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TITLE: The Impact of TCR Affinity on T-Cell Dysfunction and Immunotherapeutic Reprogramming in Solid Tumors

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Although tumor-specific CD8 T cells are found in human tumors, cancers progress, indicating that these T cells are dysfunctional; yet the regulatory mechanisms underlying tumor-specific T cell dysfunction remain poorly defined. T cell-mediated immune responses are triggered by T cell receptor (TCR) binding to peptide-major histocompatibility complex (pMHC). In acute infections, affinity of TCR:pMHC interaction is a critical determinant of T cell expansion and effector function. However, little is known about how tumor antigen affinity impacts T cell differentiation and dysfunction in progressing tumors. To investigate the functional and molecular programs determined by affinity, we generated an in vivo tumor model expressing altered peptide ligands (APL) derived from SV40 large T antigen epitope I (TAG) and recognized by TAG-specific transgenic CD8 T cells (TCR$_{TAG}$) with varying functional avidity. While affinity did not impact T cell activation and differentiation in tumor draining lymph nodes, it drove distinct functional and molecular programs at the tumor site. Interestingly, key transcription factors and effector molecules were regulated by signal strength, preserving a cell-intrinsic functional program in T cells with low-affinity interactions. In contrast, certain hallmarks of T cell dysfunction, including the expression of inhibitory receptors (e.g. PD1 and LAG3), were affinity-independent, revealing that even very weak TCR ligations can induce a typical exhaustion phenotype. Ongoing studies will define the transcriptional and epigenetic programs underlying the distinct dysfunctional T cell states in tumors driven by TCR signal strength.

15. SUBJECT TERMS
T cell, TCR, affinity, tumor

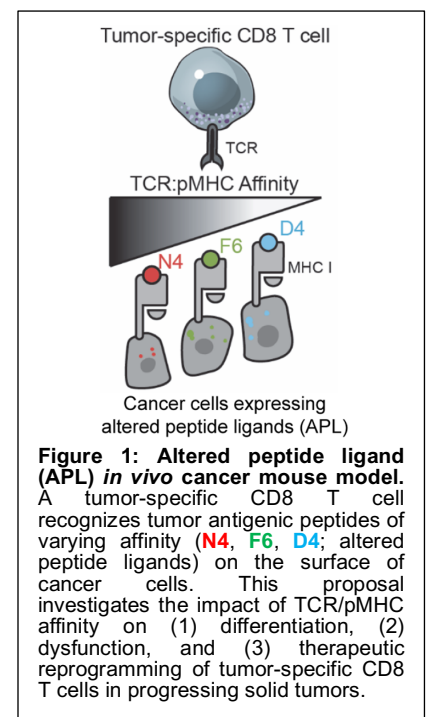| 16. SECURITY CLASSIFICATION OF: U			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
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INTRODUCTION

Tumor-specific CD8 T cells within solid tumors are dysfunctional [1-5], because despite their presence, tumors progress. Hallmarks of tumor-specific T cell dysfunction in mice and humans include the inability to produce effector cytokines (e.g. interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα)) and the expression of inhibitory receptors (e.g. programmed cell death receptor 1 (PD1), leukocyte-associated gene 3 (LAG3)) [4, 6]. While recent clinical observations and responses to immunotherapeutic strategies (e.g. blocking inhibitory receptors with monoclonal antibodies (checkpoint blockade), adoptive T cell therapy, etc.) demonstrate the power of harnessing tumor-specific CD8 T cells to fight cancers, fundamental challenges and questions remain. Durable clinical responses are only observed in a subset of patients and cancer types. Importantly, the precise immune events that are triggered by checkpoint blockade, factors that determine success or failure of immunotherapy and identification of immune and T cells that execute the anti-cancer response remain unclear [7]. This information is critical for the design of predictably effective treatments for solid tumors that are prevalent in military service members and the general public.

Tumor-specific T cells are a heterogeneous population with diverse antigen specificities and T cell receptor (TCR) affinities. T cell-mediated immune responses are initiated through the binding of the TCR to antigenic peptides presented on major histocompatibility complexes (MHC) on the surface of cells. The affinity of this interaction is a critical determinant of T cell differentiation and function; for example it was demonstrated that in settings of acute infections CD8 T cells with high-affinity interactions generally show superior effector functions [8-12]. However, little is known about the impact on TCR/tumor antigen affinity on differentiation, dysfunction and susceptibility to immunotherapeutic reprogramming of tumor-specific CD8 T cells in the context of progressing solid tumors (**Fig. 1**). To investigate this critically important question, I developed a novel mouse tumor model, in which tumor cells express antigens of varying affinity for the same TCR (altered peptide ligand (APL) *in vivo* tumor model) (**Fig. 1**). My preliminary data, which form the basis for this proposal, reveal the unexpected finding that low-affinity interactions enter a less profound state of dysfunction in progressing solid tumors, challenging the prevailing paradigm that higher-affinity TCR:pMHC interactions lead to superior T cell effector function.
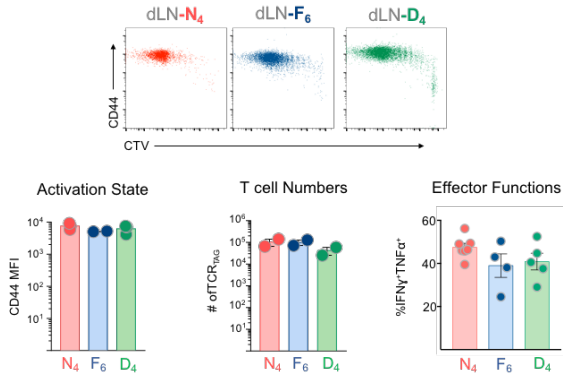

Figure 1: Altered peptide ligand (APL) *in vivo* cancer mouse model. A tumor-specific CD8 T cell recognizes tumor antigenic peptides of varying affinity (**N4**, **F6**, **D4**; altered peptide ligands) on the surface of cancer cells. This proposal investigates the impact of TCR/pMHC affinity on (1) differentiation, (2) dysfunction, and (3) therapeutic reprogramming of tumor-specific CD8 T cells in progressing solid tumors.

The ***specific hypothesis*** of this proposal is that low-affinity TCR:pMHC interactions delay or prevent differentiation of tumor-specific T cells to the dysfunctional state, making T cells with low-affinity TCRs more susceptible to immunotherapeutic reprogramming. I propose to investigate the differentiation and induction of dysfunction of tumor-specific T cells of varying TCR affinity, identify affinity-sensitive TCR signaling pathways underlying dysfunction, and determine the amenability for therapeutic reprogramming of T cells encountering tumor antigens of varying affinities. Defining the role of TCR:pMHC affinity in tumor-specific T cell dysfunction and rescue will reveal novel insights into the regulatory mechanisms of T cell function and elucidate powerful strategies for cellular reprogramming of T cells for cancer immunotherapy.

KEYWORDS
T cell, T cell receptor, TCR, tumor, antigen, affinity, dysfunction

ACCOMPLISHMENTS

Specific Aim 1 To determine the impact of TCR:pMHC affinity on the differentiation and dysfunction of tumor-specific CD8 T cells in developing solid tumors.	**Status**
Local IACUC approval	Completed
ACURO Approval	Completed 6/12/2017
Major Task 1: Does lowering TCR:pMHC affinity delay or prevent differentiation of tumor-specific CD8 T cells to the dysfunctional state?	90% Completed
Subtask 1 Tumor model establishment: Murine methycholantrene-induced fibrosarcoma cells expressing each of the 3 different SV40-I altered peptide ligands (MCA- APL) in to OTI TCR transgenic mice.	Completed
Subtask 2 Adoptive transfer of SV40-I-specific T cells: SV40-I specific T cells were collected from SV40-I TCR transgenic mice and transferred at 2 million per mouse into the APL-tumor bearing mice at 14 days post-tumor implantation.	Completed
Subtask 3 Analysis of tumor-specific T cells: At days 3 (Figure 2), 9 and 20 (Figure 3) post T cell transfer, tumors, spleens and tumor-draining lymph nodes (dLN) were excised for post-mortem analysis for the activation, proliferation, phenotypic and functional features of tumor-specific T cells. We found that at the early time point during the priming stage in the dLN, all T cells were equally activated and proliferated robustly. T cells accumulated to similar numbers in the dLN and and were similarly functional regardless of TCR:pMHC affinity (Figure 2). *(5 mice per MCA-APL type= 3 groups of 5 = 15 mice total)* *(For T cell collection: 1 donor mouse per 3 recipients = 5 donor mice)* *(repeated 3 times)* **Figure 2.** Phenotypic and functional assessment of tumor-specific T cells at 3 days post-AT from tumor-draining lymph nodes (dLN) of the APL tumor-bearing mice. T cells were labeled with cell-trace violet (CTV) prior to transfer and proliferation was assessed by CTV dilution. Activation was assessed by expression of CD44. Cells were counted using 123 count beads (Invitrogen) and effector cytokine production was assessed after 4h incubation with peptide-pulsed APCs.	Completed

At later time points (day 9-20), we found that in spite of the fact that all T cells expressed similar levels of inhibitory receptors PD1 and LAG3, T cells within the high affinity tumors were completely dysfunctional, while those within the low-affinity tumors remained functional (Figure 3)
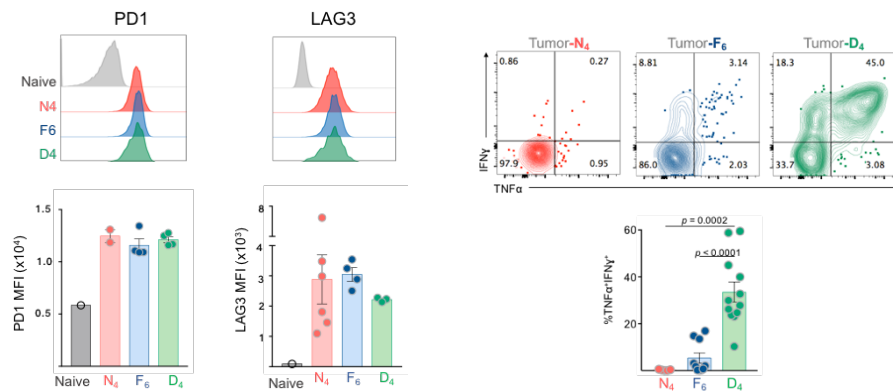

Figure 3. Phenotypic and functional analysis of T cells in the APL tumors at Day 14 post-AT. All T cells express similar levels of inhibitory receptors PD1 and LAG3, but T cells in the low-affinity D4 tumors remain functional, while those in the high affinity N4 and F6 tumors lose the ability to produce effector cytokines.

Subtask 4 Assessment of tumor outgrowth in mice with and w/o T cell transfer: Tumor models developed in Subtask 1 and 2 and tumor size was measured with a caliper every 2-3 days for 30 days total or as long as the animal's health status is normal. All tumors were found to grow equally well (Figure 4).

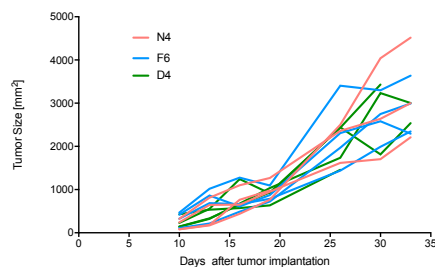*(3 mice per MCA-APL type= 3 groups of 3 = 9 mice total)*
(repeated 3 times)

Figure 4. Outgrowth of N4, F6 and D4 tumors in OTI TCR mice.

Subtask 5 Assessment of *in vitro* generated effector T cells: Tumor models will be developed as in Subtask 1 and tumor-bearing mice injected with SV40-I-specific T cells expanded *in vitro*. Tumor-specific T cells will then be assessed as in Subtask 3 *(5 mice per MCA-APL type= 3 groups of 5 = 15 mice total)* *(For T cell expansion: 2 donor mice)* *(repeated 3 times)*	Completed 2/2019
Specific Aim 2: To identify the molecular mechanisms downstream of the TCR underlying the functional differences in T cells encountering low-versus high-affinity tumor antigens.	Completed
Major Task 2 Do low-affinity TCR:pMHC interactions preserve the activity of the calcium/NFAT signaling pathway in tumor-specific CD8 T cells?	Completed

(The row for Subtask 4 / Figure 4 is marked "Completed" in the right column.)

Subtask 1Establish protocol to monitor calcium flux: SV40-I-specific T cells expanded *in vitro* were assessed for calcium response to various stimulations using calcium-sensing dye Indo-I and/or Fura-2 and analyzed with FACS or fluorescence microscopy. Fluorescence microscopy was found to yield the most reproducible results, most likely due to the ability to keep the cells at 37C throughout the assay, which was not possible on the FACS.	Completed 10/2017
Subtask 2 Analysis of calcium signaling and NFAT nuclear localization of TILs isolated from APL tumors: Tumor models were developed as in Major Task 1 Subtask 1 and 2. Since T cells were only found in the tumors after day 9 post T cell transfer, TILs were isolated and loaded with Fura-2. They were then imaged in wells seeded with MCA205-N4 cells for 30 min. Calcium flux was measured as the ratio between the 340nm and 380nm emission channels. All TILs were similarly capable of fluxing calcium (Figure5). As NFAT nuclear localization could not be measured due to technical difficulties in making this measurement from primary cells, we used ERK phosphorylation as a proxy for AP-1 activation, the transcriptional partner of NFAT required for much of T cell functional programming upon TCR stimulation. We found that indeed ERK was only phosphorylated in low-affinity TILs following TCR stimulation, indicating that a TCR signaling defect in high-affinity TILs could underlie their observed functional defect. **Figure 5.** e) Calcium flux of TILs isolated from N4 (red), F6 (blue) and D4 (green) tumors. Right panel shows representative images from cells contacting tumor cells. f) ERK phosphorylation in TILs isolated from N4 (red), F6 (blue) and D4 (green) tumors	Completed 8/2018
Major Task 3 Which TCR-driven genes expression programs are regulated by TCR:pMHC affinity and lead to dysfunction in tumor-specific CD8 T cells?	Completed
Subtask 1 RNA-seq analysis of TILs: Tumor models were developed as in Major Task 1 Subtask 1 and 2. At day 10-14 post T cell transfer, TILs from tumors were isolated and transcriptomic analysis carried out. In addition, naïve T cells and T cells isolated from the draining lymph nodes at Day 14 post-AT were used as comparison. As shown in figure 6, we found that TILs encountering low- vs. high-affinity TCR exhibit a distinct transcriptional program, expressing higher levels of markers associated with T cell effector function and memory formation. *(3 mice per MCA-APL type= 3 groups of 3 = 9 mice total)* *(For T cell collection: 1 donor mouse per 3 recipients = 3 donor mice)* **Figure 6.** b) Principle component analysis (PCA) of RNA-seq data. Each symbol represents a biological replicate. **c,** MA plot of RNA-seq data showing differentially expressed genes between high-affinity TIL-N_4/F_6 and low-affinity TIL-D_4.	

Specific Aim 3: Does lowering TCR:pMHC affinity improve susceptibility to therapeutic reprogramming?	
Major Task 4 Are T cells exposed to low TCR:pMHC affinity more amenable to rescue with PD1/PDL1 checkpoint blockade?	Completed 2/2020
Subtask 1: Analysis of functional rescue of dysfunctional TILs following *in vivo* administration of checkpoint inhibitor therapy: Tumor models will be developed as in Major Task 1 Subtask 1 and 2. Animals will be treated +/- checkpoint inhibitor therapy. Tumors will be monitored for size. Upon experimental completion, TILs will be isolated and assessed as in Major Task1 Subtask 3. If T cells with low-affinity TCR are found to be rescued, I will determine the underlying molecular mechanism for response by comparing the gene expression profile in the treated vs. untreated group as in Aim 2 Major Task 3. *(6 mice per MCA-APL type= 3 groups of 6 = 18 mice total)* *(For T cell collection: 1 donor mouse per 3 recipients = 6 donor mice)* *(2 repeats)*	

TRAINING AND PROFESSIONAL DEVELOPMENT

Work was presented at the internal research in progress meeting at MSKCC for the Immunology and Cancer Biology programs and at the Immunology Retreat 2019. I received weekly guidance from my mentor, Andrea Schietinger, and met with my co-mentor and members of my thesis committee to discuss my progress, to plan future experiments and to determine the course of my career.

DISSEMINATION OF RESULTS TO THE COMMUNITY
The results were submitted and are under revision at the Journal of Experimental Medicine.

GOALS FOR NEXT REPORTING PERIOD
N/A

IMPACT
The current clinical observations and responses with checkpoint blockade antibodies and adoptive T cell transfers have reinvigorated the field of cancer immunotherapy, and demonstrate the power of harnessing the adaptive immune system to fight cancers. However, fundamental challenges and questions remain. Significant clinical responses are only observed in a subset of patients and cancer types (e.g. liquid tumors such as leukemia and lymphoma, or some solid cancer types with higher mutational load). Moreover, the precise immune events that are being triggered by immunotherapeutic strategies, the identification of immune and T cells that execute the anti-cancer response, as well as the genetic basis and molecular makeup of their targets, remain unclear. Thus, it is currently not possible to predict which patients will respond to immunotherapeutic strategies, information that would be immensely helpful to guide clinicians. Thus, *identifying the factors that predict and/or define success of failure of immunotherapeutic interventions is fundamentally important to improve cancer immunotherapeutic strategies.*

Tumor-specific T cells are a heterogeneous population of cells, with diverse antigen specificities and T cell receptor (TCR) affinities to tumor antigens. Current immunotherapeutic strategies build on the assumption that high-affinity TCR/antigen interactions mediate superior effector function and efforts, for example, focus on the isolation and/or generation of tumor-reactive T cells with high(er) affinity TCR for adoptive T cell therapies. This assumption is largely based on studies from acute infections, but, in tumors, where antigens are generally presented in a non-inflammatory, chronic context the impact of TCR affinity in shaping anti-tumor immune responses is less clear. In fact very little is known about how TCR/tumor antigen affinity impacts (i) the activation of tumor-specific T cells, (ii) induction of T cell dysfunction in solid tumors, and (iii) a T cell's susceptibility to immunotherapy. My research on the role of TCR/antigen affinity in tumor-specific T cell dysfunction and immunotherapeutic reprogramming is the first study of this kind and provides a unique opportunity to obtain novel insights into the regulatory factors determining T cell immune responses in solid tumors and amenability to immunotherapeutic strategies. My findings that high-affinity interactions lead to complete loss of effector function in tumor-infiltrating T cells, in contrast to T cells encountering low-affinity antigens are potentially paradigm shifting, questioning the long-standing notion that higher affinity leads to superior anti-tumor effector function and could provide new insights into the identification of T cells which mediate a clinical response, e.g. by checkpoint blockade immunotherapy. My studies will elucidate the molecular signaling pathways and gene expression programs that maintain effector function in T cells with low affinity TCR/antigen interactions within solid tumors, and thus could reveal critical genes and pathways that could be targeted by pharmacological or genetic strategies which will be readily translatable into the clinic to enhance the effectiveness of immunotherapy of solid tumors.

CHANGES AND PROBLEMS
Nothing to report.

PRODUCTS
Nothing to report.

PARTICIPANTS

Name:	*Mojdeh Shakiba*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*11*
Contribution to Project:	*Mojdeh designed and performed all experiments and data analysis.*
Funding Support:	*N/A*

Name:	Andrea Schietinger
Project Role:	Mentor
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	0.5
Contribution to Project:	Designed experiments and guided Mojdeh
Funding Support:	N/A

Name:	Jedd Wolchok
Project Role:	Co-Mentor
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	0.5
Contribution to Project:	Discussed results and guided Mojdeh on experiments
Funding Support:	N/A

Name:	Steven Camara
Project Role:	Technician
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	3
Contribution to Project:	Assisted with the maintenance of the mouse colony and with experimental procedures.
Funding Support:	N/A