study.

4.5 Discussion

In this study, we characterized pre-existing immune memory to over 200 cancer-expressed phosphopeptides in a cohort of healthy donors. We also examined the possibility of ongoing effector activity over time against a subset of memory targets in 2 donors. Our analyses suggest that most phosphopeptide-specific immunity is due to less ubiquitous exposures, in that 90% of the memory targets were recognized by fewer than 30% of donors. Immunodominance was limited to 3 HLA-A2-restricted phosphopeptides and was not seen among the HLA-B7-restricted phosphopeptides. At the same time, however, there was evidence of recent or ongoing exposures to multiple phosphopeptides in 2 donors over a relatively short timeframe. To the extent that we analyzed them, robust responses to phosphopeptides in healthy donors were not due to elevated precursor frequencies of naïve cells, as with MART-1 [358]. Most pre-existing immunity was due to T_{CM}, and we also observed significant contribution from T_{SCM}. Their presence establishes the likely longevity of phosphopeptide-specific memory. However, melanoma and ovarian cancer patients showed significantly diminished responsiveness to immunodominant peptides, and in most cases (for ovarian cancer patients), also did not show reactivity to phosphopeptides displayed on their tumors. Promising results from a phase I clinical trial demonstrated the ability of a vaccine to induce or augment responses to phosphopeptides in melanoma patients, demonstrating a therapeutic opportunity for patients or healthy individuals with low phosphopeptide immunity.

The presence of phosphopeptide-specific memory T cells likely reflects immunogenic exposures through cellular transformation and/or infections that alter the phosphoproteome, generating new or over-expressed MHC-presented antigens [338, 399–

402]. In keeping with this, the phosphopeptides chosen for analysis were identified on patient-derived tumors, cancer cell lines, and EBV-transformed B-cell lines. In addition, while pre-existing phosphopeptide-specific memory was evident in most healthy donors, the range of phosphopeptides recognized was highly variable, suggesting that the range of immunogenic exposures to different phosphopeptides varies among individuals. It is possible that additional memory targets and immunodominant phosphopeptides could have been identified if more donors, PBMC collection timepoints, or effector functions had been evaluated. With the exception of exposures that give rise to immunodominant memory targets, this donor-to-donor variation is consistent with the hypothesis that pre-existing immunity is predominantly driven by infection with less common viruses or bacteria. It is also consistent with immunosurveillance of pre-malignant cancer cells, whose underlying kinase and phosphatase dysregulations differ among individuals.

It is conceivable that some donor-to-donor variation in phosphopeptide-specific memory could be due to underlying immunological differences. Isoforms of the TAP transporter, Tapasin, TAP-binding protein-related protein, and proteasomes have all been shown to restrict or enable peptide presentation [404–407]. There could also be constraints in the T cell receptor repertoire that limit phosphopeptide recognition; however, the great diversity and high number of T cell receptors in any individual make this unlikely [408]. While age is a reasonable influence on the number of exposures in an individual, it did not correlate with broader phosphopeptide immunity in our study. It is conceivable that some phosphopeptide-specific memory T cells have been generated by cross-reactivity to other peptides; however, we and others have previously demonstrated that several phosphopeptides are directly immunogenic in mice and humans [1, 3–5, 337, 352].

Additionally, phosphopeptide-specific responses are highly dependent on the phosphate moiety and exhibit negligible cross-reactivity on the unmodified peptide (here and [1–3, 45, 337, 348, 349, 352, 354–356]. We think it unlikely that an un-phosphorylated peptide can mimic the bulkiness and charge of the phosphate group. It remains possible that the phosphopeptide-specific T cells we observed were elicited by another phosphopeptide with a distinct peptide sequence. On the other hand, donor-to-donor differences in self-tolerance based on presentation of phosphopeptides or cross-reactive self-antigens could limit the ability to generate phosphopeptide-specific T-cell responses. All of the above hypotheses may explain donor-to-donor variation in responsiveness. Regardless, donors responded to an average of only 7/51 HLA-A2- and 6/154 HLA-B7-restricted phosphopeptides. We think this again suggests that the immunogens generating phosphopeptide-specific memory are rare infectious agents or incipient cancer cells with distinct dysregulations.

The T-cell subsets specific for most phosphopeptides at most timepoints in cultured responses were predominantly T_{CM} and T_{SCM}. This pattern is similar to Response Pattern 1 for Influenza $M1_{58\text{-}66}$ in the absence of recent vaccination or infection, and EBV $LMP2A_{426\text{-}434}$, which is expressed persistently at low-level. This suggests low or negligible phosphopeptide exposure at the time of PBMC collection. However, for some phosphopeptides, we also observed timepoints at which T_{EM} and T_{EMRA} were represented at much higher levels. These patterns were similar to Response Pattern 2 for EBV $BMLF1_{300\text{-}308}$, which is expressed at high-level, suggesting an active phosphopeptide response. However, while direct responses to Pattern 2 viral peptides were predominantly mediated by T_{EM} and T_{EMRA}, direct responses to phosphopeptides were considerably more variable. While T_{EM} and/or T_{EMRA} were dominant at some time points, T_{CM} were dominant

at others, and these were sometimes associated with a Pattern 2 cultured response. This suggests that immunogenic phosphopeptide exposure may induce distinct differentiation of effectors, and that this also varies over time. Nonetheless, some of these active responses occurred repeatedly within a donor, suggesting recurrent exposures to the same immunogen, while active responses to individual phosphopeptides occurred at different times. This suggests that expression of different phosphopeptides may be driven by distinct immunogens.

We also demonstrated that, in a small subset of healthy donors and most ovarian cancer patients, phosphopeptide-specific IFNγ T-cell responses were lacking. In the patients this was not limited to the phosphopeptides expressed on their tumors, consistent with the possibility that phosphopeptide-specific immune tolerance was not induced by expression on their cancer cells. An obvious possibility, in patients as well as healthy donors, is they lack prior exposure to relevant infectious agents or transformed cells expressing phosphopeptides. These individuals may also express alleles of antigen-processing pathway components that alter phosphopeptide display, leading to either lack of presentation or enhanced presentation resulting in self-tolerance. It is also possible that additional responses could have been identified by cytotoxicity, TNFα production, or multimer staining. The number of phosphopeptides evaluated in patients was limited by sample availability. Importantly, a subset of patients still responded to immunodominant phosphopeptides, and a robust effector response in PBLs and TILs to a tumor-expressed phosphopeptide was evident in one patient.

These findings create a therapeutic opportunity, in that the tumors from these patients express many phosphopeptides to which they did not respond. Along with the

demonstration that phosphopeptide-specific T cell responses correlate with delayed tumor outgrowth in humanized murine models [3–5] and the results described here demonstrating that a phosphopeptide vaccine induces immune responses in melanoma patients [5], this observation supports further investigation into augmenting or inducing tumor-specific phosphopeptide immunity in patients where it is otherwise absent as well as into its ability to contribute to tumor control. Furthermore, T cells specific for tumor-expressed phosphopeptides can be collected from some patients and expanded *in vitro* into IFNγ-producing effectors. This provides a means for isolating reactive T cell receptors that could be utilized in recombinant TCR adoptive cell therapy.

Further investigation is required to elucidate mechanisms by which phosphopeptide-specific T-cell memory is limited in some individuals and many cancer patients. Based on genetic and environmental factors, some individuals have a higher risk of developing cancer, which likely coincides with a higher frequency or wider breadth of spontaneously arising pre-malignant cells. These individuals may exhibit pre-existing immune memory to a greater number of phosphopeptides. Longitudinal studies are needed to determine if the ability of patients to generate phosphopeptide-specific responses is impaired prior to the development of cancer or if pre-existing responses are lost or compromised following its development. It would be particularly interesting to investigate pre-existing immune memory to phosphopeptides in patients with genetic syndromes known to increase cancer susceptibility (such as Li Fraumeni or Lynch Syndrome), in patients with “pre-malignant syndromes” (such as Monoclonal Gammopathy of Unknown Significance), or in individuals with histories of high carcinogenic exposures (such as

smoking or working in certain industries). The lack of responses in some donors and patients identifies a therapeutic opportunity to induce otherwise absent responses.

4.6 Figures and Tables

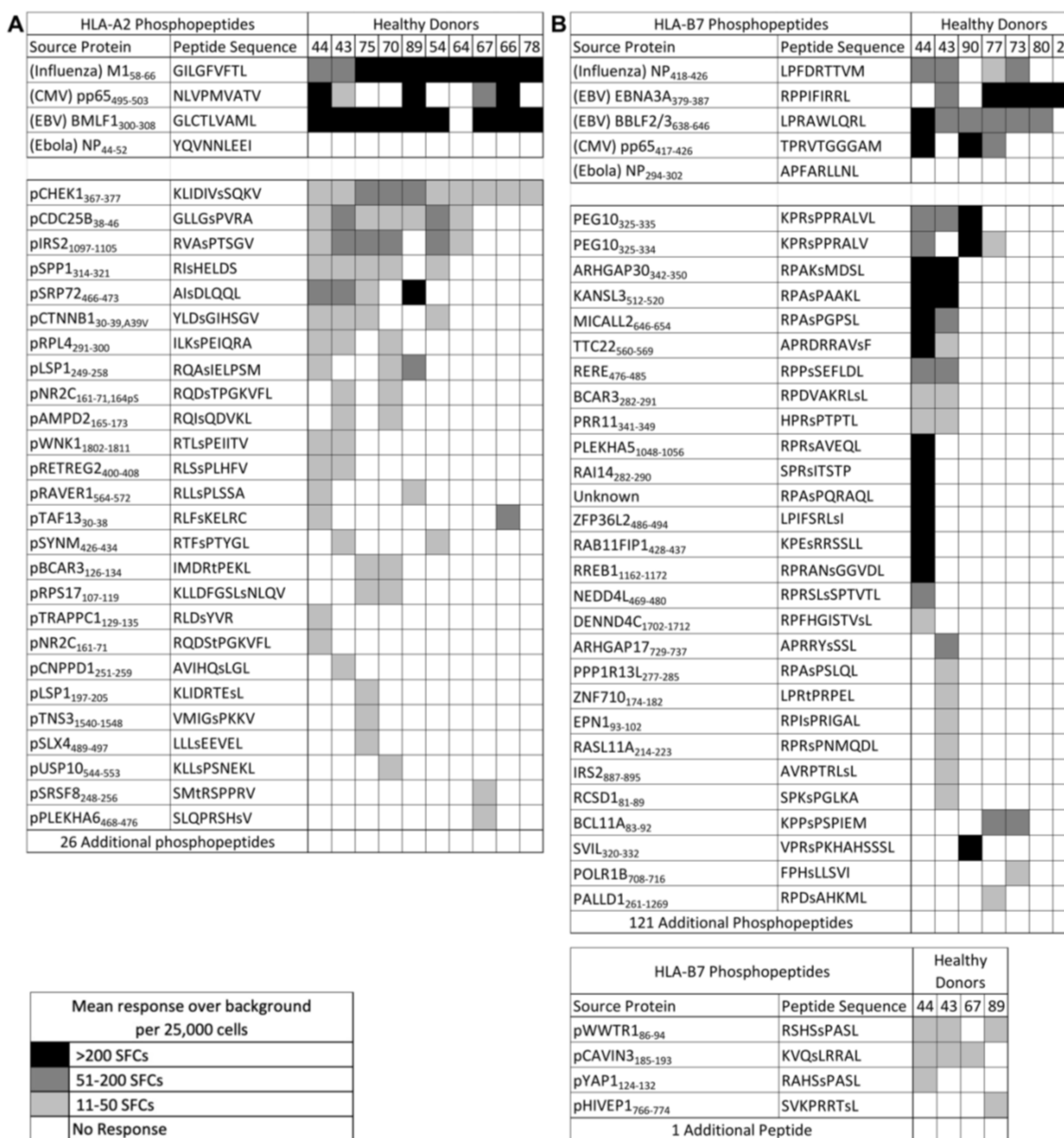


Figure 4.1: Most—but not all—healthy donors demonstrate pre-existing T cell immune memory to phosphopeptides displayed on cancer cells. Summary response data for $CD8^+CD45RO^+$ T cells from healthy donors (HD) stimulated once *in vitro* with peptide-pulsed autologous dendritic cells and cultured for 14 days, measured in triplicate wells in an IFNγ ELISpot assay. Data represent a minimum of 3 experiments for each peptide to which a response was observed in a donor. Peptides to which responses were not observed initially may have been analyzed in fewer experiments. A, Responses of HLA-$A2^+$ donors and HLA-A2-restricted peptides. B, HLA-B7+ donors and HLA-B7 restricted peptides. The lower portion of B presents data for 5 HLA-B7-restricted phosphopeptides that were analyzed in HD43, HD44, HD67, and HD89 only because of

limited sample availability. Insufficient PBMCs were available from HD67 and HD89 to assess responses against the larger cohort of HLA-B7 restricted phosphopeptides. SFCs = spot-forming cells.

	Response to phosphopeptide; No Response to unphosphorylated peptide
	Responses to both phosphopeptide and unphosphorylated peptide
	No Response to phosphorylated or unphosphorylated peptide

A

HLA-A2 Phosphopeptides	Healthy Donor			
Peptide	43	44	78	89
pCHEK1$_{367-377}$				
pIRS2$_{1097-1105}$				
pSPP1$_{314-321}$				
CDC25B$_{38-46}$				
SRP72$_{466-473}$				
LSP1$_{249-260}$				

B

HLA-B7 Phosphopeptides	Healthy Donor			
Peptide	43	44	73	77
ARHGAP30$_{342-350}$				
RERE$_{476-485}$				

HLA-B7 Phosphopeptides	Healthy Donor		
Peptide	73	77	80
POLR1B$_{708-716}$			
BCL11A$_{83-92}$			
PALLD1$_{261-1269}$			

Figure 4.2. Unphosphorylated forms of immunogenic phosphopeptides are not immunogenic. CD8^{+}CD45RO^{+} T cells were stimulated with autologous DC pulsed with either the phosphopeptide or the unphosphorylated peptide, cultured for 14 days, and responses against the same peptide measured in triplicate wells in an IFNγ ELISpot assay. Summary data of responses in (**A**) 4 healthy donors to HLA-A2 restricted and (**B**) 5 healthy donors to HLA-B7 restricted phosphopeptides and corresponding unphosphorylated peptides. Data from a minimum of 3 experiments per phosphopeptide and 1 experiment per unphosphorylated peptide.

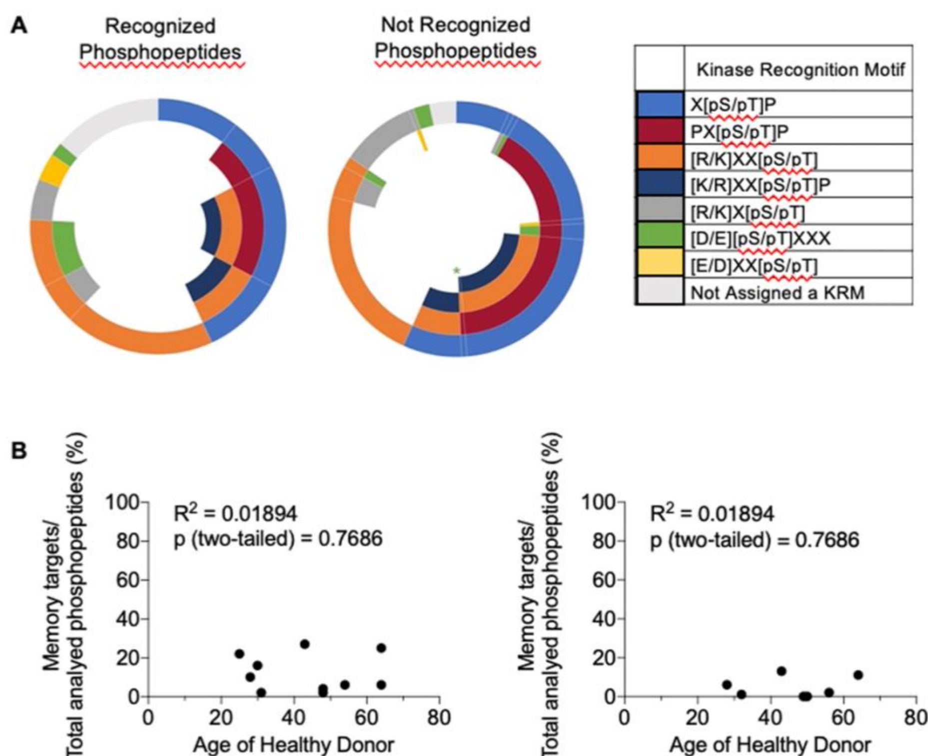
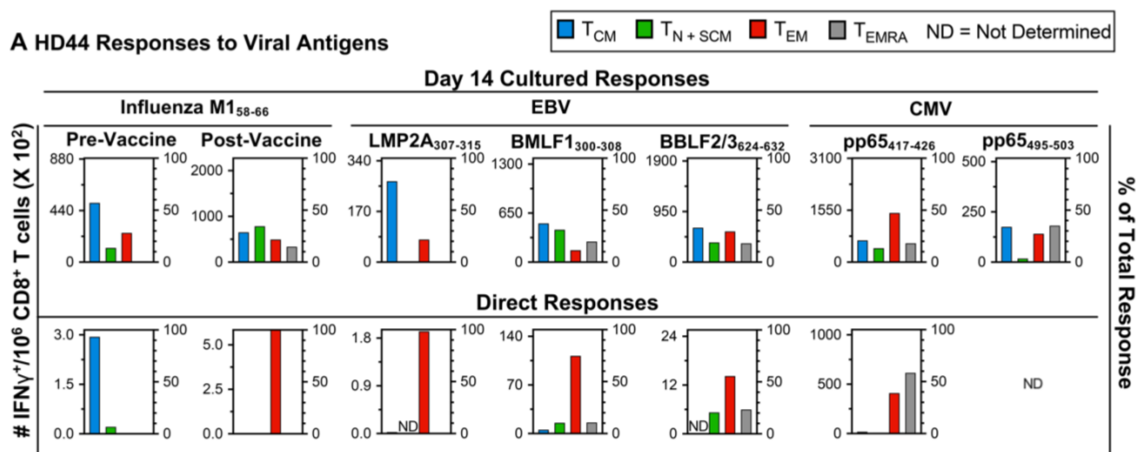
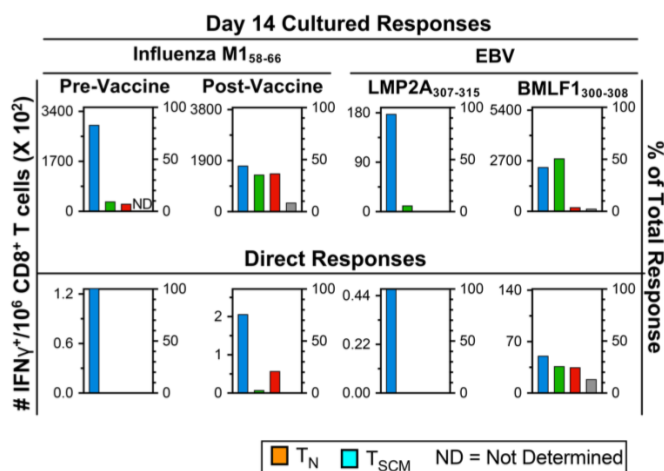
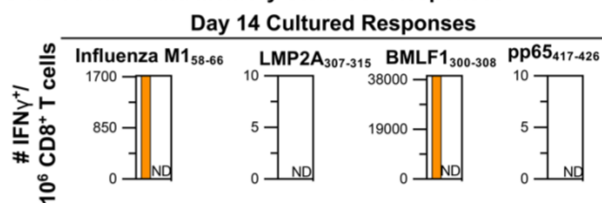


Figure 4.3. Pre-existing immune memory to phosphopeptides is not linked to kinase recognition motifs or to donor age. (A) Representation of kinase recognition motifs in analyzed HLA-A2- and HLA-B7-restricted phosphopeptide memory targets (left, n=58) and unrecognized phosphopeptides (right, n=147). Concentric arcs reflect phosphopeptides that contain multiple kinase recognition motifs. * Identifies 1 phosphopeptide containing [D/E][pS/pT]XXX and 4 other KRMs. Statistical analysis of this data is presented in **Supp. Table S6**. (**B)** Age of the donor was as of initial blood collection and the percentage of memory targets of the total phosphopeptides analyzed in 14-day cultures of $CD8^{+}CD45RO^{+}$ T cells from that donor. Number of HLA-A2-restricted phosphopeptides analyzed per donor: n=39-51; Number of HLA-B7-restricted phosphopeptides analyzed per donor: n=62-154. HD67 and HD89 were excluded from analysis in the HLA-B7-restricted phosphopeptide cohort due to the low number of phosphopeptides analyzed (5) in these donors out of the total (151). Correlations were assessed by calculating the Pearson R values.







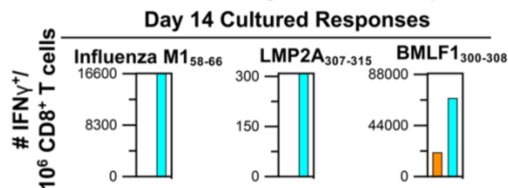


Figure 4.4. T cells responses to viral epitopes define two response patterns that distinguish recent or ongoing antigen exposure. The indicated $CD8^+$ T cell subsets from HD44 **(A, C)** and HD43 **(B, D)** were enriched by cell sorting, and analyzed in an IFNγ

ELISpot assay either immediately *ex vivo* (Direct) **(C, D)** or after one *in vitro* stimulation with the indicated viral peptide-pulsed autologous dendritic cells and a 14-day culture (Cultured) **(A, B)**. Responses were normalized for the expansion of cultured cells over 14 days and $CD8^+$ T cell subset percentages in the donor's blood, as described in Methods. Responses in each subset are reported both as the number of $IFN\gamma^+$ cells per 10^6 $CD8^+$ T cells (*left Y-axis*) and the subset percentage of the total measured response (*right Y-axis*). Responses to Influenza M1 were measured in PBMCs collected before the donor receiving the annual flu vaccine and in the absence of illness (Pre-Vaccine) or 16-17 days after receiving an influenza vaccine (Post-Vaccine). **C,** Due to low cell numbers, no data is available for T_{SCM} responses in HD44. The color coding for each subset is based on **Supplementary Fig. S1**. Most plots are representative of more than 1 experiment, except HD44 cultured and direct responses to EBV BBLF2/3, HD44 cultured response to CMV $pp65_{495\text{-}503}$, HD43 direct response to Influenza pre-vaccine, and cultured and direct response post-vaccine.

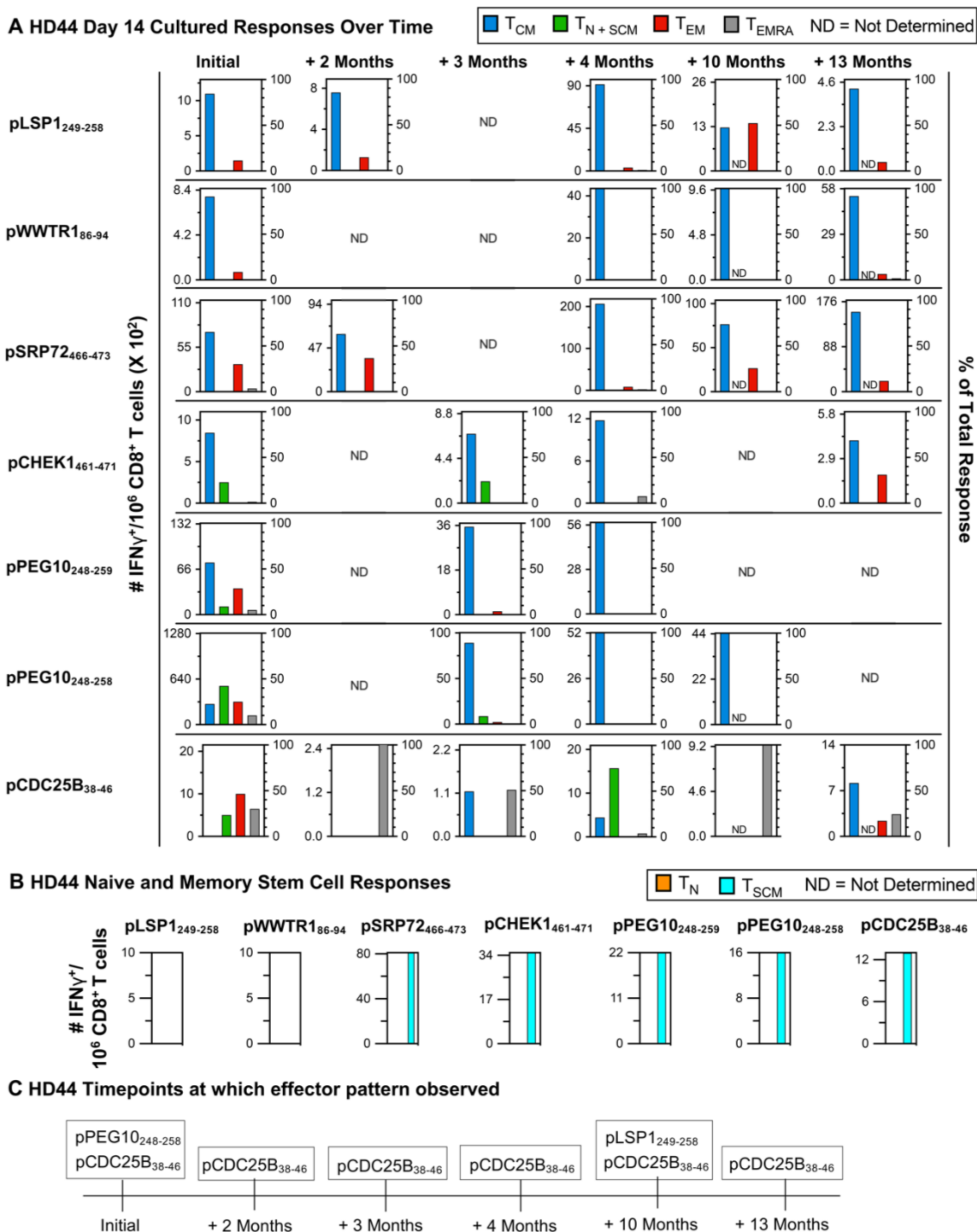


Figure 4.5. Longitudinal cultured response patterns to phosphopeptides in HD44. (A, B) Flow sorted CD8$^+$ T cell subsets from blood samples collected at different timepoints were stimulated once *in vitro* with peptide-pulsed autologous dendritic cells, cultured for 14 days, and responses to the indicated phosphopeptides measured in triplicate wells in an IFNγ ELISpot assay. Responses were normalized for the expansion of cultured cells and

subset percentages in the donor's blood, as described in Methods. Responses in each subset are reported both as the number of IFNγ^{+} cells per 10^{6} CD8^{+} T cells (*left Y-axis*) and the subset percentage of the total measured response (*right Y-axis*). The color coding for each subset is based on **Fig. 2.1**. Each timepoint reflects one analysis of PBMCs harvested at that timepoint. **A,** Longitudinal responses measured in the first collected blood sample (Initial), and in samples collected at the indicated times in relation to the Initial sample. **B,** The N + SCM subset was sorted using CD95 to evaluate responses from naïve or memory stem cells. Responses shown are from: 5 months (pWWTR1$_{86\text{-}94}$), 10 months (pPEG10$_{248\text{-}259}$, pPEG10$_{248\text{-}258}$), or 13 months (pLSP1$_{249\text{-}258}$, pCHEK1$_{461\text{-}471}$, pSRP72$_{466\text{-}473}$, pCDC25B$_{38\text{-}46}$) and are representative of at least 2 examined timepoints. **C,** Responses to the peptides in each box were considered Response Pattern 2 related and evidence of active immunogenic exposure at the indicated timepoints.

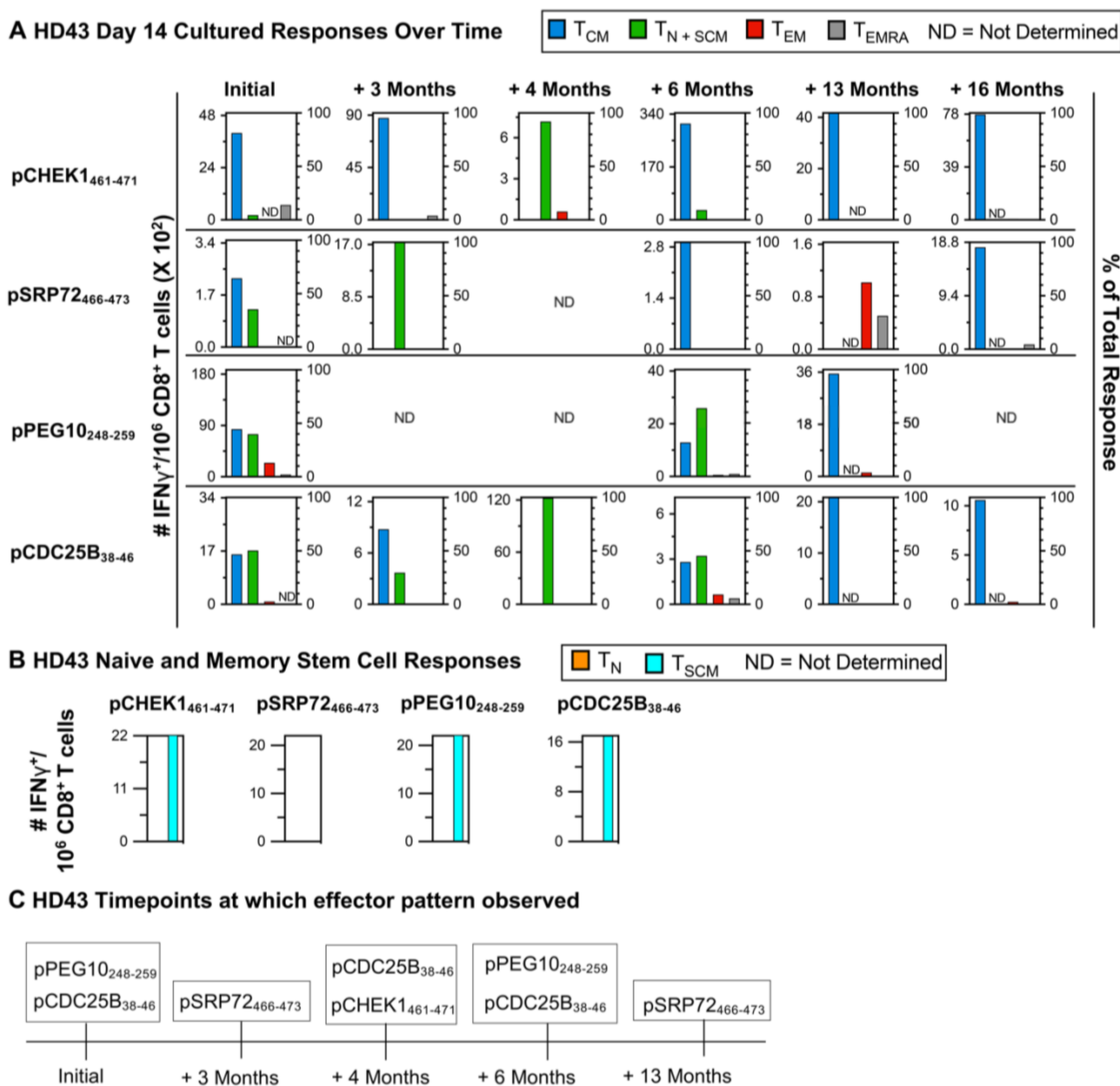
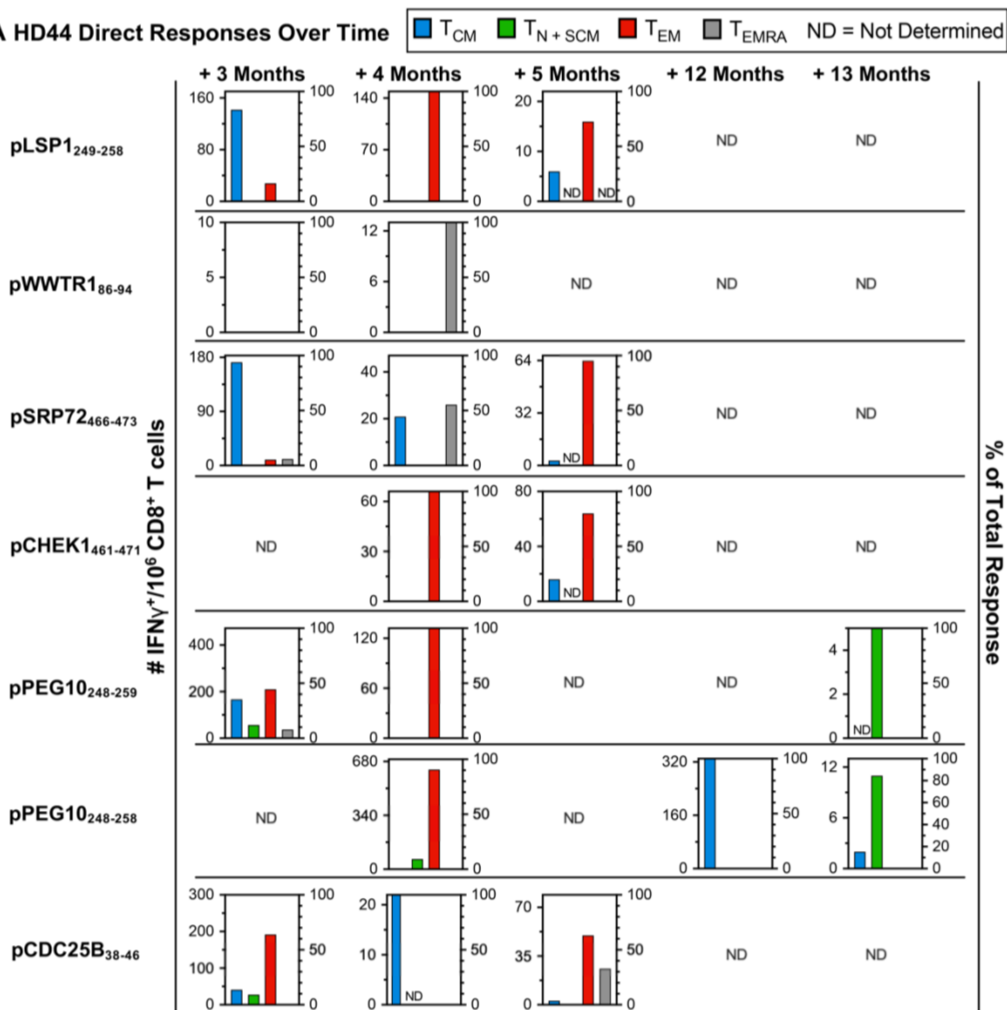


Figure 4.6. Longitudinal cultured response patterns to phosphopeptides in HD43. (A, B) Flow sorted $CD8^+$ T cell subsets from blood samples collected at different timepoints were stimulated once *in vitro* with peptide-pulsed autologous dendritic cells, cultured for 14 days, and responses to the indicated phosphopeptides measured in triplicate wells in an IFNγ ELISpot assay. Responses were normalized for the expansion of cultured cells and subset percentages in the donor's blood, as described in Methods. Responses in each subset are reported both as the number of $IFN\gamma^+$ cells per 10^6 $CD8^+$ T cells (*left Y-axis*) and the subset percentage of the total measured response (*right Y-axis*). The color coding for each subset is based on **Fig. 2.1**. Each timepoint reflects one analysis of PBMCs harvested at that timepoint. **A,** Longitudinal responses measured in the first collected blood sample (Initial), and in samples collected at the indicated times in relation to the Initial sample. **B,** The N + SCM subset was sorted using CD95 to evaluate responses from naïve or memory stem cells. Responses shown are from: 9 months ($pPEG10_{248\text{-}259}$), 13 months ($pCDC25B_{38\text{-}46}$), or 16 months ($pCHEK1_{461\text{-}471}$, $pSRP72_{466\text{-}473}$) and are representative of at least 2

examined timepoints. **C,** Responses to the peptides in each box were considered Response Pattern 2 related and evidence of active immunogenic exposure at the indicated timepoints.


A HD44 Direct Responses Over Time
TCM
TN + SCM
TEM
TEMRA
ND = Not Determined
+ 3 Months
+ 4 Months
+ 5 Months
+ 12 Months
+ 13 Months
pLSP1249-258
pWWTR186-94
pSRP72466-473
pCHEK1461-471
pPEG10248-259
pPEG10248-258
pCDC25B38-46
IFNγ+/106 CD8+ T cells
% of Total Response

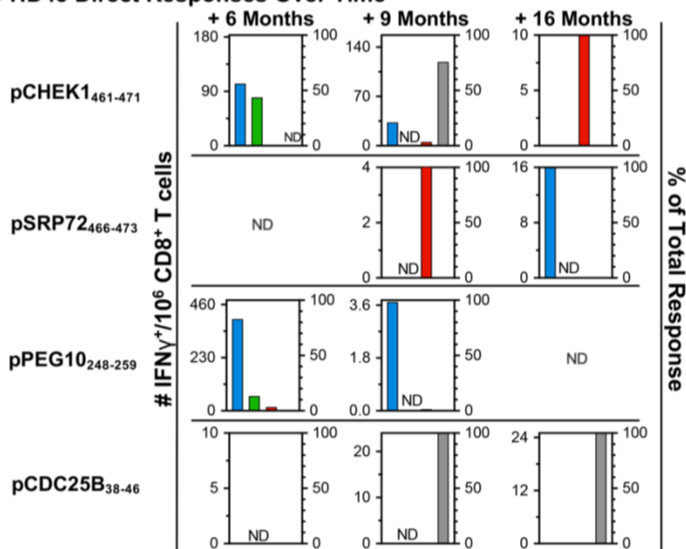

B HD43 Direct Responses Over Time
+ 6 Months
+ 9 Months
+ 16 Months
pCHEK1461-471
pSRP72466-473
pPEG10248-259
pCDC25B38-46
IFNγ+/106 CD8+ T cells
% of Total Response

Figure 4.7. Direct responses to phosphopeptides in healthy donors are largely consistent with cultured response patterns. Responses of flow sorted $CD8^+$ T cell subsets from HD44 **(A)** and HD43 **(B)** to the indicated phosphopeptides were measured directly *ex vivo* and normalized for the subset percentages in the donor's blood, as described in Methods. Responses in each subset are reported both as the number of $IFN\gamma^+$ cells per 10^6 $CD8^+$ T cells (*left Y-axis*) and the subset percentage of the total measured response (*right Y-axis*). The color coding for each subset is based on **Fig. 2.1**. Each timepoint reflects one analysis of PBMCs harvested at that timepoint.

		VTB239			VTB241			VTB285			VTB280		
		PBLs Pre-op	PBLs Recur*	TILs	PBLs Pre-op	PBLs Post-op	TILs	PBLs Pre-op	PBLs Post-op	TILs	PBLs Pre-op	PBLs Post-op	TILs
HLA-A2 Viral Peptides	Influenza M1$_{58-66}$												
	EBV BMLF1$_{300-308}$												
	EBV LMP2A$_{426-434}$												
	CMV pp65$_{495-503}$												
	Ebola NP$_{44-52}$												
Immuno-dominant Peptides	pIRS2$_{1097-1105}$	T	T	T									
	pCHEK1$_{367-377}$												
	pCDC25B$_{38-46}$												
HLA-A2 Other Peptides	pLSP1$_{249-258}$	T	T	T	T	T	T						
	pAMPD2$_{165-173}$	T	T	T	T	T	T						
	pTAF13$_{30-38}$	T	T	T									
	pSYNM$_{426-434}$				T	T	T						
	pRETREG2$_{400-408}$												
	pWWTR1$_{86-94}$												
	pYAP1$_{124-132}$												
	pLSP1$_{249-260}$												
	pSRP72$_{466-473}$												
	pSPP1$_{314-321}$												
HLA-B7 Other Peptides	pMAP3K11$_{755-763}$							T	T	T			
	pSIK1$_{430-439}$							T	T	T			
	pNEDD4L$_{469-480}$							T	T	T			
	pBCAR3$_{282-291}$							T	T	T			
	pSFRS7$_{159-168}$							T	T	T			
	pDENND4C$_{1702-1712}$												

Total # IFNγ+ SFCs/ 10^6 CD8+ T cells
100,000 +
10,001-100,000
1,001-10,000
100-1,000
<100
Not Determined

Figure 4.8. Immune responses to viral and phosphorylated peptides in ovarian cancer patients. Flow sorted $CD8^+$ T cell subsets (T_{CM} + T_N and T_{EM} + T_{EMRA} + T_{SCM}) **(Fig. 2.2)** from 4 identified ovarian cancer patients **(Table 2.3)** were stimulated once *in vitro* with the indicated viral or phosphorylated peptide-pulsed autologous dendritic cells and irradiated $CD4^-CD8^-$ PBLs as antigen presenting cells, cultured for 14 days, and responses to the same peptides measured in triplicate wells in an IFNγ ELISpot assay. Responses were normalized for the expansion of cultured cells and subset percentages in the donor's blood, as described in Methods. Each data point reflects one analysis of PBMCs or TILs harvested at the identified timepoint, and is the sum of responses from both sorted subsets. T = Phosphopeptide expressed on the patient's tumor as identified by mass spectrometry.

Total # IFNγ+ SFCs/ 10^6 CD8+ T cells
- 100,000 +
- 10,001-100,000
- 1,001-10,000
- 100-1,000
- <100
- Not Determined

		VTB246				VTB247				VTB288			VTB 291	VTB279					VTB269		
		PBLs Pre-op	PBLs Post-op	PBLs Post-op*	TILs	PBLs Pre-op	PBLs Post-op	PBLs Recur*	TILs	PBLs Pre-op	TILs Ovary	TILs Ascites	PBLs Pre-op	PBLs Pre-op*	PBLs Post-op*	PBLs Post-op*	PBLs Post-op*	TILs*	PBLs Post-op*	PBLs Post-op*	TILs*
HLA-A2 Viral Peptides	Influenza M1$_{58-66}$																				
	EBV BMLF1$_{300-308}$																				
	EBV LMP2A$_{426-434}$																				
	CMV pp65$_{495-503}$																				
	Ebola NP$_{44-52}$																				
Immuno-dominant Peptides	pIRS2$_{1097-1105}$																				
	pCHEK1$_{367-377}$									T	T	T									
	pCDC25B$_{38-46}$																				
HLA-A2 Other Peptides	pLSP1$_{249-258}$	T	T	T	T					T	T	T									
	pAMPD2$_{165-173}$																				
	pSYNM$_{426-434}$																				
	pTAF13$_{30-38}$					T	T	T	T										T	T	T
	pRETREG2$_{400-408}$									T	T	T									
	pWWTR1$_{86-94}$																				
	pYAP1$_{124-132}$																				
	pLSP1$_{249-260}$																				
	pSRP72$_{466-473}$																				
	pSPP1$_{314-321}$																				
HLA-B7 Other Peptides	pMAP3K11$_{755-763}$																				
	pSIK1$_{430-439}$																				
	pNEDD4L$_{469-480}$																				
	pBCAR3$_{282-291}$																				
	pSFRS7$_{159-168}$																				
	pDENND4C$_{1702-1712}$																				

Figure 4.9. Immunity to phosphopeptides is less evident in most ovarian cancer patients, regardless of expression on tumor cells. FACS sorted CD8$^+$ T cell subsets, T_{CM} + T_N and T_{EM} + T_{EMRA} + T_{SCM} (as shown in **Fig. 2.2**), from ovarian cancer patients were *in vitro* stimulated with peptide-pulsed autologous dendritic cells and irradiated CD4$^-$CD8$^-$ PBLs as antigen presenting cells, cultured for 14 days, and analyzed in an IFNγ ELISpot assay. Data are from 6 patients with no evident responses to the indicated phosphopeptides. Each specimen was analyzed once. * Patient had received or was receiving chemotherapy at the time of tissue collection. T = Phosphopeptide was expressed on the patient's tumor as identified by mass spectrometry.

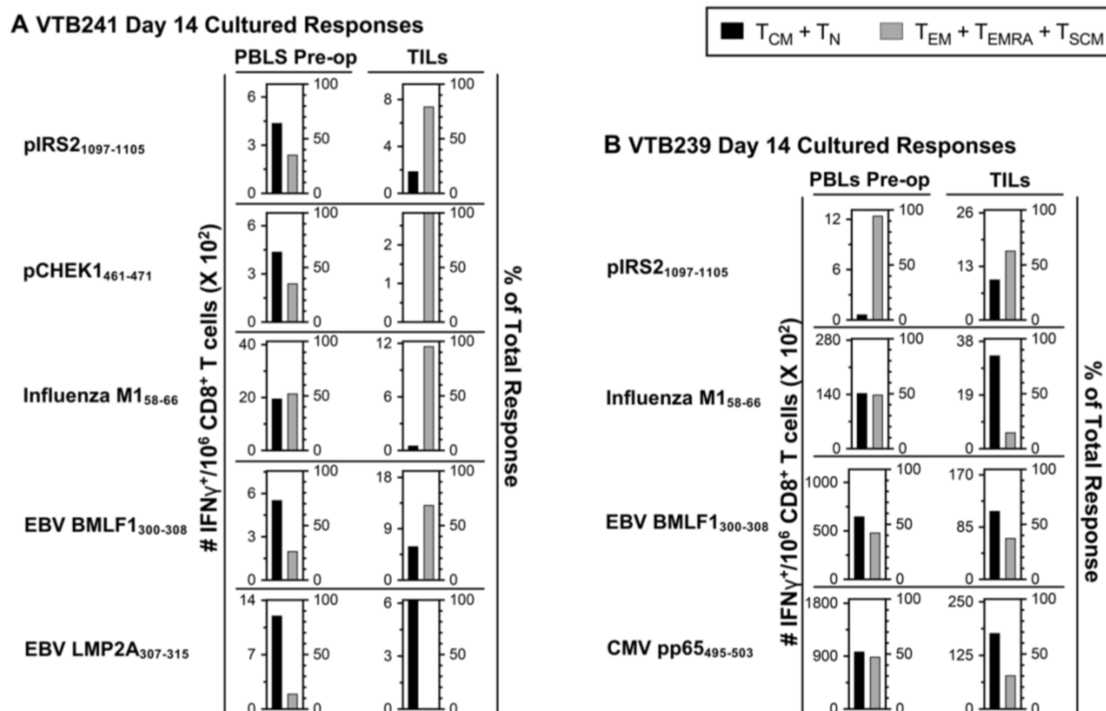


Figure 4.10. Phosphopeptide expression on patient tumor is associated with an active immune response. Flow sorted $CD8^+$ T cell subsets (T_{CM} + T_N and T_{EM} + T_{EMRA} + T_{SCM}) (**Fig. 2.2**) from ovarian cancer patients VTB241 (**A**) and VTB239 (**B**) (**Table 2.3**) were stimulated once *in vitro* with the indicated viral or phosphorylated peptide-pulsed autologous dendritic cells and irradiated $CD4^-CD8^-$ PBLs as antigen presenting cells, cultured for 14 days, and responses to the same peptides measured in triplicate wells in an IFNγ ELISpot assay. Responses were normalized for the expansion of cultured cells and subset percentages in the donor's blood, as described in Methods. Responses in each subset are reported both as the number of $IFN\gamma^+$ cells per 10^6 $CD8^+$ T cells (*left Y-axis*) and the subset percentage of the total measured response (*right Y-axis*). The color coding for each subset is based on **Fig. 2.2.** Each graph represents one analysis of PBLs or TILs.

	VMM1156	VMM1188	VMM1190	VMM728	VMM728	VMM728	VMM728	VMM934	VMM934	VMM934	VMM934
	Week 0	Week 0	Week 0	Week 0	Week 0	Week 7	Week 7	Week 0	Week 0	Week 6	Week 6
Peptide Sequence	IL-7/15	IL-7/15	IL-7/15	IL-7/15	IL-2	IL-7/15	IL-2	IL-7/15	IL-2	IL-7/15	IL-2
IMD	ND	ND	ND								
GILGFVFTL											
YQVNNLEEI				ND	ND	ND	ND	ND	ND	ND	ND
PMA/Iono											
RVAsPTSGV											
KLIDIVsSQKV											
KLLDFGSLsNLQV				ND	ND	ND	ND	ND	ND	ND	ND
YLDsGIHSGA				ND	ND	ND	ND	ND	ND	ND	ND
KLLsPSNEKL				ND	ND	ND	ND	ND	ND	ND	ND
RLDsYVR				ND	ND	ND	ND	ND	ND	ND	ND
RLFsKELRC				ND	ND	ND	ND	ND	ND	ND	ND
RTLsHISEA				ND	ND	ND	ND	ND	ND	ND	ND
RTFsPTYGL				ND	ND	ND	ND	ND	ND	ND	ND
YLDsGIHSGV				ND	ND	ND	ND	ND	ND	ND	ND
KLAsPELERL				ND	ND	ND	ND	ND	ND	ND	ND
VMIGsPKKV				ND	ND	ND	ND	ND	ND	ND	ND
RQLsSGVSEI				ND	ND	ND	ND	ND	ND	ND	ND
AMAAsPHAV				ND	ND	ND	ND	ND	ND	ND	ND
KLFPDtPLAL				ND	ND	ND	ND	ND	ND	ND	ND
RLSsPLHFV				ND	ND	ND	ND	ND	ND	ND	ND
RQDsTPGKVFL				ND	ND	ND	ND	ND	ND	ND	ND
RQIsQDVKL				ND	ND	ND	ND	ND	ND	ND	ND
ILKsPEIQRA				ND	ND	ND	ND	ND	ND	ND	ND
AVVsPPALHNA				ND	ND	ND	ND	ND	ND	ND	ND
GLLGsPVRA				ND	ND	ND	ND	ND	ND	ND	ND
IMDRtPEKL				ND	ND	ND	ND	ND	ND	ND	ND

Figure 4.11. Responses to phosphopeptides are impaired in melanoma patients. Summary response data to HLA-A2 restricted phosphopeptides and viral control peptides from melanoma patients' PBMCs after one-time *in vitro* peptide stimulation and 14 days in culture, as measured in triplicate wells in an IFNγ ELISpot assay, reported as SFCs/25,000 cells over background (target T2-B7 cells plus DMSO). For 2 patients, two timepoints were analyzed as was culturing with IL-2 instead of IL-7 and IL-15. Summary data reflect 1 experiment per peptide per patient/condition/timepoint. IMD = gp100$_{209-217}$ (IMDQVPFSV). VMM = Virginia Medical Melanoma patient ID number. ND = No Data (insufficient numbers of PBMCs to analyze).

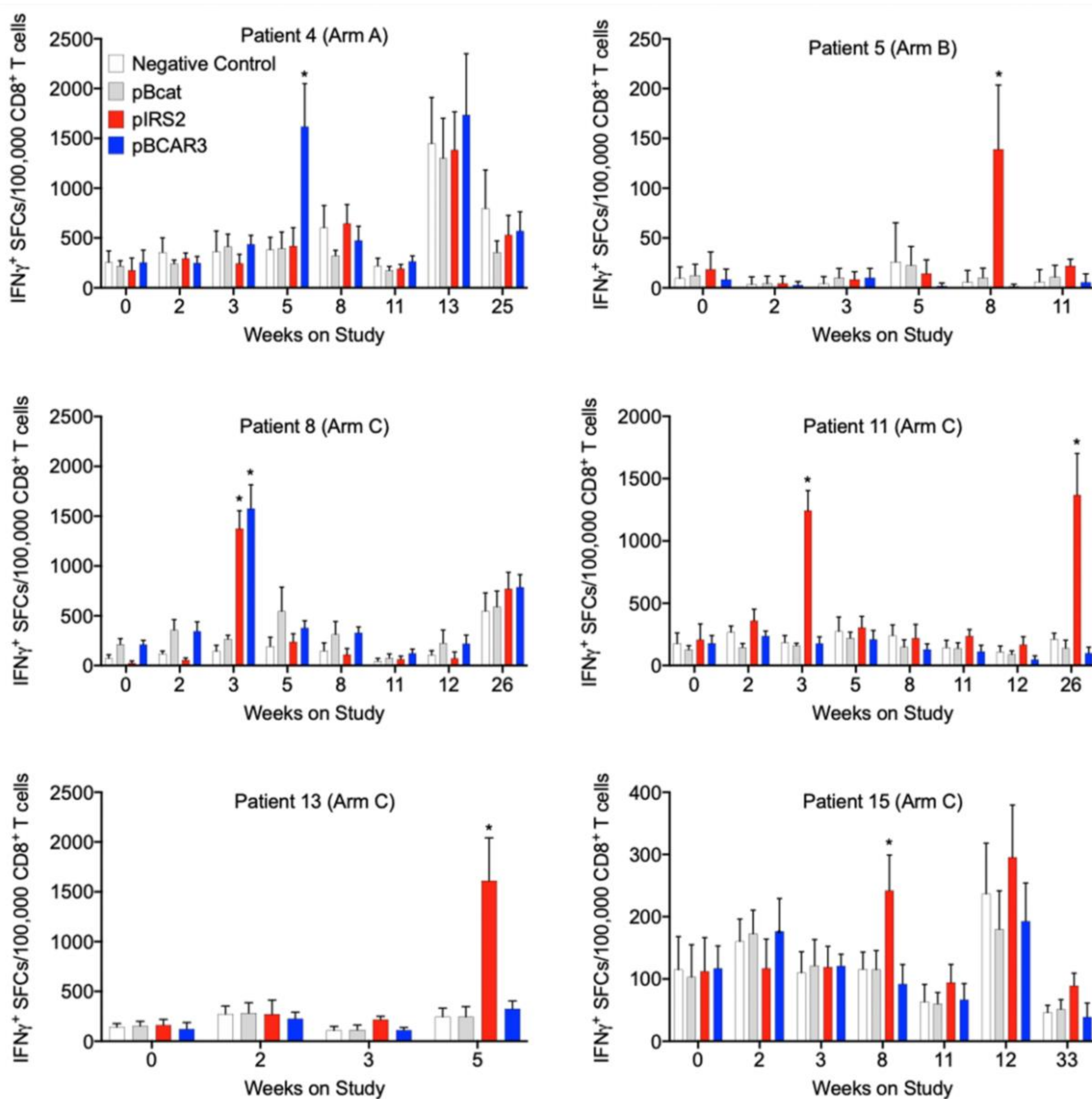
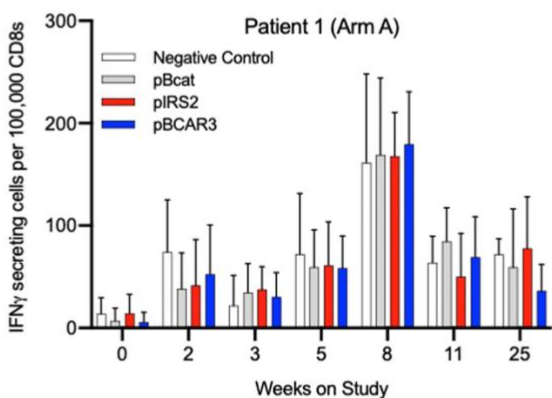
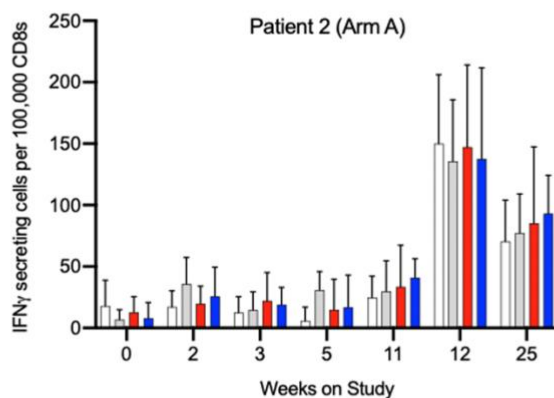
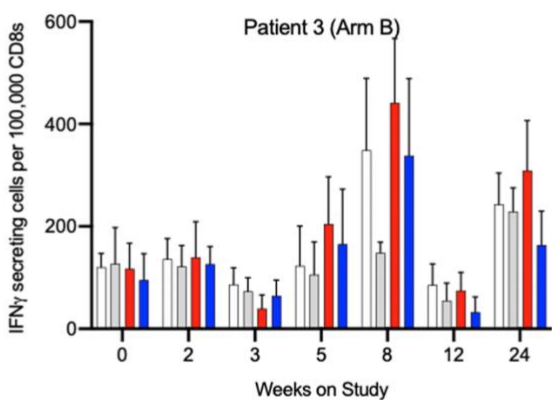
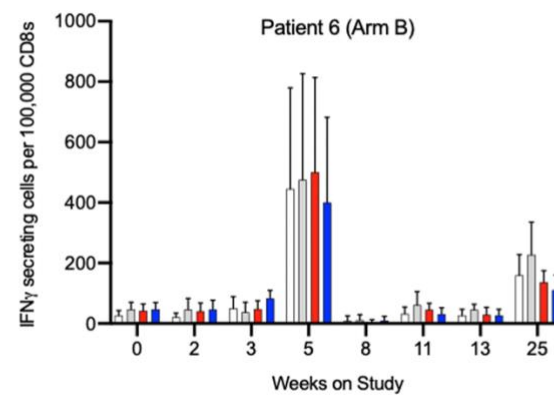


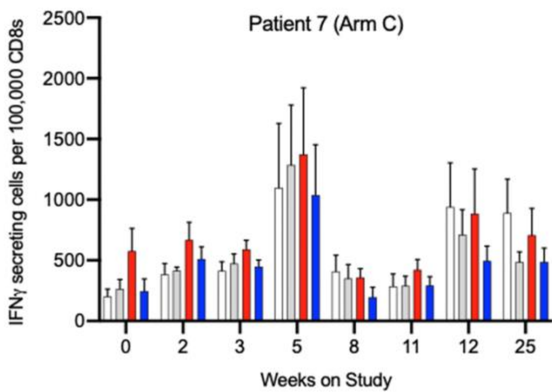
Figure 4.12. Phosphopeptide responses can be induced or augmented in some melanoma patients after peptide vaccination. Patients enrolled in clinical trial MEL59 received peptide vaccine of pIRS2, pBCAR3 or pIRS2 and pBCAR3. Responses to target T2-B7 cells pulsed with peptides were recorded as SFCs per 100,000 cells in an IFNɣ ELISpot assay. Error bars represent 1 standard deviation of the mean.

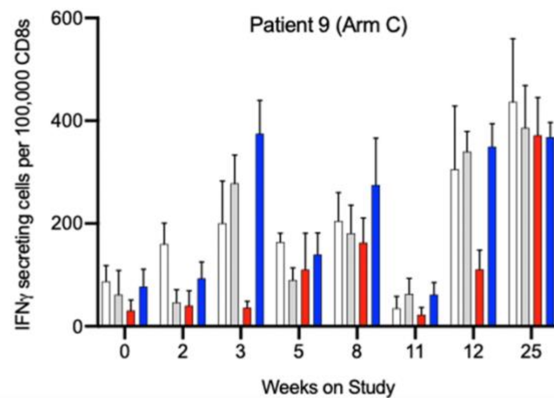

Patient 1 (Arm A)
Negative Control
pBcat
pIRS2
pBCAR3
IFNγ secreting cells per 100,000 CD8s
Weeks on Study


Patient 2 (Arm A)
IFNγ secreting cells per 100,000 CD8s
Weeks on Study


Patient 3 (Arm B)
IFNγ secreting cells per 100,000 CD8s
Weeks on Study


Patient 6 (Arm B)
IFNγ secreting cells per 100,000 CD8s
Weeks on Study


Patient 7 (Arm C)
IFNγ secreting cells per 100,000 CD8s
Weeks on Study


Patient 9 (Arm C)
IFNγ secreting cells per 100,000 CD8s
Weeks on Study

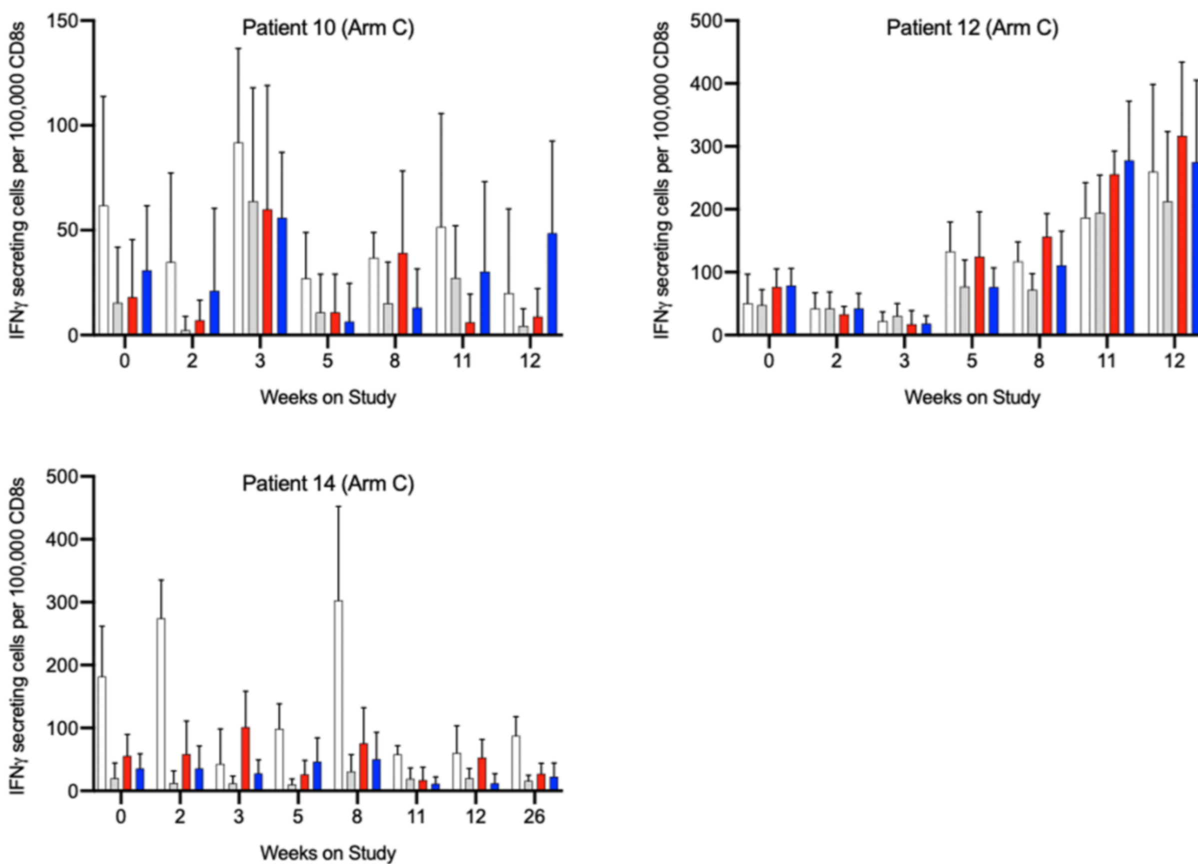


Figure 4.13. ELISpot data for non-responders. Patients enrolled in clinical trial MEL59 received peptide vaccine of pIRS2, pBCAR3 or pIRS2 and pBCAR3. Responses to target T2-B7 cells pulsed with peptides were recorded as SFCs per 100,000 cells in an IFNɣ ELISpot assay. Error bars represent 1 standard deviation of the mean.

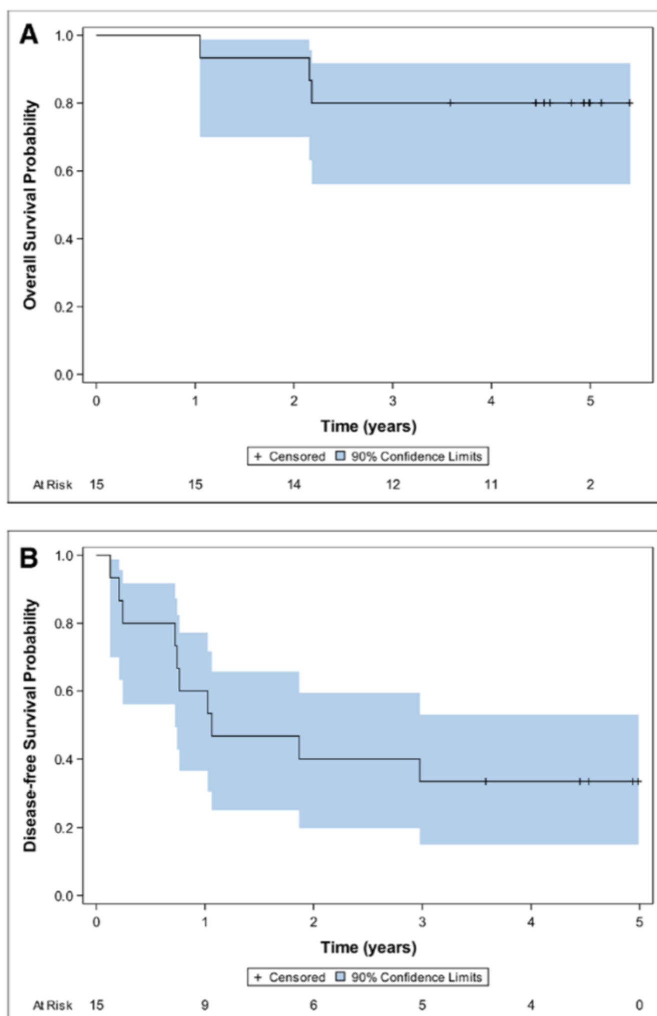


Figure 4.14. Clinical outcomes of vaccinated patients. Kaplan-Meier estimates of **A,** overall survival and **B,** disease-free survival outcomes are shown for all 15 patients as of October 2019.

Table 4.1: Additional HLA-A*0201-restricted phosphopeptides analyzed by donor

Sequence	UniProt #	Gene	Healthy Donors									
			44	43	75	70	89	54	64	67	66	78
ALDsGASLLHL	P57078	RIPK4										
AMAAsPHAV	Q13151	HNRNPA0										
FLDtPIAKV	Q969G9	NKD1										
ILDsGIYRI	Q9UPZ3	HPS5						/				
KAFsPVRSV	Q02363	ID2						/				
KLAsPELERL	P05412	JUN										
KLFPDtPLAL	Q12906	ILF3										
KLMsPKADVKL	Q86T90	KIAA1328						/				
LMFsPVTSL	Q9C0A6	SETD5						/				
RLAsLNAEAL	Q8TBE0	BAHD1						/				
RLAsYLDRV	P05783	KRT18										
RLDsYVRSL	Q9Y5R8	TRAPPC1						/				
RLQsTSERL	Q96TA2	YME1L1						/				
RQAsIELPSMAV	P33241	LSP1										
RQLsSGVSEI	P04792	HSPB1										
RTLsHISEA	Q6ZS17	RIPOR1										
RVLHsPPAV	Q9Y4B5	MTCL1										
SLLTsPPKA	Q14669	TRIP12						/				
SMTRsPPRV	Q9BRL6	SRSF8										
TLAsPSVFKST	Q6PGQ7	BORA										
VLLsPVPEL	Q9H1A4	ANAPC1						/				
VMFRtPLASV	Q9UKT4	FBXO5						/				
YLDsGIHSGA	P35222	CTNNB1										
YQRsFDEVEGVF	Q6Y7W6	GIGYF2			/	/	/	/	/	/	/	/
RRSsLDAEIDSL	Q93052	LPP			/	/	/	/	/	/	/	/

	No response
/	Not analyzed

Table 4.2: Additional HLA-B*0702-restricted phosphopeptides analyzed by donor

Sequence	UniProt #	Gene	Healthy Donors								
			44	43	90	77	73	80	29	67	89
APDsPRAFL	Unknown									/	/
APRKGsFSAL	Q13619	CUL4A			/					/	/
APRKGsFSALM	Q13619	CUL4A			/					/	/
APSVRsLSL	Q9Y446	PKP3								/	/
EPKRRsARL	P05114	HMGN1			/	/			/	/	/
EPRsPSHSM	Q03164	KMT2A			/				/	/	/
GAQPGRHsV	Q96IF1	AJUBA			/				/	/	/
GPRPGsPSAL	Q9UJJ7	RPUSD1			/				/	/	/
GPRsAsLLSL	Q9Y4H4	GPSM3			/	/			/	/	/
GPRSAsLLSL	Q9Y4H4	GPSM3			/					/	/
GPRSASLLsL	Q9Y4H4	GPSM3								/	/
GPRSAsLLsL	Q9Y4H4	GPSM3								/	/
GPRsPKAPP	Q6PJ34	ARHGAP4			/	/			/	/	/
GPRsPPVTL	Q15735	INPP5J			/	/			/	/	/
HPKRSVsL	O60238	BNIP3L			/				/	/	/
HPRSPtPTL	Q96HE9	PRR11			/				/	/	/
KARsPGRAL	Q14767	LTBP2			/	/			/	/	/
KPAsPARRL	P78559	MAP1A			/				/	/	/
KPAsPKFIVTL	Q6PJT7	ZC3H14	/		/				/	/	/
KPLIRSQsL	Q9H6H4	REEP4			/	/			/	/	/
KPPHsPLVL	P01106	MYC								/	/
KPPsPEHQSL	Q9Y6X9	MORC2			/				/	/	/
KPPtPGASF	Q96T58	SPEN			/				/	/	/
KPPYRSHsL	Q96GE4	CEP95			/				/	/	/
KPQTRGKtF	Q8IV04	TBC1D10C			/				/	/	/
KPRPLsMDL	Q9BY89	KIAA1671			/				/	/	/
KPRPPPLsP	Q15642	TRIP10			/				/	/	/
KPRRFsRsL	Q7L4I2	RSRC2			/				/	/	/
KPRsPDHVL	Q9UPN3	MACF1			/				/	/	/
KPRsPFSKI	Q9BXF6	RAB11FIP5			/	/			/	/	/
KPRsPPRAL	Q86TG7	PEG10			/	/			/	/	/
KPRsPVVEL	P25098	GRK2			/				/	/	/
KPYsPLASL	Q13469	NFATC2			/				/	/	/
LPAsPRARL	Q3KQU3	MAP7D1								/	/
LPKsPPYTAF	P23588	EIF4B			/				/	/	/
LPRGSsPSVL	Q9GZN2	TGIF2			/				/	/	/
LPVsPRLQL	P13688	CEACAM1			/				/	/	/
MPRQPsATRL	Q6NZ67	MZT2B			/	/	/	/	/	/	/
QPQRRsLRL	Q9ULW0	TPX2			/			/	/	/	/
QPRsPGPDYSL	Q99684	GFI1			/				/	/	/
QPRtPSPLVL	P33241	LSP1			/				/	/	/

QPRtPsPLVL	P33241	LSP1			/					/	/
RAPsPSSRM	Q9UQ35	SRRM2			/				/	/	/
RARGIsPIVF	Q96MU7	YTHDC1			/					/	/
RPAsAGAML	Q14814	MEF2D			/				/	/	/
RPAsARAQPGL	Q9NPB0	SAYSD1			/	/	/	/	/	/	/
RPAsRFEVL	Q8IZ52	CHPF			/	/	/		/	/	/
RPAtGGPGVA	Q86TW6	N/A			/	/			/	/	/
RPAtPTSQF	Q13115	DUSP4			/					/	/
RPDsPTRPTL	Q7RTP6	MICAL3			/					/	/
RPDsRLGKTEL	Q9BYW2	SETD2			/					/	/
RPFsPREAL	Q86V48	LUZP1			/					/	/
RPGsRQAGL	Q96JY6	PDLIM2			/					/	/
RPHsPEKAF	Q53F19	NCBP3			/					/	/
RPIsPGLSY	Q16204	CCDC6			/					/	/
RPItPPRNSA	P62136	PPP1CA			/					/	/
RPKLsSPAL	Q09472	EP300			/					/	/
RPKPSSsPV	Q15366	PCBP2			/					/	/
RPKsPLSKM	Q9HCD6	TANC2			/					/	/
RPKsVDFDSL	Q9Y5K6	CD2AP			/					/	/
RPNsPSPTAL	Q9UKI8	TLK1			/					/	/
RPPPPPDtPP	Q9Y5W3	KLF2			/					/	/
RPPsPGPVL	Q12770	SCAP			/					/	/
RPQRAtSNVF	P24844	MYL9			/					/	/
RPQRATsNVF	P24844	MYL9			/					/	/
RPRAAtVV	P10644	PRKAR1A			/					/	/
RPRAAtVVA	P10644	PRKAR1A			/					/	/
RPRARsVDAL	Q86X29	LSR			/					/	/
RPRGsQSLL	P21860	ERBB3			/					/	/
RPRPHsAPSL	Q5JXC2	MIIP			/					/	/
RPRPVsPSSL	P57059	SIK1			/					/	/
RPRRsSTQL	P28908	TNFRSF8			/					/	/
RPRsAVLL	Q12802	AKAP13			/					/	/
RPRsLEVTI	O15553	MEFV			/					/	/
RPRsMTVSA	O43312	MTSS1			/					/	/
RPRsPAARL	Q9P2Y4	ZNF219			/					/	/
RPRsPGSNSKV	P78347	GTF2I			/					/	/
RPRsPPGGP	Q86UZ6	ZBTB46			/					/	/
RPRsPPPRAP	O43900	PRICKLE3			/					/	/
RPRsPRENSI	Q99700	ATXN2			/					/	/
RPRsPRQNSI	Q99700	ATXN2			/					/	/
RPRsPSPIS	P41594	GRM5			/					/	/
RPRsPTGP	Q96I25	RBM17			/					/	/
RPRsPTGPsNSF	Q96I25	RBM17			/					/	/
RPSRSsPGL	Q8N3V7	SYNPO			/					/	/
RPSsLPDL	Q8NFD5	ARID1B			/					/	/

RPsSPALYF	Q9Y3Q8	TSC22D4			/					/	/
RPTKIGRRsL	Q96HN2	AHCYL2			/					/	/
RPTsRLNRL	Q15788	NCOA1			/					/	/
RPVsPFQEL	Unknown				/					/	/
RPVsPGKDI	P31629	HIVEP2			/					/	/
RPVtPVSDL	Q13118	KLF10			/					/	/
RPWsPAVSA	P12755	SKI			/					/	/
RPYsPPFFSL	Q9NYF3	FAM53C			/					/	/
RSLsPGGAA	Q96T37	RBM15			/					/	/
RTRsPSPTL	Q86UU1	PHLDB1			/					/	/
RVRsPTRSP	Q03164	KMT2A			/					/	/
SPAsPKISL	Q8WWM7	ATXN2L			/					/	/
SPEKAGRRsSL	A6NC98	CCDC88B			/					/	/
SPFKRQLsL	P49757	NUMB			/					/	/
SPGLARKRsL	Q9H2Y7	ZNF106			/					/	/
SPKsPTAAL	Q53EZ4	CEP55			/					/	/
SPRAPVsPLKF	Q9UBS0	RPS6KB2			/				/	/	/
SPRRsRSISL	Q16629	SRSF7			/				/	/	/
SPRsPDRTL	Q9UKN1	MUC12			/	/			/	/	/
SPRsPGKPM	Unknown				/				/	/	/
SPRSPsTTYL	Q13111	CHAF1A			/				/	/	/
SPRsPSTTYL	Q13111	CHAF1A			/				/	/	/
SPRTPVsPVKF	P23443	RPS6KB1								/	/
SPSsPSVRRQL	O75179	ANKRD17			/				/	/	/
SPSTSRSGGsSRL	Q9BUV0	RSRP1								/	/
TPAQPQRRsL	Q9ULW0	TPX2			/				/	/	/
TPIsPGRASGM	Q01196	RUNX1			/				/	/	/
TPRsPPLGL	Q16584	MAP3K11			/				/	/	/
TPRsPPLGLI	Q16584	MAP3K11								/	/
VLKGsRSSEL	Q96B45	BORCS7			/	/	/	/	/		
VPKsPAFAL	Q9ULW0	TPX2								/	/
VPRPERRsSL	Q6UWJ1	TMCO3			/	/	/		/	/	/
YPGGRRsSL	P22897	MRC1			/	/	/		/	/	/
YPSFRRsSL	O95071	UBR5			/			/	/	/	/
YPSsPRKAL	O43166	SIPA1L1			/				/	/	/
RSHsSPASL	Q9GZV5	WWTR1			/	/	/	/	/		

	No response
/	Not analyzed

Table 4.3: Analysis of Phosphopeptide Responses by their Kinase Recognition Motifs

Kinase Recognition Motif(s)	Response	No Response	Total	Fraction Response	Upper Limit[a]	Lower Limit[a]
N/A	8	5	13	0.615	0.823	0.355
x[pS/pT]P all	25	84	109	0.229	0.317	0.160
x[pS/pT]P only	6	10	16	0.375	0.614	0.185
x[pS/pT]P, [R/K]x[pS/pT]	0	1	1	0.000	0.949	0.000
x[pS/pT]P, [D/E][pS/pT]xxx	0	1	1	0.000	0.949	0.000
x[pS/pT]P, Px[pS/pT]P	4	23	27	0.148	0.325	0.059
x[pS/pT]P, Px[pS/pT]P, [E/D]xx[pS/pT]	0	1	1	0.000	0.949	0.000
x[pS/pT]P, Px[pS/pT]P, [D/E][pS/pT]xxx	0	3	3	0.000	0.561	0.000
x[pS/pT]P, Px[pS/pT]P, [K/R]xx[pS/pT], [K/R]xx[pS/pT]P	9	33	42	0.214	0.359	0.117
x[pS/pT]P, Px[pS/pT]P, [K/R]xx[pS/pT], [K/R]xx[pS/pT]P, [D/E][pS/pT]xxx	0	1	1	0.000	0.949	0.000
x[pS/pT]P, [K/R]xx[pS/pT], [K/R]xx[pS/pT]P	6	11	17	0.353	0.587	0.173
[K/R]xx[pS/pT] all	34	86	120	0.283	0.370	0.210
[K/R]xx[pS/pT] only	11	33	44	0.250	0.394	0.146
x[pS/pT]P, [K/R]xx[pS/pT], [K/R]xx[pS/pT]P	6	11	17	0.353	0.587	0.173
x[pS/pT]P, Px[pS/pT]P, [K/R]xx[pS/pT], [K/R]xx[pS/pT]P	9	33	42	0.214	0.359	0.117
x[pS/pT]P, Px[pS/pT]P, [K/R]xx[pS/pT], [K/R]xx[pS/pT]P, [D/E][pS/pT]xxx	0	1	1	0.000	0.949	0.000
[K/R]xx[pS/pT], [D/E][pS/pT]xxx	5	2	7	0.714	0.949	0.359
[K/R]xx[pS/pT], [K/R]x[pS/pT]	3	6	9	0.333	0.646	0.121
[K/R]x[pS/pT] all	6	22	28	0.214	0.395	0.102
[K/R]x[pS/pT] only	3	14	17	0.176	0.410	0.062
x[pS/pT]P, [R/K]x[pS/pT]	0	1	1	0.000	0.949	0.000
[K/R]x[pS/pT], [K/R]xx[pS/pT]	3	6	9	0.333	0.646	0.121
[K/R]x[pS/pT], [E/D]xx[pS/pT]	0	1	1	0.000	0.949	0.000
[D/E]xx[pS/pT] all	2	2	4	0.500	0.911	0.089
[D/E]xx[pS/pT] only	2	0	2	1.000	1.000	0.178
x[pS/pT]P, Px[pS/pT]P, [D/E]xx[pS/pT]	0	1	1	0.000	0.949	0.000
[K/R]x[pS/pT], [D/E]xx[pS/pT]	0	1	1	0.000	0.949	0.000
[D/E][pS/pT]xxx all	6	10	16	0.375	0.614	0.185

[D/E][pS/pT]xxx only	1	3	4	0.250	0.699	0.013
x[pS/pT]P, [D/E][pS/pT]xxx	0	1	1	0.000	0.949	0.000
x[pS/pT]P, Px[pS/pT]P, [D/E][pS/pT]xxx	0	3	3	0.000	0.561	0.000
x[pS/pT]P, Px[pS/pT]P, [K/R]xx[pS/pT], [K/R]xx[pS/pT]P, [D/E][pS/pT]xxx	0	1	1	0.000	0.949	0.000
[K/R]xx[pS/pT], [D/E][pS/pT]xxx	5	2	7	0.714	0.949	0.359

[a] Ninety-five percent confidence intervals were determined using the Wilson Brown Method.

Table 4.4: Number of phosphopeptides identified by mass spectrometry on patient tumors

Tumor Specimen	HLA alleles			# Phosphopeptides Identified
	A locus	B locus	C locus	
VTB239 Ovarian Ascites	02:01, 68:01	39:01, 40:01	03:04, 07:02	67
VTB239 Ovarian Omentum[a]	02:01, 68:01	39:01, 40:01	03:04, 07:02	23
VTB241 Ovary	02:01, 11:01	07:02, 44:02	05:01, 07:02	61
VTB246 Ovary	01:01, 02:01	15:01, 57:01	03:03, 06:02	16
VTB247 Ovarian Omentum	02:01, 03:01	44:02, 51:01	01:02, 05:01	5
VTB269 Ovary[a]	02:01, 03:01	14:01, 44:02	08:02, 05:01	5
VTB269 Omentum[a]	02:01, 03:01	14:01, 44:02	08:02, 05:01	4
VTB279 Ovarian Omentum	01:01, 02:01	08:01, 40:01	07:01, 03:04	5
VTB280 Ovary[a]	02:01, 29:02	27:05, 35:01	01:02, 04:01	2
VTB285 Pelvic Mass	02:01, 11:01	07:02, 44:02	05:01, 07:02	20
VTB288 Ovary	02:01, 02:01	15:01, 44:02	03:03, 07:04	18
VTB291 Pelvic Mass	02:01, 24:02	07:02, 51:01	07:02, 15:02	16
			Total	242
			Total Unique	180

[a] Sample was collected while patient was receiving neoadjuvant chemotherapy.

Table 4.5. Treatment-related adverse events.

		Arm A pBCAR3 n=3			Arm B pIRS2 n=3			Arm C Both n=9			Total n=15		
Category	**Adverse event**	**G1**	**G2**	**G3**	**G1**	**G2**	**G3**	**G1**	**G2**	**G3**	**G1**	**G2**	**G3**
Gastrointestinal	Constipation							1			1		
	Diarrhea	1						2			3		
	Mucositis oral							1			1		
	Nausea	2						1			3		
General and administration site	Chills	1	1		1			3	1		5	2	
	Edema limbs							1			1		
	Fatigue	2	1		2			5	1		9	2	
	Fever	1			1			1			3		
	Influenza like symptoms		1					1			1	1	
	Injection site reaction		3			3			9			15	
	Pain							2			2		
Immune system	Autoimmune disorder	2			1						3		
Investigations	Lymphocyte count decreased		1									1	
Metabolism/nutrition	Anorexia							1			1		
Musculoskeletal/ connective tissue	Arthralgia	1						3			4		
	Myalgia	2						4			6		
Nervous system	Dizziness	1						2			3		
	Headache		1		1			4			5	1	
Psychiatric	Agitation							1			1		
Respiratory/thoracic/ mediastinal	Cough							1			1		
	Sore throat							1			1		
Skin/subcutaneous tissue	Dry skin							1			1		
	Skin induration		3		1			4	5		5	8	
	Skin ulceration		1					1			1	1	
Vascular	Flushing	1									1		
	Hot flashes							1			1		
Participant highest grade*	Any		3			3			9			15	

*There were no grade 3–5 treatment-related adverse events.
pBCAR3, phosphopeptide from breast cancer antiestrogen resistance 3; pIRS2, phosphopeptide from insulin receptor substrate 2.

Table 4.6. IFNγ T-cell responses, autoimmune toxicities and clinical outcomes by patient and in aggregate

		Vaccine with:		T-cell response detected to:			Baseline* ANA/RF+	On-treatment ANA+	Melanoma clinical outcome October 2019	
Patient	Study arm	pBCAR3	pIRS2	pBCAR3	pIRS2	Either			Recurrence	Died
1	A	+						+	+	+
2	A	+								
3	B		+					+	+	
4	A	+		+		+		+	+	
5	B		+		+	+			+	+
6	B		+						+	
7	C	+	+				+			
8	C	+	+	+	+	+			+	
9	C	+	+						+	
10	C	+	+				+		+	
11	C	+	+		+	+			+	
12	C	+	+							
13	C	+	+		+	+			+	+
14	C	+	+							
15	C	+	+		+	+	+			
Total		12	12	2	5	6	3	3	10	3
# vaccinated with that peptide				12	12	15				
% T-cell response to peptide				17%	42%	40%				
90% CI T-cell responses				3% to 44%	18% to 68%	19% to 64%				

ANA, antinuclear antibody; IFN-γ, interferon-γ; pBCAR3, phosphopeptide from breast cancer antiestrogen resistance 3; pIRS2, phosphopeptide from insulin receptor substrate 2; RF, rheumatoid factor

Chapter 5: Overall Conclusions & Future Directions

5.1 Overall conclusions for healthy donors and cancer patients

$CD8^+$ T cell responses are vital for the anti-tumor response [326, 328, 329, 362, 409, 410]. Identification of relevant MHC-I presented antigens on tumor cells can improve vaccines or adoptive cell therapies aimed at enhancing $CD8^+$ T cell anti-tumor responses. MHC-expressed phosphopeptides have been identified and characterized by us and others as potential cancer immunotherapy targets [1–4, 347, 353, 356, 411]. One interesting finding from these previous studies was that healthy donors with no history of clinically-evident malignancy had robust responses against cancer-expressed phosphopeptides [2, 3]. Robust responses are suggestive of immune memory, which would indicate that these donors had previously encountered phosphopeptides in an immunogenic insult. Phosphopeptide-specific T cells have been shown to contribute to tumor control in humanized murine models [3, 4]. Although their impact on tumor control in human patients has not yet been assessed, pre-existing phosphopeptide specific memory T cells in otherwise healthy individuals could be protective against the development of cancer.

In the current study we explicitly assessed $CD8^+$ T cell memory in 15 healthy donors against HLA-A2- or HLA-B7-restricted phosphopeptides previously identified on solid malignancies and/or leukemias. Most of the 15 healthy donors had evidence of some pre-existing immune memory to phosphopeptides. However, the breadth of responses and the particular phosphopeptides recognized varied highly from donor-to-donor, suggesting that numerous discrete immunogenic sources of exposure drive expression of phosphopeptides in healthy donors. Three phosphopeptides were identified as immunodominant memory targets that were recognized by most $HLA\text{-}A2^+$ donors,

suggesting a common immunogen that induces their expression. Aside from these, most memory targets were recognized in only 1-3 donors. This recognition pattern is more consistent with exposure to rare pathogens or incipient cancer cells.

We characterized the responsive T cells to a subset of phosphopeptides in two healthy donors to make inferences about the nature of the immunogens that induced phosphopeptide immunity. Responses in both donors were predominantly derived from T_{CM}, consistent with resting memory against a previously encountered immunogen. However, there were also several blood draws showing responses with enhanced representation of T_{EM} and/or T_{EMRA}, consistent with active immune responses due to recent re-exposure to an immunogen that caused expression of the phosphopeptide. Our results also demonstrated a significant contribution of T_{SCM} to some responses. Because T_{SCM} are long lived, multipotent, memory cells [82, 85, 89, 412], we believe their contribution to day 14 responses most likely reflects the persistence of T_{SCM} rather than being indicative of an active response. During an active response, T_{SCM} can give rise to T_{CM}, T_{EM}, and/or T_{EMRA}, but a population of progenitor T_{SCM} memory cells still remains. T_{CM} and T_{SCM} are self-renewing, long-lived and multi-potent [82, 85, 89, 412], supporting the likely durability of phosphopeptide immune memory. Both T_{CM} and T_{SCM} have also been demonstrated to have superior anti-tumor effects compared to T_{EM} and effectors in murine and human studies [413–419]. This suggests that phosphopeptide-reactive T_{CM} and T_{SCM} could also be more effective in eradicating phosphopeptide-expressing incipient cancer cells.

We observed considerable heterogeneity in the activated and resting memory characteristics, with variations from donor to donor and even between phosphopeptides in

the same donor. Although we cannot be sure of the underlying causes for this heterogeneity, it does identify an opportunity for future investigation. The use of additional markers to further distinguish between T cell subsets, combined with more detailed longitudinal analysis with regular biweekly blood collections, could provide better resolution of distinctions in response variability. Another opportunity for future investigation would be to conduct similar analyses of the longitudinal response patterns in multiple donors with the immunodominant memory targets to compare the exposure patterns.

Patients with melanoma or ovarian cancer had limited phosphopeptide immunity compared to healthy donor PBMC responses, consistent with previous findings in patients with AML or CLL [2]. If phosphopeptide immunity in patients was impaired due to tumor-induced exhaustion or suppression, we would have expected to see limited responses only to tumor-expressed phosphopeptides. We would have expected to see responses to the 3 immunodominant phosphopeptides at a similar frequency in the patients as that which we observed in the healthy donors. It was striking that most responses to tumor-expressed phosphopeptides were absent, suggesting either that those T cells had been lost or that T cells had not been primed by DC cross-presentation. Altogether these results are consistent with the hypothesis that limited responses in cancer patients are due to lack of immunogenic exposure. These results suggest that, despite the phosphopeptides' presentation on the surface of tumor cells, demonstrating that they can be processed and presented, there may be obstacles to their cross-presentation by professional APCs. These results identify an opportunity to investigate the ability of responses to tumor-expressed phosphopeptide induced by vaccination or adoptive cell therapy to augment tumor control. They also identify an opportunity to uncover why some patients and healthy donors have

limited phosphopeptide immunity in order to determine ways to boost responses or overcome the restrictions.

Despite the generally limited phosphopeptide immunity in the ovarian cancer patients, 4 of the 10 patients responded to 1-2 immunodominant phosphopeptides. Two of the 4 patients had responses from TILs, including one patient whose tumor expressed the phosphopeptide of interest. Responses in this one patient to the tumor-expressed phosphopeptide were predominantly T_{EM} and effectors, consistent with an active immune response. Therefore, even though phosphopeptide immunity is generally compromised in patients, some patients are still able to generate T cell memory responses to a subset of immunodominant phosphopeptides, and expression on one patient's tumor was associated with an effector T cell response. These results identified an opportunity to isolate phosphopeptide-specific TILs from some patients and create T cells with recombinant TCRs for adoptive cell therapy.

The phosphopeptides expressed on the tumors of melanoma patients enrolled in the vaccine clinical trial (**Chapter 4**) are unknown. Therefore, while pre-vaccine responses to the immunodominant $pIRS2_{1097-1105}$ were limited in melanoma patients relative to responses in healthy donors, we were unable to determine whether this was associated with expression on the patient's tumor. Also, immune responses to one or both phosphopeptides were observed at 1 or 2 timepoints after vaccination but were not durable. One possible explanation is that the phosphopeptide-specific T cells had become exhausted, if the phosphopeptide was expressed on the patient's tumor. This could have been identified using multimer staining. It is also possible that phosphopeptide-specific T cells were terminal effectors unable to persist for 14 days in culture, but IFNγ responses would have

been evident *ex vivo*. Limited samples precluded this analysis. Finally, it is possible that the phosphopeptide-reactive T cells had left the blood and infiltrated the tumor. This could also explain the transient responses observed following vaccination. TIL analyses were unavailable in this study, but evidence in two ovarian cancer patients demonstrated that phosphopeptide-reactive T cells can infiltrate tumors.

5.2 Limitations on conclusions

In our initial screens we enriched antigen-experienced $CD45RO^{+}CD8^{+}$ T cells to offer the best possibility of detecting phosphopeptide-specific memory cells, which eliminated $CD45RO^{neg}CD8^{+}$ T_{EMRA} and T_{SCM}. Later analyses revealed a role for these subsets in the response, depending on the donor and the phosphopeptide. This suggests the possibility of false negatives in our “no response” category, although most analyzed responses in HD43 and HD44 were not exclusively made up of T_{EMRA} or T_{SCM}, and so we are less concerned with this possibility.

Other factors may have also contributed to false-negatives. Some phosphopeptides that were not recognized as memory targets by early donors were excluded from analyses of later donors. Responses to some phosphopeptides were analyzed only once, with T cells from a single blood collection. As there was considerable experiment-to-experiment and blood collection-to-collection variation in the responses of several donors, it is possible that a one-time response analysis was not representative. Finally, response analyses were limited to detection of the T cells that could persist in culture for 14 days with support of IL-7 and IL-15 and produce IFNγ upon re-exposure to the target antigen. If antigen-specific T cells were present in the initial culture but unable to survive and replicate for 14 days in culture, or if the T cells were unable to produce IFNγ at that time, they would have not

been identified in our assay. The combination of *ex vivo* assays along with the 14 day stimulated assays would have identified T cells that had not yet formed long-term memory. In parallel analyses utilizing IL-2 and shorter culture times, such as 5 and 10 days, may have identified additional effector-like cells that would not have been supported by IL-7/15. As described in chapter 3, phosphopeptide-MHC multimer technology would be a useful tool to assess for dysfunctional antigen-specific T cells, but this technique requires further development. In lieu of multimer staining, flow cytometry analysis after re-stimulation could potentially identify phosphopeptide-reactive T cells by staining for Nur77, CD69, IL-2, TNFα, and IFNγ. However, ELISpot assays generally have better detection of low-frequency cell subsets than flow cytometry; therefore, flow cytometry may have only been able to detect antigen-specific cells in select cases of high-frequency antigen-specific T cells.

Our study included analyses in 10 HLA-A2$^+$ and 7 HLA-B7$^+$ healthy donors. However, with so much natural variation in humans, as well as the donor-to-donor variation in responses we observed, response analyses in additional donors would be required to determine the possibility of additional immunodominant memory targets. These limitations are particularly true for the HLA-B7-restricted phosphopeptides. Therefore, it is possible, and perhaps even probable, that additional memory targets and immunodominant memory targets could be identified with expanded analyses, additional blood collections, and larger cohorts of HLA-A2$^+$ and especially HLA-B7$^+$ healthy donors. Furthermore, while MHC-II-restricted phosphopeptides have been identified [347], CD4$^+$ T cell effector and memory responses to them have largely not been investigated. Lastly, it is known that MHC polymorphism influences the peptide repertoires presented by any one HLA type [420,

421], and, therefore, a more complete understanding of a person's responsiveness to phosphopeptides should encompass multiple alleles of MHC-I and MHC-II presented phosphopeptides.

The biggest limitation in our response analyses in ovarian cancer patients was the low PBMC and TIL numbers, which limited the number of responses that could be evaluated in any patient. Based on the high donor-to-donor-variability in phosphopeptide immunity, future studies should prioritize collecting larger patient specimens for response analyses. Another limitation was that the ovarian tumor-expressed phosphopeptides were identified from one biopsy site and from one disease timepoint in most patients. It has been demonstrated that antigens identified in singular biopsy samples are often not reflective of the overall tumor mass [422–424], that primary and metastatic tumors differ in their antigen repertoires, and that tumor antigen display evolves over time and in response to treatment [422–427]. In the present study, two tumor specimens were collected from two patients for phosphopeptide identification by mass spectrometry. In VTB239, 72 MHC-I-expressed phosphopeptides were identified on tumor cells from the ascites compared to 23 phosphopeptides on tumor cells from the omentum, only 5 of which were shared. In VTB269, 5 MHC-I-expressed phosphopeptides were identified on tumor cells from the ovary compared to 4 phosphopeptides on tumor cells from the omentum, 2 of which were shared. Therefore, the phosphopeptides identified from two samples from sites in the same patient may not reflect the phosphopeptides displayed at an earlier point in tumor outgrowth, which may have affected the patient's immunity to them. We had evidence that there was at least some degree of mosaicism in the clonality of the tumor cells in the two

patients for whom two tumor specimens were analyzed, although the additional influence of chemotherapy cannot be ruled out for VTB239.

Further investigation is required to determine why phosphopeptide immunity is limited in some patients but not all, similarly to the observation that phosphopeptide immunity is limited in some healthy donors as well. This investigation can be combined with the interrogation of healthy donors. In both cases, expression of the phosphoproteomes and the MHC-phosphopeptides on their EBV-transformed B cells would be compared to determine whether there are limitations in the ability of that person's cells to present phosphopeptides. If there is discordance between the phosphoproteome and MHC-phosphopeptide expression, then the expression of the proteasome and isoforms of TAP would be identified.

5.3 Future Directions for Healthy Donors – factors influencing phosphopeptide display and recognition in healthy donors

5.3.1 Overall rationale

While many healthy donors demonstrated some degree of pre-existing immune memory to phosphopeptides, the breadth of phosphopeptide-specific immunity varied significantly from donor to donor. There are several possible explanations for this. The proposed future directions evaluate each of these possibilities.

The most obvious possibility for high donor-to-donor variation is that it reflects unique exposure histories to the immunogens that induce expression of the phosphopeptides. Therefore, an obvious possibility for the absence of memory to a phosphopeptide is the lack of exposure to the immunogen that drove its expression. This cannot be directly assessed because it is impossible to know a human's comprehensive

exposure history. However, we can test a related hypothesis: that healthy donors with limited phosphopeptide memory can respond to MHC-phosphopeptides when they are exposed to them. The alternative explanation is that there are differences in the donor that limit the available T cell receptor repertoire capable of recognizing phosphopeptides. Any deficiencies in the TCR repertoire would likely be attributable to tolerance or deletion of phosphopeptide-specific T cells that are specific to phosphopeptides that are expressed in the thymus in that donor or are cross-reactive to MHC-self-antigens in that donor [428]. If this is the reason for the lack of response in a donor to a particular phosphopeptide, the inability for endogenous T cells to recognize that phosphopeptide would be irreversible. We find this explanation plausible to explain the absence of responses to one or a few phosphopeptides but highly unlikely to account for impaired responsiveness to all phosphopeptides in a donor. This possibility will be assessed in **Future Direction 1**.

Another possible explanation is donor-to-donor variation in immunological processes, such as the components of the MHC-I antigen processing and presentation pathway. Numerous proteins, chaperones, etc. are involved in this pathway, some of which have multiple allelic isoforms that have been described as having SNPs that alter their functions or specificities. One component of the MHC-I antigen processing and presentation pathway that influences that presented peptide repertoire is degradation by the constitutive proteasome or by the immunoproteasome. Therefore, depending upon allelic variations and the involved cell type, a person may have encountered that immunogenic exposure but was unable to present the phosphopeptide of interest. We can test the hypothesis that expression of the immunoproteasome alters the display of phosphopeptides

on cells from expression of the constitutive proteasome alone. This possibility will be assessed in **Future Direction 2**.

5.3.2 Future Direction 1 - To determine whether donors with limited pre-existing immune memory are also limited in naïve T cell responses to phosphopeptides

Naïve $CD45RO^{-}CCR7^{+}CD8^{+}$ T cells would be isolated from healthy donors with broad phosphopeptide memory and from healthy donors with limited phosphopeptide memory. A 4-6 week culture system using peptide-pulsed autologous mature DCs, IL-12, and IL-2 would be used to generate *de novo* responses. To establish confidence that the culture system is capable of inducing naïve T cell responses in that donor, control cultures with MART-1, Yellow Fever, or Ebola peptides would also be generated.

If a response to the MHC-phosphopeptide can be generated, this would be consistent with the absence of memory reflecting absence of exposure. However, we also could not exclude the possibility that there had been an exposure and limitation in expression of the MHC-phosphopeptide precluded generation of a response. If the donor responds to some MHC-phosphopeptides but not to others, this is consistent with the lack of phosphopeptide-specific TCRs due to tolerance. However, if negligible or few responses can be generated to MHC-phosphopeptides, and the donor also had limited phosphopeptide memory, this suggests there is something restrictive in their MHC presentation and/or TCR repertoire that limits broad recognition of phosphopeptides. If responses can be generated from naïve T cells against MART-1, Yellow Fever, and/or Ebola peptides but not to any phosphopeptides, it is highly unlikely that the person's entire TCR is incapable of recognizing phosphopeptides given the high number and diversity of TCRs. Therefore, this

is more consistent with a restriction in the person's ability to generate or present MHC-phosphopeptides.

To test the hypothesis that limited immune memory reflects restrictions in MHC-I-phosphopeptide presentation, donor PBMCs could be isolated and EBV used to transform the enriched B cells. A large number of EBV-transformed donor B cells would then be grown in culture and assessed for MHC-phosphopeptide expression. We would expect that most HLA-A2^{+} healthy donors would express a similar quantity and repertoire of phosphopeptides. Variations in the repertoires would likely be a result of heterogeneous MHC alleles. This could be assessed by comparing the phosphopeptides identified by W6/32 immunoprecipitation of all MHC-I and BB7.2 immunoprecipitation of HLA-A2-restricted phosphopeptides. If the donor B cells can present MHC-phosphopeptides, then an inability to generate a T cell response against it is most likely due to tolerance. Some phosphopeptides, although identified on cancer cells, may be presented at high levels in the person's periphery or in the thymus. High expression on peripheral tissues could tolerize phosphopeptide-reactive T cells. On the other hand, central tolerance could be induced during thymic selection. Central tolerance could be induced in response to high affinity interaction with the phosphopeptide itself or by cross-reactivity with another phosphopeptide. However, it remains unknown whether phosphopeptides are even expressed in thymus. Therefore, while tolerance cannot be proved, it would be the most likely explanation if the person is capable of presenting MHC-phosphopeptides but there are no evident responsive TCRs among the naïve T cell subset. On the other hand, if the donor's EBV-transformed B cells were unable to present or were severely restricted in the number of MHC-phosphopeptides, this suggests an MHC-processing or presentation issue.

5.3.3 Future Direction 2 - To determine whether expression of the immunoproteasome alters the display of phosphopeptides

While phosphopeptide presentation has been demonstrated to be TAP-dependent [337, 359], it is unknown whether allelic isoforms of TAP are equally capable of transporting phosphopeptides into the ER for MHC-loading. Furthermore, differences in MHC-presented phosphopeptides due to protein degradation by the immunoproteasome versus the constitutive 20S proteasome are also unknown. To test the hypothesis that expression of the immunoproteasome alters the display of phosphopeptides on cells from expression of the constitutive proteasome alone, we will utilize HLA-A2^{+} and HLA-B7^{+} K562 cell lines to identify the MHC-phosphopeptide repertoires under different proteasomal conditions [429–431]. K562 cells express the B1, B2, and B5 subunits of the constitutive proteasome. Upon IFNγ treatment, they upregulate LMP2, LMP7, and LMP10 of the immunoproteasome [430]. Western blotting will assess the expression levels under both conditions for all proteasomal subunits. When assessing the immunoproteasome, the B1, B2, and B5 subunits can also be knocked down using siRNA, knocked out using CRISPR, or inhibited using proteasome (such as Bortezomib) and immunoproteasome inhibitors (such as KZR-616 [432]) to specifically identify immunoproteasome-generated epitopes. In this way, the MHC-phosphopeptide repertoires under 3 conditions would be assessed: constitutive proteasome, immunoproteasome, or both proteasomes present. Large numbers of K562 cells will be grown in culture and then treated (or not) with IFNγ. The additional control of an immunoproteasome inhibitor with IFNγ would identify phosphopeptides induced by IFNγ treatment alone. Mass spectrometry will identify the phosphopeptide products generated by the constitutive proteasome and by the immunoproteasome. The

MHC-phosphopeptide repertoires on healthy donor EBV-transformed B cells can similarly be assessed under 3 conditions: resting state, IFNγ treatment, and immunoproteasome inhibition. Healthy donors with abundant phosphopeptide memory responses would be chosen for their presumed broad ability to present phosphopeptides.

To determine whether phosphopeptides expressed on cancer cells are generated by either the constitutive proteasome or by the immunoproteasome, we will carry out similar studies in cancer cell lines known to express phosphopeptides. This would be carried out in SLM2, VMM39, SKOV3, COV413, and JY cell lines, as their HLA-A2-restricted phosphopeptide repertoires have already been determined by our lab. From these cell lines, separate CRISPR knock out lines would be generated, targeting the subunits of either the constitutive proteasome or of the immunoproteasome. Immunoproteasome inhibition with KZR-616 would also be utilized. Upon successful knock out or inhibition, confirmed by western blotting, the HLA-A2-restricted phosphopeptides would be identified using immunoprecipitation and mass spectrometry. If sufficient tumor cells are available from a patient, CRISPR or immunoproteasome inhibition could similarly be employed to determine the immunoproteasome-dependence of phosphopeptides' expression. This would require a sufficient number of tumor cells to grow out and analyze under the different conditions.

5.4 Future Directions in studying phosphopeptide immunity in cancer patients – Investigating mechanisms for limited phosphopeptide responses and identifying potential new therapeutic strategies

5.4.1 Overall rationale

The limited responses to tumor-expressed phosphopeptides in patients identifies opportunities to 1) induce or augment responses and to 2) enhance phosphopeptide expression for enhanced T cell targeting of the tumor cells. In this and previous studies, immune responses can be induced in patients with vaccination [5] and phosphopeptide-specific T cells demonstrate tumor control in two humanized murine models [3, 4]. These data support further work into inducing phosphopeptide responses in cancer patients as part of immunotherapy strategies. It would be important to isolate naïve T cells from the ovarian cancer patients and attempt to generate responses to phosphopeptides, as described for healthy donors in **Future Direction 1**. The ability to generate responses *in vitro* to tumor-expressed phosphopeptides would demonstrate that responses in the patient can be generated, suggesting that either the conditions of presentation in the tumor were insufficient or that the responses had been there but were lost over time. We believe one of these last two possibilities most likely, particularly because responses could be induced by vaccination in melanoma patients. Regardless, the results of our study demonstrate that additional investigation into vaccine and adoptive cell therapies inducing or augmenting phosphopeptide immunity are warranted in patients with cancer. This will be further explored in **Future Direction 3**.

One question then is whether the patients' tumor-expressed phosphopeptides were immunogenic. It is possible that the phosphopeptides were subdominant tumor epitopes,

due to perhaps the timing of expression or relative abundance. It is also possible that the phosphopeptides, despite their expression on the tumor cells, are not cross-presented by an immunogenic mature APC. DC-mediated cross-presentation of tumor antigens is required to prime $CD8^+$ T cells [93, 433–439]. A deficiency in cross-presentation would restrict T cell priming [440, 441]. One possible explanation for this is that suppressive or tolerogenic factors from the tumor have restricted proper maturation and activation of DCs. The maturation markers acquired by the immature DCs could be assessed by flow cytometry to determine whether the engulfment of apoptosed tumor cells induced DC activation. This could be overcome with a DC-based vaccine or by treating with a DC modulator such as a CD40 agonist [92, 442]. It is also possible that the conditions created by the tumor in the draining lymph node have impaired DCs' ability to cross-present tumor antigens in general. This has been described in a murine stress model in which cross-presentation was restricted due to stress-induced proteasomal dysfunction in the DCs [93]. Another possible explanation is that healthy, immunogenic DCs are inherently limited in their ability to cross-present phosphopeptides. To the best of our knowledge, this has not yet been evaluated and will be assessed in **Future Direction 4**.

The etiological origins of MHC-expressed phosphopeptides in heathy donors are unknown, but one proposed source for generating phosphopeptide-specific T cell responses is viral infection. There is evidence that infection plays a role in inducing immune responses to tumor-associated antigens (TAAs) [289] beyond virally-driven cancers. As described in **Chapter 1**, epidemiologic studies have shown correlations between enhanced history of viral infections, or, specifically of febrile infections and lower risk of cancer [277, 278, 280, 281]. It is possible that some of this protection involves phosphopeptide-

specific T cell responses. Many viruses dysregulate the same signaling pathways as in cancer including some of the same kinases and phosphatases for their self-propagation, likely generating an overabundance of phosphorylated proteins [280, 400–402]. However, beyond EBV-transformed B cells, it is unknown whether viral infection induces the expression of MHC-phosphopeptides and to what extent these repertoires overlap with cancer-expressed phosphopeptides. Innate immune sensing of pathogens triggers a number of signaling pathways containing serine/threonine kinases and activation of these pathways alone may be sufficient to enhance MHC-phosphopeptide expression. These issues will be investigated in **Future Direction 5**. Furthermore, two limiting factors in all T cell-based immunotherapies are the percentage of antigen positive tumor cells and the expression of the antigen on cells. It is likely that therapies that augment antigen expression level on a cell and increase the number of tumor cells expressing the antigen would enhance antigen-dependent T cell targeting. Investigation into combination therapies that could augment T cell responses as well as augment antigen availability are needed. Vaccines use adjuvants to promote a pro-inflammatory response [443]. Some innate immune pathways activated by vaccines involve kinase signaling [401, 402, 444, 445] (and reviewed in [400, 446]). Similarly, many immunotherapies in clinical trials involve oncolytic viruses. Oncolytic viruses efficiently traffic to the tumor. One reason oncolytic viruses are used in immunotherapy is for their induction of innate sensing pathways [447, 448], many of which result in kinase activation. Therefore, it is reasonable to hypothesize that innate immune signaling or oncolytic viruses can upregulate MHC-phosphopeptide expression, which could be combined synergistically with T cell-based immunotherapy. This will also be investigated in **Future Direction 5**.

Lastly, the phase I clinical trial described here was not powered to detected changes in progression free survival or overall survival, and it is unknown whether the induced phosphopeptide-specific responses could lead to a clinical response. While it is beyond the scope of these current studies, future clinical trials will be needed to address the association between phosphopeptide responses and PFS or OS in patients. Despite this, an outstanding question remains as to when phosphopeptide immunity becomes restricted in cancer patients. Results from this study suggest a general limitation in phosphopeptide immunity in patients. This may suggest that phosphopeptide responses failed to develop in the first place in patients with cancer. However, there is still the possibility that responses were evident and became lost over time. This will be explored in part in **Future Direction 6**, examining phosphopeptide antigen expression and immunity over time in patients who are at high risk of developing cancer.

5.4.2 Future Direction 3: Vaccination and adoptive cell therapy strategies to enhance T cell responses against tumor-expressed phosphopeptides

In this study we have identified three phosphopeptides that are recognized by most healthy donors. Their immunodominance supports their immunogenicity, the lack of tolerance, and the absence of autoimmunity. Responses to one of the immunodominant phosphopeptides, $pIRS2_{1097\text{-}1105}$, can be induced by vaccination in melanoma patients [5]. In a humanized murine model $pIRS2_{1097\text{-}1105}$-specific T cells were able to delay tumor outgrowth [3]. These data support pursuing the use of T cells specific to $pIRS2_{1097\text{-}1105}$, as well as $pCHEK1_{461\text{-}471}$ and $pCDC25B_{38\text{-}46}$ in patient therapy.

Vaccination (as demonstrated here) or adoptive cell therapy can induce or augment the patient's phosphopeptide-specific T cell responses. While phosphopeptide-specific

responses were observed in some melanoma patients after vaccination, these responses were not persistently observed from PBMCs. One possible explanation for this is that the vaccine failed to induce a robust, durable response. Therefore, it is prudent to investigate and compare multiple vaccine formulations to induce a robust and durable T cell response that contributes to tumor control. It is possible that DCs in the patients were not properly activated or matured. This could be assessed with characterization of patient-derived DCs and assessment of levels of co-stimulatory molecules, MHC, and cytokine production. If this was found to be the case, alternative vaccine formulations or platforms, such as vaccines that include CD40 agonist [442] or DC-based vaccines [320–322], could overcome those restrictions and result in robust, durable T cell responses. These of course are general concerns for vaccine strategies and not specific to phosphopeptide vaccination.

Using multimers, phosphopeptide-specific TCRs could be isolated from responsive healthy donors. The TCRs would then be identified by sequencing and used to generate recombinant TCRs for use in adoptive cell therapy. As alluded to in **Chapter 3**, MHC-phosphopeptide multimer technology must be further developed and optimized to support future studies. This may require a collaboration with a structural biochemist. Alternatively, limiting dilutions of phosphopeptide-reactive T cells could allow for TCR sequencing from single cells. TCRs among patient TILs have been shown to have highest affinity [151]. Therefore, it would be most useful to find patients with intratumoral phosphopeptide-specific T cells, such as VTB239 in this study (**Chapter 4**) as a source for identifying useful, high affinity TCRs. One interesting possibility is collecting TILs from high-risk patients' benign masses to determine whether tumor-specific phosphopeptide-reactive T cells were already present and able to infiltrate the benign mass. If benign tumors are also

enriched for higher affinity TCRs, this could offer a real opportunity to prophylactically induce a robust anti-tumor antigen T cell response in high-risk patients. *In vitro* binding assays would determine the MHC-TCR affinity of the TCRs isolated from benign mass TILs and compare that to those of PBMC-derived and malignant tumor-derived TCRs from the same patient. T cells with genetically engineered TCRs will be assessed for their effector function, high affinity for the MHC-phosphopeptide, sensitivity to MHC-phosphopeptide expression levels found on cancer cells, and specificity for targeting cancer cells expressing the phosphopeptide of interest without affecting normal cells and tissues. Pre-clinical studies of tumor control by transfected phosphopeptide-specific human $CD8^+$ T cells will be assessed against human tumors implanted in NOD/SCID/IL-2R$\gamma c^{-/-}$ (NSG) mice, using decreasing numbers of T cells to assess the T cell number-dependency of tumor-control, as described [3].

5.4.3 Future Direction 4: To determine whether MHC-I phosphopeptides can be cross-presented by DCs.

For tumor-expressed antigens, including MHC-phosphopeptides, to be targets of $CD8^+$ T cell targeting, $CD8^+$ T cells first need to be primed/activated by a professional APC. This could have occurred in the past, if the person previously saw the MHC-phosphopeptide on a professional APC and generated memory to it. T cell priming can also occur when immature DCs (iDCs) acquire antigens from apoptotic tumor cells. The iDCs carry the antigen load to the tumor-draining lymph node, where the iDC matures, becomes immunogenic, and cross-presents the acquired phosphopeptide to naïve T cells [433–435, 449]. If the person does not have pre-existing memory to the antigen prior to malignancy,

cross-presentation by DCs is critical to prime the T cell response [92, 449–451]. It is currently unknown whether DCs can cross-present phosphopeptides.

To test this, iDCs from healthy donors with broad phosphopeptide immunity, such as HD43 or HD44, would be assessed for their ability to cross-present phosphopeptides. The mDC maturation protocol used in this current study generates iDCs from $CD14^+$ monocytes at day 9 [452]. The SLM2 tumor cell line is known to express pIRS2 [3]. These tumor cells would be killed *in vitro* with oxaliplatin treatment [453]. Apoptosis of the tumor cells would be confirmed by Annexin V staining. Next, engulfment of the apoptotic cells by the iDCs would be assessed by immunohistochemistry, flow cytometry, or ImageStream analysis. If iDCs engulf apoptotic cells, and subsequently mature and express co-stimulatory markers (including high levels of MHC-I and MHC-II, CD80, and CD86), as assessed by flow cytometry, then they should be able to cross-present tumor cell antigens. This would be assessed by an *in vitro* cross-presentation assay as described [93, 453]. The apoptotic tumor cells would be pelleted to remove soluble antigen and co-cultured with iDCs and either previously re-stimulated $CD45RO^+CD8^+$ T cells or naïve T cells from healthy donors known to respond to the phosphopeptide. In addition to testing cell-delivered phosphopeptide antigen, dilutions of soluble synthetic long phosphopeptide and synthetic long phosphopeptide conjugated to beads would also be assessed. The newly primed T cells would be cultured for 18-20 hours (in the case of the previously re-stimulated $CD45RO^+CD8^+$ T cells) or 4-6 weeks (in the case of the naïve T cells) and then assessed for antigen responses by ELISpot assay. The tumor cells used would be $SLM2^{WT}$ and $SLM2^{\beta 2m-/-}$ to eliminate HLA-A2 expression and redressing of the MHC-pIRS2 complex from the cell surface of the SLM2 cells to the surface of the DCs. If T cell

responses are induced after DC exposure to treated SLM2$^{\beta2m-/-}$ cells, this would demonstrate that DCs are able to effectively cross-present phosphopeptides from apoptotic tumor cells. If T cell responses are not induced, this suggests a limitation in the phosphopeptide entering the DC's MHC-I cross-presentation pathway. If only cell-derived antigens fail to be cross-presented, it might suggest that there is an insufficient abundance of the phosphopeptide. An alternative, and we believe more likely, possibility is that these results suggest that the phosphopeptide is unstable once apoptosis is induced. Due to the active role serine/threonine phosphatases play in apoptosis [454, 455], the phosphorylated protein may not persist long enough for the phosphopeptide to be generated in the DC cross-presentation pathway. To assess this possibility, phosphatase inhibitors could be added during tumor cell killing, although it is unclear how this treatment might affect apoptosis. Hydrolysable and non-hydrolysable synthetic long phosphopeptides could also be introduced with treated tumor cells to determine if the non-hydrolysable, but not the hydrolysable, long phosphopeptide can be processed and presented by DCs, which would support the possibility that phosphatases limit the stability of phosphorylated proteins during apoptosis.

If T cell responses cannot be generated by tumor cell-derived DC cross-presentation, this suggests that priming of naïve phosphopeptide-specific T cells can only occur when a hematopoietic cell is infected or transformed and directly presents the phosphopeptide. Furthermore, this finding would suggest that phosphopeptides first encountered on the tumor would be insufficient to prime T cells, and it would support the continued investigation of vaccine strategies or adoptive cell therapy with recombinant TCRs to induce immune responses against tumor-expressed phosphopeptides.

5.4.4 Future Direction 5: To determine the influence of inflammatory signaling on display of MHC-restricted phosphopeptides

Infections, such as those with the Influenza virus or Rhinovirus, have been shown to not only induce tumor antigen expression but more specifically, to activate many of the same kinases and phosphatases as in cancer cells [280, 400–402]. Some of this can be attributed to the recognition of viruses triggering innate immune signaling cascades that activate serine/threonine kinases [401, 402, 444, 445] (and reviewed in [400, 446]). For example, early recognition of Rhinovirus (RV) occurs through endosomal TLR3/TRIF sensing of viral dsRNA, and later through the TLR3/TRIF-induced RNA helicases RIG-I and MDA-5 [456]. Activation of these pathways signals through MyD88, TBK1 or IKK-I, and IRF3 to induce the expression of IFNβ and IFNλ [456, 457]. IKK and TBK1 are both serine/threonine kinases that phosphorylate IRF-3 [457, 458]. TLR3 sensing of dsRNA also induces autocrine EGFR/ERK signaling by the induced production of EGFR ligands (amphiregulin, epiregulin, and HB-EGF) [445, 459]. MEK and ERK signaling downstream of EGFR engagement are heavily dependent on multiple serine/threonine phosphorylation events [460]. Therefore, to test the hypothesis that TLR agonists enhance MHC-phosphopeptide expression, cells expressing HLA-A2 would be grown up in culture and treated with individual TLR agonists. Mass spectrometry would be performed on the eluted, enriched MHC-phosphopeptides as described [366]. K562 cells are commonly used to assess MHC-restricted T cell responses due to their MHC^{neg} status and well-established lentiviral or retroviral transfectants with HLA molecules of interest [461–464]. Once K562 cells are transfected to express an HLA allele, TAP-dependent MHC antigen processing and presentation are intact [465]. K562 cells would be incubated for 6 hours with PolyIC,

IFNα, IFNβ, IFNγ, or LPS. PolyIC signals through TLR3 and TRIF and induces the upregulation of RIG-I and MDA5 [456]. IFNα and IFNβ signal through the Type I IFN receptor (IFNR), activating JAK/STAT kinases in the canonical pathways or MAPK or PI3K/mTOR non-canonical pathways [466]. IFNβ can also be used to activate RIG-I and MDA-5 [456]. IFNγ signals through the Type II IFNR to activate JAK/STATs, IFN-inducible genes, and a host of signaling pathways [467]. LPS signals through TLR4, activating TRIF, TBK1, and IRF3 [458]. The MHC-phosphopeptides induced in response to these innate immune stimulants could also be assessed on healthy donors' PBMCs.

The same studies proposed to assess MHC-phosphopeptides upon TLR signaling could also be completed with Influenza or Rhinovirus infections. Influenza virus is a common virus and is also known to activate host kinases through innate immune signaling cascades, including RIG-I/MAVS/TRAF3/TBK1 and IKKe/IRF3 [468]. Influenza viral infection also activates signaling through the RAF/MEK/ERK pathway [402, 469, 470]. The MHC-phosphopeptide repertoire on HLA-A2$^+$ K562 cells would be identified upon Influenza infection. Lastly, as the genetically-modified herpes simplex virus 1 (HSV) is an FDA-approved oncolytic virus and could also potentially enhance the expression of MHC-phosphopeptides due to innate immune sensing [153], treatment of cells with oncolytic virus could similarly be assessed.

The distinct HLA-A2-restricted phosphopeptide repertoires induced by TLR signaling would be compared to the repertoires induced by infection with Influenza or treatment with oncolytic virus. The first question is whether either Influenza infection or oncolytic virus induces different or additional phosphopeptides than TLR signaling alone. The next question is whether the innate immune signaling-induced and/or Influenza-

induced phosphopeptides are induced in the pathologically-relevant cell type as well as to confirm that the phosphopeptides detected are not artifacts, augmented from underlying signaling of the K562 CML cancer cell line. Primary alveolar epithelial cell lines can be purchased from ATCC for these experiments, as they are susceptible to and physiologically relevant for Influenza infection. Next, if the phosphopeptides were not assessed in this current study, responses to the TLR- or Influenza-induced phosphopeptides should be assessed in a group of at least 10 HLA-A2$^+$ healthy donors, ideally the same cohort as in this current study since a baseline has already been established. Lastly, we would determine whether there are shared phosphopeptides that are expressed by innate immune signaling and/or Influenza infection as well as on tumor cells. T cell recognition of TLR-, Influenza-, or oncolytic virus-induced phosphopeptides would create an opportunity to boost phosphopeptide immunity by vaccines or by oncolytic viral therapy, particularly if they are also relevant to cancer immunity.

If phosphopeptide expression is increased by TLR agonists, their inclusion in intratumoral vaccines or nanoparticles that traffic to the tumor may enhance expression of MHC-phosphopeptides on the tumor cells through the activation of specific innate signaling pathways. Similarly, Influenza vaccination or oncolytic viral therapy could also be used to enhance MHC-phosphopeptide expression on the tumor cells. There is a safe, FDA-approved Influenza vaccine that many people receive annually. Due to the prevalence of vaccination or infection, Influenza-specific responses are robust in most people. Other groups have recently identified viral-specific T cells among TILs [280, 471], suggesting they may be exploited in the anti-tumor response. Therefore, the Influenza vaccine could induce a robust memory response against the viral antigens, as well as enhance MHC-

phosphopeptide display. T-VEC is a genetically-modified herpes simplex virus 1 (HSV) FDA-approved oncolytic virus used for advanced melanoma [153], and could also be assessed for its ability to enhance T cell targeting of tumor cells. Pairing one of these phosphopeptide antigen expression enhancing therapies with a T cell-based therapy, such as vaccination or adoptive cell therapy, could potentially optimize the ability of the phosphopeptide-specific T cells to target the tumor.

To assess the potential additive or synergistic effect of combination therapy, phosphopeptide-specific recombinant TCR T cells (as described in [3]) could be assessed for their ability to control humanized murine models of melanoma with or without the proposed treatments. The efficacy of tumor control by the adoptive transfer of phosphopeptide-specific T cells would be assessed alone and when combined with the intratumoral injection or delivery of 1) TLR agonists, 2) Influenza vaccination, or 3) oncolytic viral therapy. First, SLM2 cells *in vitro* would be assessed for the changes in MHC-phosphopeptide expression upon treatment with 1) TLR agonists, 2) Influenza vaccination, or 3) oncolytic virus. Next, SLM2 tumors implanted in NOD/SCID/IL-2Rγc$^{-/-}$ (NSG) mice would be treated *in vivo* with one of the three agents or vehicle controls. Then, dilutions of pIRS2-specific T cells would be transferred into the mice as described [3]. To assess the efficacy of such treatments, expression of MHC-phosphopeptides on the tumor cells, activation of innate signaling pathways, and tumor control relative to number of pIRS2-specific T cells would be evaluated. This dual therapy, augmenting both antigen display as well as the T cell response, could potentially enhance anti-tumor responses synergistically.

5.4.5 Future Direction 6: Determine if patients with high pre-disposition to develop cancer have restricted phosphopeptide immunity.

It remains unclear whether limited phosphopeptide immune memory was present in patients prior to cancer development or whether it occurred as a consequence of cancer development. As described in **Chapter 1** (**section 1.5**), some people have genetic conditions that cause a high pre-disposition to develop cancer. This includes patients with Lynch, Li Fraumeni, Familial Adenomatous Polyposis, or PTEN Hamartoma Tumor Syndromes. Patients with high pre-dispositions to develop cancer provide an opportunity to analyze changes in phosphopeptide immunity and expression during the progression from pre- to post-cancer diagnosis. These patients undergo extensive cancer screening, including multiple biopsies of concerning masses. Approximately 20 HLA-A2$^+$ patients with one of these syndromes but without clinically-evident malignancy would be enrolled in a longitudinal study with the expectation that a majority of them will go on to develop cancer. The specimen collection period of this study would be expected to take a number of years for completion, and, therefore, would be most appropriate to be carried out in a lab with a strong clinical focus. Patient specimens (PBMCs and tumors) would be banked long-term to identify the patients who develop cancer and to have side-by-side analyses of benign and malignant tumors and responses pre- and post-cancer diagnosis.

First, to test the hypothesis that patients have a basic ability to express MHC-phosphopeptides, EBV-transformed B cells from each patient would undergo mass spectrometric analysis, as described for healthy donors in **Future Direction 1**. If there are negligible MHC-phosphopeptides expressed on the cell surface, this would suggest that processing and/or presentation of phosphopeptides on MHC molecules is impaired. The

patient's benign and malignant tumors would undergo mass spectrometric analyses to identify their MHC-phosphopeptide repertoires. High-risk patients have genes variants that dysregulate normal kinase or phosphatase cell signaling pathways. For this reason, one hypothesis to test is that benign masses in high-risk patients express an abundance of MHC-phosphopeptides, similar to levels seen on malignant tumors, including some of the same phosphopeptides. This analysis would demonstrate the similarity or the changes of the MHC-phosphopeptide repertoire before and after the transition to malignancy in the same patient. If possible, analyses of multiple masses or at the least multiple biopsy samples from the same mass would provide evidence of the highly conserved phosphopeptides as well as the breadth of phosphopeptides expressed.

Phosphopeptides unique to the malignant tumor and not seen on the same patient's benign tumors may reflect pathway dysregulations that are contributing to the cell's malignancy. Identifying phosphopeptides that are unique to malignancy may provide opportunities to explore cell signaling changes that resulted in malignant transformation. These studies could start with western blotting and immunohistochemistry of suspected or known signaling pathways in which the phosphorylated protein becomes generated. The pathways explored could be based on existing literature or on results from the proposed kinase/phosphatase identification study. Over-expression or inhibition via small molecule inhibitors or siRNA will identify the components of the dysregulated pathway. Identifying the cell signaling pathways altered during the transition from the pre-malignant to malignant stages could potentially identify intracellular or cell receptor targets that could be inhibited therapeutically to stall progression in the patient to malignancy.

The next question to address is whether T cell responses can be generated against the HLA-A2-restricted phosphopeptides displayed on the patients' benign and/or malignant tumors. If the identified phosphopeptides were not analyzed in this current study, then a cohort of at least 10 HLA-A2^{+} healthy donors would be assessed for T cell responses. If responses are not observed in any of the healthy donors, naïve T cells should be isolated from the healthy donors and *de novo* responses generated to ensure that T cells at least from some people can recognize the phosphopeptide.

If T cell responses can be generated, responses will be analyzed in the patients. Direct *ex vivo* and one time *in vitro* stimulated effector and memory T cell responses to immunodominant phosphopeptides and to phosphopeptides expressed on their masses would be assessed from PBMCs and TILs collected before and after clinically-evident malignancy. One possible outcome is that patients, both pre- and post-cancer diagnosis, have limited responses to the immunodominant and tumor-expressed phosphopeptides compared to healthy donors. This result would be most consistent with our hypothesis that phosphopeptide-specific T cell memory and effector responses had not developed in these patients. If this is observed, one possible explanation would be that immunogenic exposures giving rise to phosphopeptides had not occurred in the donor. This is possible even though the phosphopeptide was identified as being expressed on their tumor. Expression of a phosphopeptide on tumor cells may not be sufficient to generate a T cell response, if, for example, their DCs are unable to cross-present tumor-expressed phosphopeptides, as described in **Future Direction 4**. It is also possible that generation of a T cell response in the patient was impeded due to a hole in the TCR repertoire, which would be tested by attempting to generate responses from the patient's naïve T cells, as

described in **Future Direction 1**. Multimer detection of phosphopeptide-specific T cells would be vital to assess whether there are dysfunctional – exhausted or tolerized – T cells to tumor-expressed phosphopeptides.

There are two last possible outcomes, although we believe these less likely given the findings of this and a previous study [2]. The first possibility is that patients have enhanced phosphopeptide immunity pre-diagnosis but it is lost after the diagnosis of cancer, both in terms of the robustness of the responses and the breadth of phosphopeptides recognized. If observed, this would suggest that the loss of phosphopeptide immunity was due to tumor-induced mechanisms of exhaustion, suppression, or induced tolerance. Since the restrictions in the current study included responses to non-tumor-expressed immunodominant phosphopeptides, we believe it is unlikely to be a tumor-specific loss of responsiveness. The second possible outcome is that patients have more phosphopeptide immunity after their cancer diagnosis than they had before. If observed, this would suggest that there was limited expression of phosphopeptides on their benign tumors or that the expression of phosphopeptides on their benign tumors was insufficient to induce T cell responses, in contrast to their enhanced immunogenicity on malignant tumors. If observed, this last result would raise additional questions about the distinctions between the tumor microenvironments in these patients compared to those in patients with ovarian cancer since we observed negligible responses from patients.

Future clinical trials could assess the effect that induced or augmented phosphopeptide responses (by vaccination, for example) in patients with benign masses could protect from the transition to malignancy.

5.5 Figures

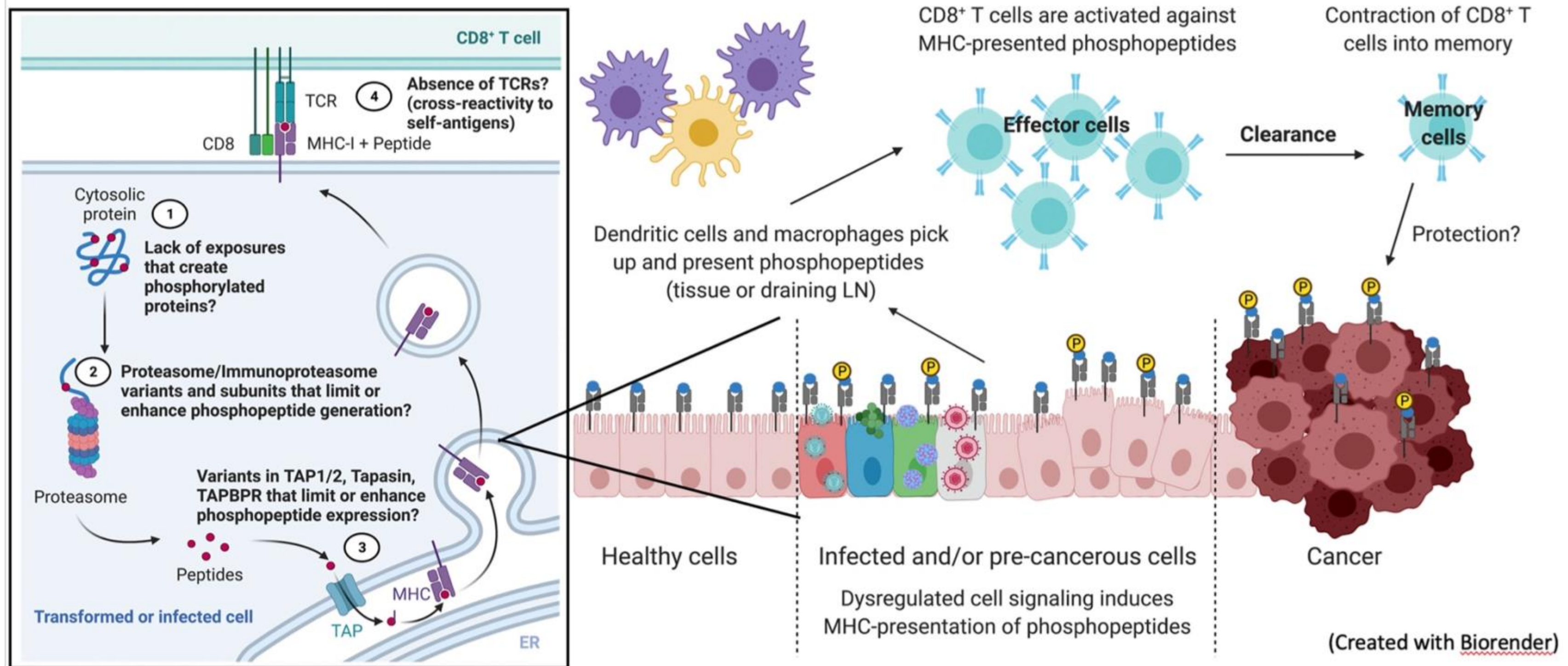


Figure 5.1. Working Model. 1) Otherwise healthy individuals are exposed to MHC-phosphopeptides upon infection and/or clearance of incipient cancer cells due to dysregulated signaling involving kinases and/or phosphatases. 2) Professional antigen presenting cells present the phosphopeptides to and activate 2) $CD8^+$ T cells. 3) Upon clearance of the insult, the $CD8^+$ T cells contract into memory cells, which 5) may be protective against the development of cancer. Inset: Four variables may inhibit the development of phosphopeptide immunity, including 1) the absence of exposures that give rise to phosphopeptides, 2) variant expression or isoforms of components of the ubiquitin-proteasome or immunoproteasome systems or of 3) the antigen processing and presentation machinery, or 4) holes in the T cell repertoire due to cross-reactivity to self-antigens that resulted in deletion of phosphopeptide-specific T cells.

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