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TITLE: Diacylglycerol Activation of T-Cell Receptor Signaling for Cancer Immunotherapy

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14. ABSTRACT We have accomplished all of our goals and milestones outlined in Year 1 of the statement of work, which has resulted in 3 peer-reviewed publications and 6 conference abstract to disseminate our research findings. As part of our research efforts, 2 of my senior graduate students received vital career development opportunities at prestigious national meetings. Our findings have impacted the chemistry and cancer immunology field by identifying novel druggable sites of diacylglycerol kinases (DGKs), which are exciting new drug targets for immunology in melanoma. We identified a lead fragment molecule (RF001) that will serve as the starting point for developing potent and selective DGK inhibitors. The broader impacts of our findings is to inform the general public on the significance of fat metabolism, which is greatly influenced by food choices in society, in our immune system's ability to fight cancer.						
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1. **INTRODUCTION:** The overall goal of this proposal is to understand how lipid kinase diacylglycerol kinase alpha (DGKA) modulates diacylglycerol lipid signaling pathways to influence T cell function and related disorders. Proper immune response to infection and disease requires that T cells are active and responsive. Activation of T cells occurs by presentation of Ag-peptides to naïve T cells. Ag-specific T cells become activated and can specifically fight foreign material by rapid growth, moving to sites where the Ag is present, and producing molecules that serve as signals of cytotoxicity.

Diacylglycerols (DAGs) play a prominent role in T cell activation by serving as messengers to recruit key proteins involved in signaling pathways that can control T cell function. Termination of DAG signaling is important because it prevents overactive immune response and the onset of autoimmunity. Diacylglycerol kinases (DGKS) are enzymes that prompt the conversion of DAG lipids to phosphatidic acid (PA). DGKA plays a critical role in immunity by regulating DAG signaling events. Strict balance between lipid, DAG, and enzyme, DGKA, is important in the regulation of proper T cell function. Excessive amounts of DGA can result in T cell anergy, where cells become only partially activated and unresponsive. This condition is highly noted in tumor microenvironments. We seek to identify the precise role of DGKA in T cells in order to translate DGK biology into novel therapies.

2. **KEYWORDS:** immunology; immunotherapy; lipid metabolism; T cell; melanoma; tumor; kinase; cancer; diacylglycerol kinase; phosphatidic acid; chemical proteomics

3. **ACCOMPLISHMENTS:**

- **What were the major goals of the project?**

1. To map the ritanserin binding site of DGKA using competitive ABPP and quantitative LC-MS.
2. To evaluate the selectivity of ritanserin against the kinase superfamily.
3. To determine the endogenous substrates and products regulated by DGKA.
4. To test whether ritanserin modulates T cell biology and enhances anti-tumor immunity.

- **What was accomplished under these goals?**

A. Specific Aim 1 - Experiment 1: We identified ritanserin-binding sites of DGKA at the atypical C1 and accessory (DAGKa) sites.

We hypothesized that the serotonin receptor antagonist ritanserin could function as a DGKA inhibitor based on high structural similarity between this compound and established DGKA inhibitors¹. This compound was shown to be safe in human trials and demonstrated no clinically relevant negative effects, providing a potential opportunity for rapid translation of our findings via drug repurposing². To rapidly evaluate compound selectivity in complex lysates, we developed a novel activity-based protein-profiling (ABPP) assay to assess kinase activity. The assay utilizes an ATP acyl-phosphate nucleotide probe (**Figure 1**) to detect recombinant and endogenous kinase activity by binding to the ATP binding pocket resulting in covalent modification of the target kinase. We identified bands corresponding to DGKA (~80kDa) in our ABPP assay, which matches the molecular weights of recombinant proteins detected in our Western blot analysis. To confirm that ritanserin could block DGKA catalytic activity we included DGKA-inactive serotonin receptor antagonist ketanserin as a negative control for our studies. Pretreatment of recombinant DGKA lysates with compounds followed by addition of acyl-phosphate ATP probe resulted in blockade of probe labeling with ritanserin but not ketanserin. Thus, our ABPP assay results match those observed in traditional substrate assays and demonstrates that the acyl-phosphate ATP probe can report authentic DGKA activity.

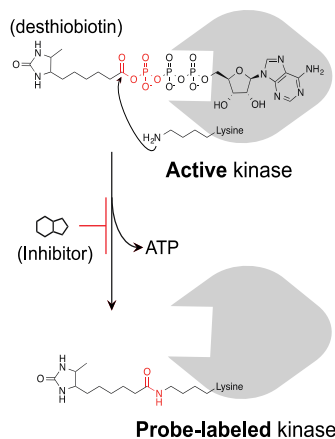


Figure 1. Acyl-phosphate nucleotide probe. The ATP probe binds to the kinase active site and covalently modifies active kinases. Probe labeling is blocked after pretreatment with inhibitor.

Next, we used liquid chromatography-mass spectrometry (LC-MS) to evaluate the site of labeling and selectivity of ritanserin against DGKA. We used stable isotopic labeling of amino acids in cell culture (SILAC) methodology³ to permit quantitation at the peptide level (**Figure 2A**). The binding sites for compounds are readout as light/heavy peptide ratios > 5 ($SR = \text{SILAC Ratio}$), which indicates $> 80\%$ inhibition of probe labeling by respective compound. We identified peptides that were sensitive to ATP and ritanserin competition (**Figure 2B**). Ritanserin inhibits DGKA predominantly through binding interactions at the atypical C1 (K237, $SR = 7.0$) and accessory (DAGKa) sites (K539, $SR = 7.0$) (**Figure 2B**). Surprisingly,

we observed minimal competition at the catalytic (DAGKc) domain (K377, $SR = 2.0$), which helps to explain previous kinetic assays describing a mixed competitive mechanism of inhibition for this inhibitor; ritanserin is hypothesized to bind a DGKA-ATP complex through an unidentified binding site⁴.

B. Specific Aim 1 - Experiment 2: We determined that ritanserin is selective for DGKA among DGK isoforms, but displays kinase off-target activity. We then assessed selectivity of ritanserin against representative DGKs of each subtype (kappa, type 2; epsilon, type 3; zeta, type 4; and theta, type 5) using the methodology described above. Profiling revealed that ritanserin showed minimal activity against other isoforms ($SR < 2$ at all binding sites detected) (**Figure 2B**). Finally, we measured the selectivity of ritanserin against >50 native kinases and found

that the most potent targets of ritanserin were DGKA and the non-receptor tyrosine protein kinase FER ($SR = 7.9$). Our studies demonstrate the use of chemical proteomics to elucidate the binding mode and selectivity of ritanserin, resulting in discovery of the C1 domain as a novel ligand binding site and FER as an unanticipated off-target⁵.

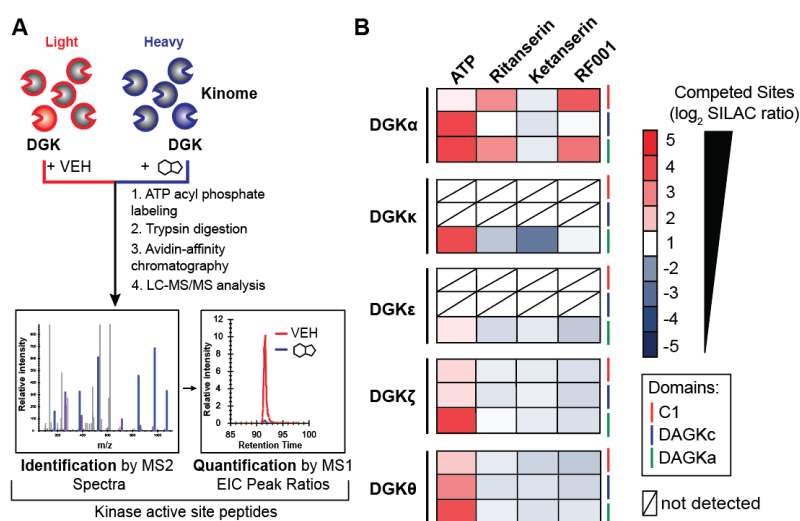


Figure 2. Profiling of the DGK superfamily. (A) Schematic of quantitative LC-MS proteomics workflow to identify ligand binding sites of recombinant DGKs using ATP acyl phosphate probe. (B) Heatmap showing SILAC ratios for probe-binding sites of respective DGK isoforms in ATP- (1 mM), ritanserin- (100 μ M), ketanserin- (100 μ M), or RF001- (1 mM) versus vehicle-treated proteomes. Binding sites are defined by $SR > 5$. ATP interacts with binding sites for all isoforms. Ritanserin and RF001 selectively bind DGKA.

C. Specific Aim 1 - Experiment 2: We used fragment-based inhibitor screening to discover a lead fragment inhibitor of DGKA. In an effort to improve selectivity of ritanserin for DGKA, we explored ligand deconstruction strategies to evaluate the contributions of representative fragments for binding affinity and selectivity⁶⁻⁸. We hypothesized that a

fragment molecule derived from ritanserin (designated RF001)⁵ can serve as a DGKA inhibitor due to conservation of functional groups across several DGKA inhibitors⁹. Treatment of DGKA with RF001 resulted in potent competition at C1 (*SR* = 12.6) and DAGKa sites (*SR* = 9.1) while showing mild activity at the DAGKc site, similar to ritanserin. We also confirmed that RF001 was largely inactive against other DGK subtypes as determined by low SILAC ratios at all detected DGK probe-modified sites (average *SR* ~1). A striking finding from our studies is the dramatic improvement in selectivity against the kinome observed with RF001 compared with ritanserin. Specifically, the potent FER off-target activity observed with ritanserin was largely eliminated using RF001 (*SR* = 1.2). In fact, RF001 showed potent activity (*SR* = 5) against a single kinase target, DGKA, across all detectable kinases (native and recombinant DGKs) quantified in our chemical proteomics studies and, unlike ritanserin, RF001 maintained good selectivity, even for kinase targets that show moderate to weak inhibition.

D. Specific Aim 2 - Experiment 1: We determined that Type 1 DGK isoforms exhibit DAG substrate specificity.

Previous studies indicate that only type 3 DGK-epsilon exhibits substrate specificity¹⁰. We have collected new, unprecedented preliminary data that indicates DAG substrate specificity for type 1 isoforms DGK-alpha (DGKA), -beta (DGKB), and

DAG species	rDGK α		rDGK β		rDGK γ	
	log2 fold change	degree of significance	log2 fold change	degree of significance	log2 fold change	degree of significance
DG(16:0/16:0)	-1.37	ns	-1.08	*	-1.82	***
DG(16:0/16:1)	-1.09	ns	-0.19	ns	-0.46	*
DG(16:0/18:1)	-0.59	ns	-1.07	*	-0.56	ns
DG(16:0/20:1)	-0.70	ns	-1.34	**	-0.58	**
DG(16:0/20:2)	-0.78	ns	-0.75	*	-0.91	**
DG(16:0/20:3)	-0.73	ns	-0.76	ns	-0.77	**
DG(16:0/22:1)	-0.68	ns	-2.03	**	-1.11	**
DG(16:0/22:4)	-0.23	ns	-0.44	ns	-0.64	*
DG(16:0/24:1)	-1.42	**	-3.06	***	-2.25	**
DG(16:1/18:1)	-0.75	ns	-0.62	ns	-0.38	*
DG(18:0/16:0)	-1.28	**	-2.47	**	-1.71	*
DG(18:0/18:0)	-1.05	***	-1.36	*	-1.80	*
DG(18:0/18:1)	-0.47	ns	-1.07	**	-0.23	ns
DG(18:0/18:2)	-0.39	*	-1.26	*	-0.19	ns
DG(18:0/20:2)	0.05	ns	-2.10	*	-0.09	ns
DG(18:1/18:1)	-0.79	*	-0.70	*	-0.90	*
DG(18:1/18:2)	-0.64	ns	-0.72	ns	-0.54	*
DG(18:1/20:1)	-0.77	ns	-0.79	*	-0.85	**
DG(18:1/20:2)	-0.71	ns	-0.81	**	-0.65	*
DG(18:1/20:3)	-0.50	ns	-0.74	*	-0.72	*
DG(18:1/22:1)	-0.63	ns	-1.07	*	-0.50	*
DG(18:1/22:3)	-0.99	ns	-1.51	*	-1.41	*
DG(18:1/22:5)	-0.91	*	0.78	ns	-1.12	*
DG(18:1/22:6)	-0.92	ns	-0.48	ns	-0.73	*
DG(20:1/18:2)	-0.73	*	-0.63	*	-0.80	*
DG(26:1/16:0)	-1.09	ns	-2.20	*	-3.46	**

Table 1. DAG Lipid Profiling of Type 1 DGK isoforms. Type 1 DGK proteins expressed in HEK293T cells for 48h and lipid metabolites were extracted, analyzed, and compared to a non-transfected control. Color coding differentiates DAG lipids regulated by each DGK isoform. Significance calculated using two-tail student's T-test (n=3 biological replicates).

-gamma (DGKG) (**Table 1**). In order to identify lipid species regulated by individual isoforms, DGK protein was transiently expressed in HEK293T cells to drive expression and regulation. The Bligh Dyer method was used to extract lipids in 1:2 CHCl₃/MeOH + 0.1N HCl, an acidified

extraction for improved neutralization and recovery of charged phospholipids¹¹. Neutral DAG lipid species were analyzed by LC-MS in positive mode with ammonium formate to identify DAG-ammonium (M+NH₄) adducts. Relative quantitation of lipids was achieved by two methods: normalization of total lipids to protein concentration and doping in internal lipid standards at known concentrations.

Comparison of the DAGs regulated by type 1 isoforms shows that unique DAG species were significantly decreased upon expression of all three type 1 DGK isoforms (**Table 1**). This is expected as DGKs phosphorylate DAGs to produce PAs. Remarkably, out of 55 detectable DAG species, only five were utilized by all three type 1 DGKS (**Table 1**). More significantly, DGK-beta and -gamma preferentially regulate three and six DAG lipid species each, respectively. There is some overlap between the three type 1 isoforms. DGKA preferentially regulates seven lipids, one shared with DGK-beta, one with DGKG, and five with both. This is the *first data* to suggest that type 1 DGKs impart DAG substrate specificity.

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E. SOW milestones proposed for Year 1 and completion status:

<i>Specific Aim 1: Test our hypothesis that ritanserin binds to a novel allosteric site(s) of DGKA to impart isoform specific inhibition.</i>	Timeline	
Major Task 1: Mapping the ritanserin binding site of DGKA using competitive ABPP and quantitative LC-MS	Months	% completed
Subtask 1: Producing recombinant DGK isoforms (5 isoforms in total: alpha, kappa, epsilon, zeta, and theta plasmids have been validated in Hsu lab) in SILAC HEK293T cells (commercial source: ATCC)	1-2	100%
Milestone(s) Achieved: <i>Generating SILAC DGK-HEK293T cells for activity-based protein profiling (ABPP) studies</i>	2-3	100%
Subtask 2: Preparing cell lysate treated +/- compounds for ABPP studies. Compounds to be tested: ritanserin (DGK inhibitor) and ketanserin (DGK-inactive negative control probe).	2-3	100%
Subtask 3: Treating cell lysates with ATP acyl phosphate probe, enrichment, and preparation for nano LC-MS	2-3	100%
Subtask 4: nanospray LC-MS analysis of samples prepared from ABPP analysis	2-6	100%
Subtask 5: Data analysis of nanospray LC-MS results by IP2 bioinformatics software	6-12	100%

Milestone(s) Achieved: 1) <i>Identification of small molecule binding sites for all 10 DGK isoforms</i> 2) <i>Mapping the ATP-binding pocket for all 10 DGK isoforms</i> 3) <i>Showing that ritanserin binds an allosteric site on DGKA isoform</i>	12	100%
Major Task 2: Evaluating selectivity of ritanserin against kinase superfamily	Months	Key Personnel
Subtask 1: UVA IACUC & USAMRMC ACURO review for all animal studies proposed	1-4	100%
Milestone(s) Achieved: UVA IACUC & USAMRMC ACURO approval	4	100%

- **What opportunities for training and professional development has the project provided?**

Two of my senior graduate students were provided the opportunity to share and present their research via poster presentations at the Spring 2018 255th American Chemical Society National Meeting in New Orleans, LA. Both students were also provided the opportunity to present posters of their research and one student gave an oral presentation at the highly competitive 2018 Bioorganic Gordon Research Seminar and Bioorganic Gordon Research Conference in Andover, NH.

- **How were the results disseminated to communities of interest?**

Results from our studies were disseminated to the chemistry and cancer community in the form of peer-reviewed manuscripts and conference abstracts both at the American Chemical Society, Bioorganic Gordon Research Seminar, and the American Association for Cancer Research. We have also listed our publications on the lab website (hsulab.com), which provides additional opportunities for the local, national, and international general public to see the outcomes from our proposed studies.

- **What do you plan to do during the next reporting period to accomplish the goals?**

We have accomplished all of the goals proposed in Year 1. We plan to exercise the same level of commitment and dedication to accomplish the milestones and goals for Year 2.

4. **IMPACT:**

- **What was the impact on the development of the principal discipline(s) of the project?**

Our studies have impacted the chemistry and cancer immunology field by revealing new druggable sites for lipid kinases that are important targets for immuno-oncology. Our findings will provide a roadmap towards drug discovery by revealing molecular level understanding of which diacylglycerol kinase (DGK) to target in cancers but also the exact position in the active site to direct efforts for development of highly potent and selective drugs.

- **What was the impact on other disciplines?**

Our findings have impacted fields outside of chemistry and cancer immunology by showing the general chemical biology field that lipid kinases are accessible using chemical probes and mass spectrometry. The kinase field has been largely dominated by efforts towards protein kinases. We are paving the way for innovative chemical approaches to tackle lipid kinases.

- **What was the impact on technology transfer?**

We discovered a lead fragment inhibitor RF001 that shows selective blockade of DGKA activity. We believe this lead compound will enable development of drug-like DGKA inhibitors that can ultimately lead to initiation of a start-up company.

- **What was the impact on society beyond science and technology?**

We believe our studies and findings have broader impacts in society by demonstrating the importance of fat metabolism in our immune system and ability to fight cancer. Our basic studies will one day enable a mechanistic connection between healthy food choices and the impact on our immune health and susceptibility to cancers.

5. **CHANGES/PROBLEMS:**

- **Changes in approach and reasons for change**

Nothing to report.

- **Actual or anticipated problems or delays and actions or plans to resolve them**

Nothing to report.

- **Changes that had a significant impact on expenditures**

Nothing to report.

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

Nothing to report.

- **Significant changes in use or care of human subjects**

Not applicable.

- **Significant changes in use or care of vertebrate animals.**

Nothing to report.

- **Significant changes in use of biohazards and/or select agents**

Nothing to report.

6. PRODUCTS:

- **Publications, conference papers, and presentations.**

- **Journal publications.**

1. Campbell ST, Franks CE, Borne AL, Shin M, Zhang L, and Hsu KL. Chemoproteomic discovery of a ritanserin-targeted kinase network mediating apoptotic cell death of lung tumor cells. *Molecular Pharmacology*, (2018). [Accepted, in press](#). Acknowledgement of federal support? Yes.
2. Shin M, Franks CE, and Hsu KL. Isoform-selective activity-based profiling of ERK signaling. [Chemical Science 9, 2419-2431 \(2018\)](#). Published. Acknowledgement of federal support? Yes.
3. McCloud RL, Franks CE, Campbell ST, Purow BW, Harris TE, and Hsu KL. Deconstructing Lipid Kinase Inhibitors by Chemical Proteomics. [Biochemistry 57, 231-236 \(2018\)](#). Published. Acknowledgement of federal support? Yes.

- **Books or other non-periodical, one-time publications.**

Nothing to report.

- **Other publications, conference papers, and presentations.**

1. Franks CE, McCloud RL, Campbell ST, Purow BW, Harris TE, Hsu KL. Quantitative chemical proteomics to evaluate lipid kinase inhibitor binding profiles. Oral presentation delivered at the Bioorganic Chemistry Gordon Research Seminar, Andover, NH, June 2018.
2. Franks CE, McCloud RL, Campbell ST, Purow BW, Harris TE, Hsu KL. Quantitative chemical proteomics to evaluate lipid kinase inhibitor binding

profiles. Poster presentation delivered at the Bioorganic Chemistry Gordon Research Conference, Andover, NH, June 2018.

3. Campbell ST, Franks CE, Borne AL, McCloud RL, Hsu KL. Using Activity-Based Protein Profiling Proteomics to Determine Novel Pathways and Therapeutic Monitoring Targets. Oral presentation delivered at the ACLPS Annual Meeting, Houston, TX, June 2018.
4. Hsu, KL. Targeting diacylglycerol kinases for immuno-oncology. Poster presentation delivered at the American Association for Cancer Research Annual Meeting, Chicago, IL, April 2018.
5. Franks CE, Campbell ST, Purow BW, Harris TE, Hsu KL. Development of a chemical proteomic strategy to target diacylglycerol kinases. Poster presentation delivered at the 255th ACS National Meeting, New Orleans, LA, March 2018.
6. Campbell ST, Franks CE, Borne AL, McCloud RL, Hsu KL. Using Activity-Based Protein Profiling Proteomics to Determine Novel Pathways and Therapeutic Monitoring Targets. Poster presentation delivered at the MSACL - Annual Congress in Clinical Mass Spectrometry, Palm Springs, CA, January 2018.

- **Website(s) or other Internet site(s)**

1. <https://www.youtube.com/watch?v=nJULdCGgduc&feature=youtu.be>

- **Technologies or techniques**

Nothing to report.

- **Inventions, patent applications, and/or licenses**

Nothing to report.

- **Other Products**

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

Name:	<i>Ku-Lung Hsu, PhD</i>
Project Role:	<i>PI</i>
Researcher Identifier (e.g. ORCID ID):	<i>0000-0001-5620-3972</i>

Nearest person month worked:	4
Contribution to Project:	<i>Prof. Hsu is responsible for planning and direction of the project, for data analyses, and for scientific reports and publications. He is also responsible for training and mentoring of graduate students and postdoctoral fellows.</i>
Funding Support:	

Name:	<i>Caroline Franks</i>
Project Role:	<i>Graduate Student</i>
Researcher Identifier (e.g. ORCID ID):	<i>0000-0003-1728-1526</i>
Nearest person month worked:	4
Contribution to Project:	<i>Ms. Franks performed work in the area of chemical proteomics and mass spectrometry experimentation, and data analysis.</i>
Funding Support:	<i>The National Institute of General Medical Sciences (training grant)</i>

Name:	<i>Sean Campbell, PhD</i>
Project Role:	<i>Postdoctoral Fellow</i>
Researcher Identifier (e.g. ORCID ID):	<i>0000-0001-5465-5107</i>
Nearest person month worked:	1
Contribution to Project:	<i>Dr. Campbell was responsible for executing standard molecular biology, biochemistry, cell biology, and pharmacology experiments.</i>
Funding Support:	<i>The National Cancer Institute (training grant)</i>

Name:	<i>Jeffrey Brulet, PhD</i>
Project Role:	<i>Graduate Student</i>
Researcher Identifier (e.g. ORCID ID):	<i>0000-0003-1959-2475</i>
Nearest person month worked:	1
Contribution to Project:	<i>Mr. Brulet performed work in the area of design, synthesis, and evaluation of small molecules.</i>

Funding Support:	
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Name:	<i>Tao Huang, PhD</i>
Project Role:	<i>Postdoctoral Fellow</i>
Researcher Identifier (e.g. ORCID ID):	0000-0002-6147-0490
Nearest person month worked:	6
Contribution to Project:	<i>Mr. Huang performed work in the area of design, synthesis, and evaluation of small molecules.</i>
Funding Support:	

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Ku-Lung Hsu, PI

The Current Support submitted by Dr. Hsu in March 2017 is shown below. Changes and additions are in italics.

Current Support

Project Title: Functional Characterization of Diacylglycerol Lipases in Mammalian Physiology

Time Commitment: 60% effort

Supporting Agency: National Institute on Drug Abuse

Award Number: 5R00DA035864-03

Funding Agency's Contracting/Grants Officer: Debra Battle-Dudley, ddudley@nida.nih.gov

Performance Period: 07/01/2015-06/30/2018

Funding Amount: \$746,840 (total costs for 3 years)

Specific Aims: The Specific Aims of this application are to integrate genetic mouse models with novel chemical probes to functionally uncouple the central and peripheral functions of the endocannabinoid system in obesity-associated metabolic disorders.

Overlap with DoD Project: no overlap

This award ended 6/30/2018

Project Title: Endocannabinoid Biosynthesis in Inflammation and Pain

Time Commitment: 8.3%

Supporting Agency: National Institutes of Health

Performance Period: 09/15/2017-06/30/2022

Funding Amount: \$1,952,939

Specific Aims: The major goals of this project are to test whether DAGL β -mediated anti-inflammatory effects are mediated by autocrine lipid signaling pathways in macrophages to reduce local inflammatory responses in vivo.

Overlap with DoD Project: no overlap

This award was previously reported as pending. It was awarded with a slight change in start date and funding amount from that previously reported.

Project Title: Manipulating Cellular Metabolism to Promote Cancer Immunity in Melanoma

Time Commitment: 4.17% effort

Supporting Agency: Melanoma Research Alliance

Performance Period: 06/01/2018-05/31/2021

Funding Amount: \$225,000

Specific Aims: Identify serine hydrolase activities in tumor infiltrating immune cells and develop selective inhibitors to inactivate these potential therapeutic targets.

Overlap with DoD Project: no overlap

A prior submission of this proposal was previously reported as pending. A resubmission was awarded, with the period of performance and level of effort noted above.

Donald Hunt, PhD, Mentor

The current support submitted by Donald Hunt, PhD, in March 2017 is shown below.

Changes are noted in italics.

Current Support

Project Title: Protein Sequencing by Tandem Mass Spectroscopy

Time Commitment: 40% effort

Supporting Agency: National Institute of General Medical Science

Award Number: 2R01GM037537-29

Funding Agency's Contracting/Grants Officer: Dina Wilson, Dina.Wilson@nih.gov

Performance Period: 01/12/1987-08/31/2019

Funding Amount: \$474,246 (total direct costs, Year 29)

Project Goals: The goal of this research is to develop automated mass spectrometric methods for the rapid characterization of known and unknown proteins at the attomole level in complex mixtures.

Specific Aims:

- 1) Continue the development of mass spectrometry instrumentation and methods that will facilitate both identification and near complete amino acid sequence analysis of intact proteins plus characterization of their post-translational modifications on a chromatography time-scale.
- 2) Continue the development of micro-column, enzyme reactors for time limited digestion of membrane proteins and protein mixtures to generate 5 to 10 kDa fragments that can be fully characterized by FETD and IIP MS on a chromatographic time scale. Here we will explore the utility of additional immobilized enzymes, Lys-N, Lys-C, and OmpT, as well as detergents, lipids, and organic solvents that will allow us to characterize mammalian membrane proteins, routinely.
3. Continue research to characterize posttranslational modifications that constitute the histone code and the proteins that write, read, and erase them and thus underpin the whole field of epigenetics. Here we will undertake a comprehensive effort to characterize regulatory proteolytic cleavage events on histones, H4, H1, Cenp-A, three isoforms of H3, and multiple isoforms of H2A and H2B in a variety of cell types under different conditions.

4. Develop boronic acid chemistry in non-aqueous solvents for the enrichment and characterization of O- GlcNAcylated proteins and continue to characterize mammalian proteins modified by O-GlcNAcylation. Targets of this approach will be ribosomal proteins, heart-muscle mitochondrial proteins and recombinant forms of all 518 human kinases.

5. Identify and characterize binding partners of RISK 1. Proposed here are separate experiments in which RSK 1 is covalently linked to either a peroxidase or a promiscuous biotin ligase. Both enzymes tag interacting proteins with biotin. Labeled proteins are then pulled-down with streptavidin beads. We will use the new mass technology discussed above to identify the interacting proteins and characterize their posttranslational modifications.

Overlap with Awarded Project: no overlap

Project Title: Processed Antigen Characterization by Mass Spectrometry

Time Commitment: 30% effort

Supporting Agency: National Institute of Allergy and Infectious Diseases

Award Number: 5R01AI033993-22

Funding Agency's Contracting/Grants Officer: Jorge Machuca, Jorge.machuca@nih.gov

Performance Period: 09/20/1999- 11/31/2018

Funding Amount: \$409,888 (total direct costs, year 22)

Project Goals: The goal of this research is to identify posttranslationally modified peptides that are produced by dysregulated cell signaling pathways in tumors and presented to the immune system by Class I and Class II MHC molecules. These peptides should be excellent candidates for use in immunotherapy of cancer.

Specific Aims:

1. Complete research on melanoma by using the newly developed ELISpot assay to screen healthy donors for T-cell recall responses to the tumor specific HLA A*0101, HLA A*0301, HLA B*4402, and HLA B*0702 phosphopeptides described in the Progress Report.
2. Complete research on leukemia by analyzing class I MHC phosphopeptides presented uniquely by HLA A*0101, HLA A*0301, and HLA B*4402 alleles on chronic lymphocytic leukemia (CLL), acute myelogenous leukemia (AML), and acute lymphocytic leukemia (ALL).
3. Characterize class II MHC phosphopeptides presented uniquely by CLL, AML, and ALL.
4. Complete research on colorectal cancer by identifying class I MHC phosphopeptides presented on the remaining four major class I MHC molecules, HLA A*0201, HLA A*0101, HLA A*0301, and HLA B*4402.
5. Initiate research to identify class I MHC phosphopeptides on esophageal, liver and ovarian cancer.
6. Identify O-GlcNAcylated class I and class II peptides from all of the above tumor samples.
7. Identify class I HLA-E peptides that are; (a) derived from HIV proteins, (b) recognized by human CD8+ T- cells, and (c) allow a subset of the human population, elite controllers, to remain disease free following infection with the virus.
8. Characterize prescription drug- or environmental agent-induced, altered self-peptide repertoires that are presented by class I and/or class II MHC molecules and induce life-threatening autoimmune disease.

9. Develop novel mass spectrometry technology that will facilitate near-complete, amino-acid sequence coverage of intact therapeutic antibodies and other proteins at the high femtomole level on a chromatographic time scale.

This award is in a no-cost extension period that ends 11/30/2018. It will not be renewed.

Tim Bullock, PhD, Collaborator

The Current Support submitted by Dr. Bullock in March 2017 is shown below. Changes and additions are in italics

Current Support

Project Title: BLIMP-1 mediated regulation of CD8+ TIL

Time Commitment: 25% effort

Supporting Agency: National Cancer Institute

Award Number: R01CA166458-01A1

Funding Agency's Contracting/Grants Officer: Susan McCarthy mccarths@mail.nih.gov

Performance Period: 01/01/2013-12/31/2017

Funding Amount: \$1,250,000 (total direct costs for 5 years)

Project Goals: The goals of this project are to understand how the transcriptional regulator, BLIMP1, is induced in CD8+ TIL, the processes which are regulated upon induction, and approaches that abrogate its effect on T cell function in tumors.

Overlap with DoD Project: No overlap with the proposed project

This award has ended

Project Title: Bioenergetics of CD8+ TIL

Time Commitment: 30% effort

Supporting Agency: National Cancer Institute

Award Number: R01CA166458-01 S3

Funding Agency's Contracting/Grants Officer: Susan McCarthy mccarths@mail.nih.gov

Performance Period: 05/01/2015-12/31/2017

Funding Amount: \$750,000 (total direct for 3 years)

Project Goals: Ascertain basis of metabolic dysfunction of TIL

Specific Aims: Determine mechanisms to augment TIL glycolysis; define approaches to re-functionalize mitochondria in TIL

Overlap with DoD Project: None

This award has ended

Project Title: Immunotherapeutic nanoparticle delivery to melanoma with MR-guided Focused Ultrasound

Time Commitment: 15% effort (Bullock Co-Principal Investigator)

Supporting Agency: National Cancer Institute

Award Number: 1R01CA197111-01

Funding Agency's Contracting/Grants Officer: TANDON, PUSHPA
tandonp@mail.nih.gov

Performance Period: 04/01/2015-03/31/2020

Funding Amount: \$2,685,000 Z(total direct for 5 years)

Project Goals: Incorporate FUS into tumor immunotherapy

Specific Aims: Ascertain dynamics of FUS necessary to open blood-tumor barrier in brain; define whether FUS-mediated knockdown of STAT3 in TME can increase T cell

function; determine whether expression of influenza M1 protein by FUS-mediated transfection augments immunity to brain metastases.

Overlap with DoD Project: None

No change

Project Title: Deployment and development of Stereotactic Body Radiation Therapy and Immunostimulation to Augment T cell responses to Prostate Cancer

Time Commitment: 10% (Bullock Co-Investigator)

Supporting Agency: Chartis Initiative Team Award

Funding Agency's Contracting/Grants Officer: UVA School of Medicine.

Performance Period: 10/01/2013-09/30/2016

Funding Amount: \$500,000 (total direct costs for 3 years)

Specific Aims: To determine whether inclusion of irradiation augments immunity to prostate cancer. To determine whether radiation and CD27 stimulation or checkpoint blockade synergize; to define whether new or pre-existing immune responses are enhanced by radiation.

Overlap with DoD Project: None

This award has ended

Project Title: Enhancing immune therapy for brain metastases with focused ultrasound

Time Commitment: 10% effort

Supporting Agency: Melanoma Research Alliance

Funding Agency's Contracting/Grants Officer: Kristen Mueller, 1101 New York Ave. NW, Suite 620 Washington DC 20005. kmuller@curemelanoma.org

Performance Period: 05/15/2016-05/14/2019

Funding Amount: \$335,000 (total direct costs for 3 years)

Project Goals: Develop FUS for immunotherapy of melanoma brain metastases

Specific Aims: Determine parameters of FUS that augment antibody transport across BB and BT barriers; define whether FUS induces pro- or anti-inflammatory immune responses in brain mets; combine FUS with anti-tumor immunotherapy

Overlap with DoD Project: None

Project Title: Assessment of arginase inhibition for tumor immunity in NSCLC

Title Commitment: 2% effort (Principal Investigator)

Supporting Agency: Calithera

Award Number: NAFunding Agency's Contracting /Grants Officer: Susan Demos
sdemo@calithera.com

Performance Period: 8/1/2016-7/31/2018

Funding Amount: \$19,500

Project Goals: Ascertain the contribution of arginase production by MDSC to immune dysfunction in NSCLC.

Specific Aims: To characterize alterations in frequency and function of effector cells in murine models of NSCLC by co-targeting arginase and PD-1; To determine whether in situ arginase inhibition augments the effector activity of human CD8, CD4 T cell and NK cells in human NSCLC resections and cooperates with PD-1 blockade; To ascertain the impact of arginase inhibition with and without PD-1 blockade on the expansion and survival of human tumor-derived T cells.

Overlap with DoD Project: None

This award has ended

Project Title: Determination of immunogenicity of high intensity focused ultrasound in breast cancer

Time Commitment: 2% effort (Principal Investigator)

Supporting Agency: Theraclion

Award Number: NA

Funding Agency's Contracting/Grants Officer: Bernard Pau bernard.pau4@orange.fr

Performance Period: 5/9/2016-5/8/2017

Funding Amount: \$ 20,540.00

Project Goals: To develop mouse models of Focused Ultrasound treatment of breast cancer using a mouse-adapted Theraclion ultrasound unit to understand the immunologic effects of a treatment regimen currently being tested in human breast adenocarcinoma

Specific Aims: To quantify baseline and Ultrasound-induced levels of immune cells and checkpoint ligand expression in a 4T1-HA model of triple negative breast cancer; to understand how the tumor microenvironment is affected with respect to cytokine and chemokine production after ultrasound; to consider the effect for repeat treatments and alterations in duration and intensity of ultrasound treatment

Overlap with DOD Project: none

This award has ended

Project Title: Immunogenicity of focused ultrasound in GBM consortium

Time Commitment: 10% effort (Bullock Co-Principal Investigator)

Supporting Agency: Focused Ultrasound Foundation

Award Number: NA

Funding Agency's Contracting/Grants Officer: Matthew Eames/Director Extramural Research Focused Ultrasound Foundation. meames@fusfoundation.org

Performance Period: 8/1/2016-7/31/2019

Funding Amount: \$62,463

Project Goals: To determine the ability of focused ultrasound and microbubbles to constrain GBM outgrowth in a murine model.

Specific Aims: Measure the impact of inducing inertial cavitation on the growth of GBM; define whether FUS-induced opening of blood-tumor-barrier increases efficacy of anti-PD1 and anti-TIGIT antibodies.

Overlap with DOD Project: none

No-cost extension

Project Title: Ultrasound Immune Responses in Breast Cancer

Time Commitment: 0% effort (Bullock Co-Principle Investigator)

Supporting Agency: Focused Ultrasound Foundation

Award Number: NA

Funding Agency's Contracting/Grants Officer: Matthew Eames/Director Extramural Research Focused Ultrasound Foundation. meames@fusfoundation.org

Performance Period: 12/1/2016-12/1/2017

Funding Amount: \$97,832

Project Goals: To define how different ultrasound modalities influence the immune landscape in breast cancer using mouse models.

Specific Aims: To define the ability of hyperthermia, thermal ablation and histotripsy on the innate and adaptive immune response in the 4T1-HA model to select optimal treatment parameters; to perform RNA seq analysis in acute and later timepoints in tumors that respond or don't respond durably to ultrasound treatment.

This award has ended

New Current Support

Project Title: Endocannabinoid Biosynthesis in Inflammation and Pain (Hsu, K., PI)

Time Commitment: 2.0%

Supporting Agency: National Institutes of Health

Performance Period: 09/15/2017-06/30/2022

Funding Amount: \$1,952,939

Specific Aims: The major goals of this project are to test whether DAGL β -mediated anti-inflammatory effects are mediated by autocrine lipid signaling pathways in macrophages to reduce local inflammatory responses in vivo.

Overlap with DoD Project: no overlap

Craig Slingsluff, M.D.

The Current Support submitted by Dr. Bullock in March 2017 is shown below. Changes and additions are in italics.

Current Support

Project Title: Analysis of the kinetics and effects of vemurafenib on intratumoral and host immunity in patients with BRAF^{V600E} melanoma: implications for combined BRAF targeted therapy and immunotherapy (M. Atkins, PI)

Time commitment: Slingsluff, Co-I, 0.6 mos

Supporting Agency: Melanoma Research Alliance

Performance Period: 05/01/2012-04/30/2017

Funding amount: \$125,000 last year; now in no-cost extension

Project Goals: Co-investigator on clinical trial, at 3 clinical centers to evaluate changes in the tumor microenvironment after BRAF inhibitor therapy

Overlap with Proposed Project: none

This award has ended

Project Title: Melanoma vaccine for helper T cells combined with targeted or immune therapies

Time Commitment: PI, 1.6 months

Supporting Agency: NIH National Cancer Institute; R01 CA178846

Award Number: R01CA178846

Funding Agency's Grants Officer: Kimery Griffin, griffink3@mail.nih.gov

Performance Period: 07/01/2013-06/30/2019

Funding Amount: \$259,219 direct costs/year

Project Goals: Clinical trials with a helper peptide vaccine for melanoma plus TLR agonist adjuvants, BRAF inhibitor, and CTLA4 blockade.

Overlap with Proposed Project: none

No-cost extension

Project Title: Promising biomarkers for Immune signatures and response to pembrolizumab

Time Commitment: PI, 0.48 mos

Supporting Agency: Merck Sharp & Dohme

Award Number: Merck 52602

Performance Period: 03/14/2016-03/13/2018 (ongoing)

Funding amount: \$202,520 direct over two years

Project Aims: Aims: to test these hypotheses: (1) The following measures of Th1 and CD8 T cell immune infiltration of melanoma metastases and non-small-cell lung cancers (NSCLC), evaluable by immunohistologic evaluation of formalin-fixed tumor biopsies, will be positive predictors of clinical response to pembrolizumab and will complement PD-L1 assessment: [CD8+ T cells expressing retention integrins ($\alpha 1\beta 1$, $\alpha 2\beta 1$, or $\alpha E\beta 7$), Th1 transcription factor expression, • chemokines CXCL9-11, immunotype, tertiary lymphoid structures), and (2) Patients with tumors expressing high levels of barrier molecules (filaggrin (FLG), dystonin (DST, bullous pemphigoid antigen 1), and/or tumor-associated calcium signal transduction 2 (TACSTD2)) will likely fail to respond to pembrolizumab.

Overlap with Proposed Project: none

Project Title: Retention integrins: induction and function on cancer-reactive T lymphocytes

Time Commitment: PI, 0.6 mos

Supporting Agency: Cancer Research Institute (Clinic and Laboratory Integration Program (CLIP))

Performance Period: 07/01/2015-06/30/2017

Funding Amount: \$100,000 direct costs/yr

Project Aims: Aim 1. To determine the roles of cytokines, T-cell receptor (TCR) stimulation, and ligand binding on expression of retention integrins (RI) $\alpha 1\beta 1$, $\alpha 2\beta 1$, and $\alpha E\beta 7$ by human T cells.

Aim 2. To assess function of RI-expressing T cells induced in vitro or isolated from melanoma patients.

Aim 3. To obtain preliminary data on whether RI expression by CD4+ or CD8+ T cells infiltrating melanoma metastases or non-small cell lung cancers may have prognostic or predictive value.

Overlap with Proposed Project: none

This award has ended

Project Title: Designs for Phase I trials in heterogeneous groups (M. Conway, PI)

Time Commitment: Slingluff, Co-I, 0.3 mos

Supporting Agency: NIH National Cancer Institute

Award Number: R01CA142859-04A1

Performance Period: 05/01/2014-04/30/2018

Funding Amount: \$213,739 direct costs/year

Project Goals: The goal of this project is to develop and test statistical designs for dose-finding trials conducted in heterogeneous groups of patients

Overlap with Proposed Project: none
This award has ended

Project Title: Member Status in the Cancer Immunotherapy Trials Network (CITN)
Time Commitment: Slingluff, Program Director at UVA (no effort specified)
Supporting Agency: NIH National Cancer Institute
Award Number: U01CA154967
Performance Period: 09/22/2010-08/31/2016
Funding Amount: Funding to be provided based on enrollment in CITN sponsored trials
Overlap with Proposed Project: none
This award has ended

Project Title: University of Virginia Cancer Center Support Grant
Time Commitment: Slingluff co-leader of Cancer-Immunology/Immunotherapy program, 0.6 mos
Supporting Agency: NIH National Cancer Institute
Award Number: P30CA44579
Funding Amount: \$1,466,236
Performance Period: 02/01/2012-01/31/2022
Overlap with Proposed Project: none

Project Title: Deployment and development of Stereotactic Body Radiation Therapy and immune-stimulation to augment T cell responses to prostate cancer (J. Lerner, PI)
Time Commitment: Slingluff, Co-I, 0.3 mos
Supporting Agency: University of Virginia Health System (internal award)
Performance Period: 10/01/2014-9/30/2017
Project Goals: Co-investigator on clinical trial of CD27 antibody therapy for prostate cancer, and oversight of correlative studies
Overlap with Proposed Project: none
This award has ended

Project Title: Immunity to MHC-restricted phosphopeptides in healthy donors and cancer patients (V. Engelhard, PI)
Time Commitment: Co-Investigator, 0.24 mos
Supporting Agency: NIH National Cancer Institute
Award Number: R01 CA190665
Performance Period: 09/01/2014-09/30/2019
Funding Amount: \$327,936 direct costs/year
Project Goals: This project will evaluate spontaneous T-cell reactivity to phosphopeptides in melanoma, other solid tumors, and hematologic malignancies, to understand their prognostic value and biologic relevance.
Overlap with Proposed Project: none

Project Title: Immunotherapeutic Targeting Cell Surface Neoantigen SAS1B (Ovastacin, ASTL)

Time Commitment:0.6 mos

Supporting Agency: Cancer Research Institute (Clinical Strategy Team Grant)

Performance Period: 07/01/2015-06/30/2017 (incl 1 yr. no-cost extension)

Funding Amount: \$500,000 direct costs

Project Goals: Aims: (1) Develop and qualify murine monoclonal antibodies targeted to SAS1B epitopes exposed and accessible to antibody binding at the tumor cell surface [ectodomains], conjugate these antibodies with drugs and radioactive nuclides, and test these immunotherapeutic reagents for imaging and therapeutic properties. (2) Re-engineer high affinity monoclonal antibodies to SAS1B ectodomains as ScFv minigenes, purified ScFv peptides, and tested their antigen specific binding by a variety of immunochemical methods. (3) SAS1B peptide vaccines will be synthesized as long and short peptide immunogens and T-cell responses will be evaluated for efficacy and safety in HLA-A2 transgenic mice.

Overlap with Proposed Project: none

This award has ended

Project Title: Clinical trial of peptide vaccine plus CD27 agonistic antibody

Time Commitment: PI, 0.6 calendar months

Supporting Agency: Celldex Therapeutics, Inc.

Performance Period: 03/01/2017-02/29/2020

Funding Amount: \$300,000 direct costs total

Project Goals: clinical trial

Overlap with Proposed Project: none

Project Title: A randomized multicenter phase IB/II study to assess the safety and the immunological effect of chemoradiation therapy (CRT) in combination with MK-3475 (anti-PD1) to CTR alone in patients with resectable or borderline resectable pancreatic cancer (NCT02305186)

Time Commitment: 0.6 calendar months

Supporting Agency: Merck

Award Number: MISP 51527

Performance Period: 11/02/2016-

Funding Amount: approximately \$700,000 direct costs total

Project Goals: This funds a multicenter clinical trial in pancreatic cancer. Dr Slingluff is the PI and IND-holder. The trial and grant were initiated in late 2014, led by Dr. Osama Rahma. He remains in a leadership role after moving to the Dana Farber Cancer Institute. Dr Slingluff became PI in late 2016.

Overlap with Proposed Project: none

Project Title: Pilot evaluation of safety, immunological effect, and therapeutic impact of focused ultrasound ablation of advanced solid tumors alone and in combination with PD-1 antibody blockade (Dept. of Radiation Oncology Grant Program)

Time Commitment: 0.6 calendar months

Supporting Agency: University of Virginia Dept. of Radiation Oncology

Performance Period: 11/01/2016-11/30/2018

Funding Amount: \$500,000 total direct costs over 2 years

Project Goals: Clinical trial of focused ultrasound ablation of solid tumors with or without PD-1 blockade

Overlap with Proposed Project: none

Project Title: DOD – Idea Award (Andarawewa, PI)

Time Commitment: 0.6 calendar months

Supporting Agency: Department of Defense

Performance Period: 9/01/2015-08/31/2017

Funding Amount: \$200,000 direct costs/year x 2 years

Project Goals: Preclinical studies of therapeutic use of ultrasound mediated immune responses for melanoma

Overlap with Proposed Project: none

This award has ended

Project Title: Postdoctoral Training Grant for MDs in Surgical Oncology Research

Time Commitment: 0.6 months

Supporting Agency: NIH NCI

Award Number: T32 CA163177

Funding Agency's Grants Officer: Sharon Richards, sharon.richards@nih.gov

Performance Period: 09/01/2011-08/31/2022

Funding Amount: \$266,240 direct costs/year

Project Goals: Postdoctoral training grant

Overlap with Proposed Project: none

Project Title: An Open-Label, Randomized, Phase 2 Study of Nivolumab Given Sequentially with Ipilimumab in Subjects with Advanced or Metastatic Disease

Supporting Agency: Bristol-Myers Squibb Company

Award Number: CA209-064-005

Performance Period: 06/25/2013-06/25/2017

Funding Amount: \$35,083 total costs

Project Goals: Clinical Trial

Overlap with Proposed Project: none

This award has ended

New Current Support

Project Title: Antibodies to melanoma vaccine peptides

Time Commitment: 0.48 calendar months

Supporting Agency: NIH NCI

Application Number: R03 CA219715

Performance Period: 07/01/2017-06/30/2019

Funding Amount: \$50,000/year x 2 years

Project Goals: This project is for analysis of antibodies induced to peptides in melanoma vaccines, to understand how those antibodies may modulate T cell immunogenicity.

Overlap with Proposed Project: none

This award was listed as pending in the March 2017 submission. It is now active.

Project Title: Barrier Molecules and their Impact on T cell Infiltration in Melanoma

Time Commitment: 0.6 calendar months

Supporting Agency: Cancer Research Institute Clinic and Laboratory Integration Program

Performance Period: 07/01/2017-06/30/2019

Funding Amount: \$100,000/year x 2 years

Project Aims: 1. To test whether barrier molecule gene (BMG) expression can be modulated in human melanoma cells by transcription factors involved in regulation of epidermal differentiation.

2. To test whether overexpression of barrier molecule genes inhibits immune cell migration and function or improves human melanoma cell survival in novel 3-D multilayered co-cultures of human melanoma cells with fibroblasts and immune cells.

3. To test whether overexpression of barrier molecule genes interferes with immune cell infiltration and immune signatures within murine melanomas and to assess how this impacts tumor progression and survival.

Overlap with Proposed Project: none

This award was listed as pending in the March 2017 submission. It is now active.

Project Title: Correlative immune studies of blood and tumor tissue for LUD2014-011 clinical trial (intratumoral tremelimumab plus TLR3 agonist, combined with systemic PD-L1 blockade (durvalumab) in patients with advanced cancers accessible for injection and biopsy.

Time Commitment: 0.6 calendar months

Supporting Agency: Ludwig Institute for Cancer Research

Award Number: LUD2014-011 Trial

Performance Period: 4/13/2017-4/13/2022

Funding Amount: \$749,989 total direct costs

Project Title: Immune stimulating nanoparticles containing 6MHP (melanoma helper peptides) for targeted peptide vaccines.

Time Commitment: 5% effort

Supporting Agency: University of Virginia Engineering in Medicine Pilot Program

Performance Period: 7/1/2018-6/30/2019; matching funds of \$199,570 from Center for Innovative Technology-Commonwealth Research Commercialization Fund

▪ **What other organizations were involved as partners?**

Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

- **COLLABORATIVE AWARDS:** Not applicable.
- **QUAD CHARTS:** Not applicable.

9. APPENDICES: None