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14. ABSTRACT In the current funding period we have optimized phage-screens to select clones that differentially bind to either tumor infiltrating cytotoxic (CD8+) lymphocytes, activated CD8+ lymphocytes from the spleen, or un-activated naïve CD8+ T cells. We have developed a high-throughput flow cytometric approach that allows us to screen the specificity of several phage clones for each of these CD8+ populations. Using this initial approach we have identified 17 phage that selectively bind TIL rather than effector cells. However, none of these phage influenced CD8+ TILl expansion or function in vitro. Using a novel NextGeneration sequencing approach, we have further defined another 1,000,000 phage that selectively bind TIL, of which 100,000 are unique reads. Highly represented phage have been subcloned and are being tested for in vitro function. We have identified one phage that augments T cell expansion in vitro.

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1. Introduction: CD8+ T cells found within tumors are frequently dysfunctional. In many cases, dysfunction is caused by the expression of inhibitory molecules on the T cells that are designed to prevent immunopathology, but in the context of tumors, prevent T cells from performing their effector functions. Studies have shown that blocking these inhibitors with monoclonal antibodies, a process known as checkpoint blockade, can lead to the control of tumor. In melanoma patients, approximately 20% of patients make clinically relevant responses to their tumors in the presence of checkpoint blockade for the inhibitory molecule PD-1. Notably, responsiveness is strongly correlated with the expression of the ligands for PD-1. This result, while promising, begs the question why more patients do not respond. Several other inhibitory molecules have been described, suggesting that restraint of T cell function by inhibitory molecules is a multi-faceted process of critical evolutionary importance, leading to speculation that the full panoply of inhibitory molecules needs to be identified before a significantly greater proportion of patients respond to checkpoint blockade. It also raises the question as to whether different checkpoint blockade molecules are expressed by T cells that infiltrate tumors of different histology, and in different anatomical locations. Contemporary approaches to defining inhibitory molecules have tended to depend upon defining genetic lesions that result in an autoimmune phenotype in mutant mice. Other studies have focused on using gene expression profiling to identify differentially expressed molecules that have homology to identified inhibitory molecules, or contain motifs that have previously been identified to confer inhibitory function. However, this approach suffers from uncertainty about the biology of the identified transcript. To directly identify inhibitory molecules, we have proposed to use phage-display expression libraries to perform functional proteomics to identify molecules expressed on the surface of tumor infiltrating lymphocytes that are absent from resting or effector lymphocytes that are found in non-tumor settings. We then intend to use the phage to image the expression of these molecules in vivo, and determine the functional relevance of the molecules.

2. Keywords: phage-display; CD8+ T cells; checkpoint inhibition; cancer immunotherapy; in vivo imaging.

Original Statement of Work

Task 1. To identify peptides that selectively bind dysfunctional tumor-infiltrating $CD8^+$ T cells (months 1-14)

1a. Sort CD8+ T cells from B16cOVA tumors in 10 C57Bl/6 treated with anti-PD1, and 4 $PD1^-$ knockout mice, and define dysfunctional subsets (months 1-4)

1b. Perform phage screens of dysfunctional CD8+ T cells derived from 4 C57Bl/6 mice bearing B16cOVA tumors (months 1-4)

1c. Sort $CD8^+$ T cells from naïve (1), memory (4) and effector (1) control C57Bl/6 mice (months 4-5)

1d. Perform selective screens of phage against naïve, memory and effector $CD8^+$ T cells (months 4-5)

1e. Perform in vitro screens of candidate phage in $CD8^+$ T cell functional assays using B16cOVA cell lines as stimulators for CD8+ T cells sorted from 68 C57Bl/6 mice (months 6-9).

1f. Sequence peptides from selected phage; perform pull-down assays with phage from sorted $CD8^+$ T cell from B16cOVA tumors in 5 C57Bl/6 mice (months 9-12).

1g. Sequencing of pull-down products by mass spec (months 10-14).

Task 2. To determine the importance of identified cell surface proteins on CD8$^+$ T cell function (months 12-18):
2a. Define high affinity peptides for candidates that are identified during initial screens (months 12-14) using 10 C57Bl/6 mice per assay.
2b. Perform in vitro validation assays of peptide binding and activity (months 15-16).
2c. Determine the ability of in vitro defined peptides to alter CD8$^+$ T cell function in vivo (months 16-24) using CD8+ T cells from 20 C57Bl/6 mice bearing B16cOVA tumors.
2d. To image the induction of inhibitory molecule expression using labeled phage (months 16-24) using 3 C57BL/6 mice, plus 3 controls, per phage.

Task 3. To identify the ligands of inhibitory molecules expressed by melanomas (months 14-24):
2a. Perform reverse-phage screening of ORF libraries using 3T3 cells transfected with cDNA of the identified IM; determine ligand expression in B16cOVA cell lines (months 9-12).
2b. Generate ligand-negative B16cOVA tumor clones and determine importance of ligand expression to CD8$^+$ T cell function after transferring into 5 C57Bl/6 mice per IM (months 13-16).
2c. Define importance of ligand to in vivo growth of B16cOVA tumor (months 16-24), using 5 control and 5 phage-treated C57Bl/6 mice per phage to be tested.

3. Overall Project Summary:

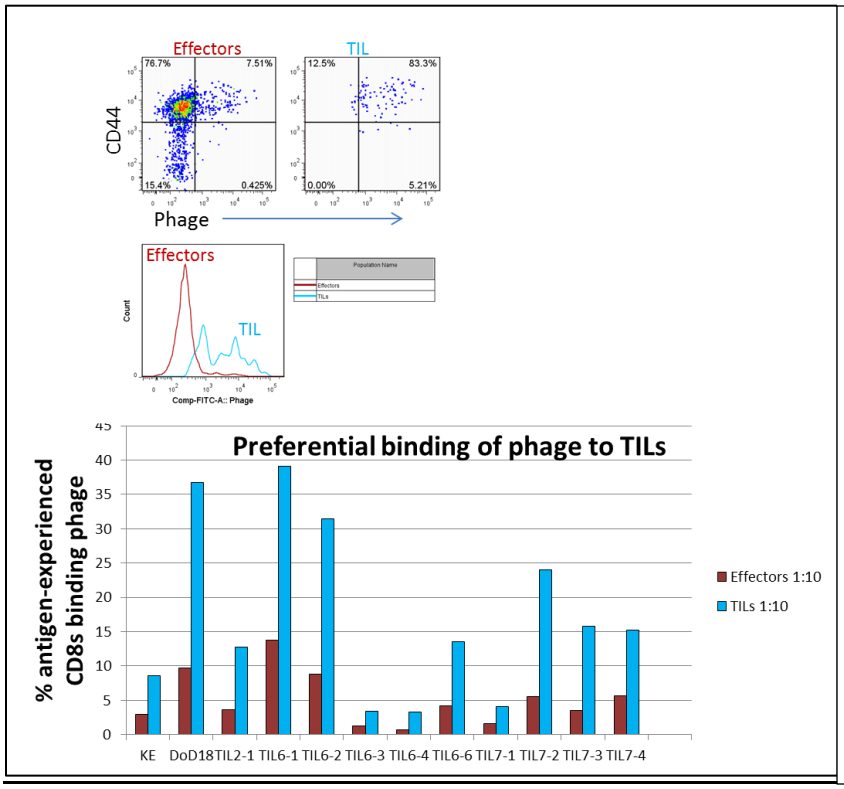

Figure 1. Selective binding of phage to CD8+ TIL. Plaque purified phage were derived from pools selected on the basis of positive binding to CD8+ TIL and panned against effector and naïve CD8+ T cells. Phage are labelled with fluorochromes and then assessed for binding to T cells excised from either the spleens of mice immunized 7d prior (Effectors, left dot plot) or from d14 CD8+ TIL populations (TIL, right dot plots). Histogram shows relative phage binding. Bar chart shows selective binding of 10 representative phage. KE is a control phage.

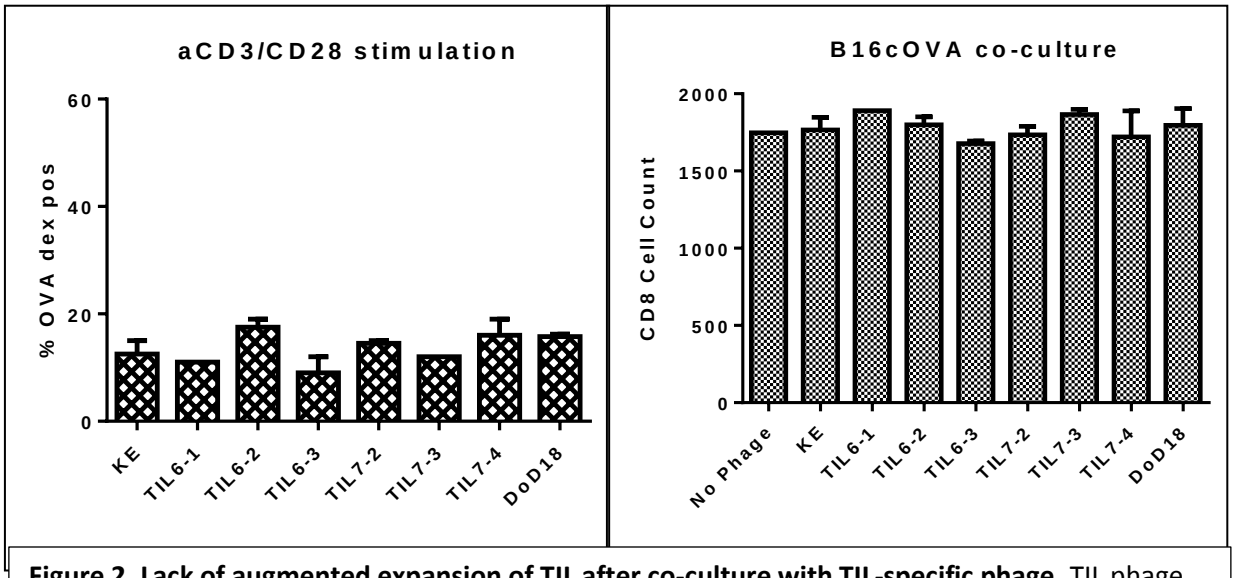

Figure 2. Lack of augmented expansion of TIL after co-culture with TIL-specific phage. TIL phage or controls (KE; DoD18) were co-cultured with CD8+ TIL isolated from d14 tumors. Co-cultures were stimulated with either agonistic antibodies for T cell activation (CD3/CD28; top chart) or tumor cells (B16cOVA, bottom chart) and % tumor specific and cell numbers and CD8+ T cell numbers quantified 5d later.

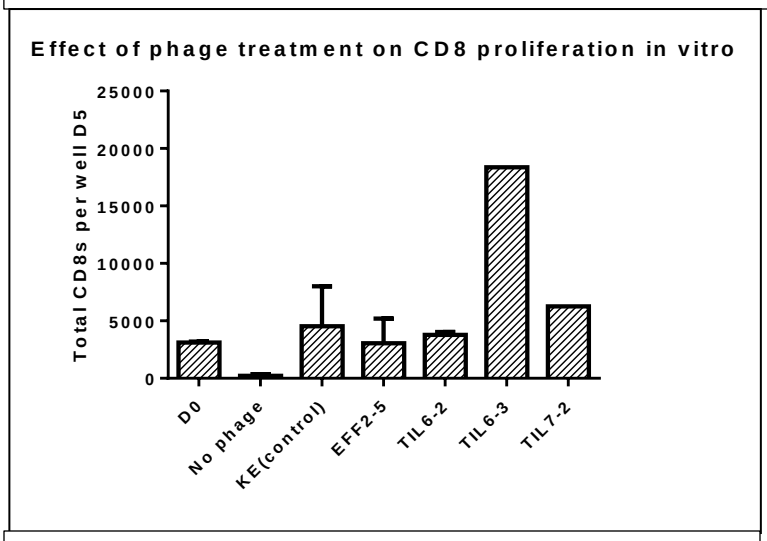

Figure 3. Phage-mediated increased expansion of effector CD8+ T cells. Naive CD8+ T cells were activated with peptide and antigen presenting cells in the presence of the indicated phage. 6d later, antigen-specific CD8+ T cells were enumerated by flow cytometry.

Task 1: We have completed Tasks 1a – 1e. Using the original approach outlined in the application, we identified a total of 17 unique phage that selectively bind CD8+ TIL but not effector or naïve CD8+ T cells (**Figure 1**). Interestingly, some of these phage also bind CD4+ TIL, and other cells in the tumor microenvironment (TME). To date, none of these phage have promoted the function (proliferation, cytokine production) of CD8+ TIL ex vivo (Task 1e) (**Figure 2**). Presumably they bind to proteins that are associated with trafficking or persistence of CD8+ T cells in peripheral tissue rather than secondary lymphoid organs. We can test this possibility by asking whether these phage bind effectors that are found in lung tissue. Alternatively, phage may not sufficiently abrogate receptor-ligand interactions. We will examine this possibility by identifying the ligands of some of the most distinctively binding phage and testing whether antibodies to these ligands influence T cell function. Pertaining to this, we have identified 1 phage that promotes the expansion of control effector CD8+ T cells (**Figure 3**). This suggests that the phage do have the ability to

engage molecules on the surface of T cells in manner that is sufficient to manipulate their function, suggesting that further screening of unique phage is warranted. We have sequenced the agonst phage (Task 1f) and forwarded it to pull-down studies and mass-spec identification (Task 1g).

Modifications to Task 1 in new SOW for no-cost extension (NCE):

A. We have determined that the complexity of the disparate phage that selectively bind to TIL (Task 1d) is compromised by three processes: first, the amplification process; second, the selection of plaques; third, serial panning against multiple subpopulations. Amplification can result in phage that have inherent amplification advantages. Plaque selection often results in the same phage sequences and is based on the frequency, not the scarcity, that a moiety is selectively expressed. Panning is compromised by the tendency of T cells to be vulnerable to cell-death during the panning process. To overcome these deficiencies in our approach, we have generated a Next Generation Deep Sequencing (NGS) approach that has allowed us to sequence all the phage that bind to TIL and to effector/memory/naïve CD8+ T cells. Rather than screening one plaque at a time for sequence information (50 phage to date, 17 unique sequences), we have acquired unique sequences from 100,000 phage via NGS! We have also developed a database of phage sequences that are known to bind to other tissues. We can now use in silico approaches to identify all the sequences that bind to CD8+ TIL, subtract those that are known to bind to other cell populations, and subclone differentially expressed sequences the phage backbone for functional assessment (or have peptides synthesized). This approach provides two further advantages: we can sequence phage that bind to knockout mice and thus identify those sequences that selectively bind to those molecules; we can use antibodies against known inhibitory molecules to elute phage, thus removing them from the repertoire of molecules. We anticipate testing the first round of NGS defined sequences in task 1e in the next two weeks.
   Timeline of modified Task 1d during NCE: Months 1-3.
B. We proposed a phage-directed pull-down approach to allow mass-spectrometry driven identification of the molecules selectively bound by phage (Task 1g). Traditionally, we have identified the phage peptide binding partner through indiscriminate modification of the phage coat protein with a photo-activatable cross-linker. However, data interpretation has been hampered through non-specific binding. Therefore, we are introducing modifications into the phage coat protein to allow site specific modification, which we theorize will reduce non-specific binding. This derivation should also reduce the number of CD8+ TIL needed to execute the pull-downs, thus reducing the number of mice needed for experiments. We are currently testing this principle with the phage identified as promoting CD8 T cell function in Task 1g, and will use this to identify the molecules that demonstrate highly selective phage binding even if they do not promote TIL function. This will provide us some idea of the molecules that phage are binding to, which in turn will help our in silico subtraction approaches.
   Timeline of modified Task 1g during NCE: Months 4-6
C. We have introduced an alternative approach to facilitate the identification of phage-selective molecules. We will introduce pools of siRNA, derived from comprehensive libraries, into TIL and identify siRNA sequences that lead to selective loss of phage binding.
   Timeline of modified Task 1g during NCE: Months 4-6

Task 2: As we have only identified one sequence that influences T cell function, we have made limited progress on this Task. The peptide has been synthesized (2a) and validated as binding independently from the phage backbone in vitro (2b). We are currently assessing the ability of this peptide to bind CD8+ T cells in vivo and examine impact on in vivo CD8+ TIL function (2c).

No modifications of this Task are expected in the NCE.

Timeline of continuation of Task 2 during NCE: Months 1-6

Task 3: No ligands have been identified to date due to insufficient candidates.

No modifications of this Task are expected in the NCE.

We do not anticipate being able to advance new candidates identified using modified Task 1 to Task 3 by the end of the NCE. If we identify the target for the currently being assessed in Task 2, and it's binding partner is not currently known, we will pursue its partner's identify during months 3-6 of the NCE.

4. Key Research Accomplishments:

- 50 phage identified by selection and negative subtraction approaches
- 17 “panned” phage shown to selectively bind to TIL
- 1 “panned” phage demonstrates agonistic activity in vitro.
- 100,000 novel TIL-selective phage sequences identified by NextGen sequencing
- Development of in silico approaches and accompanying bioinformatics to apply NextGen sequencing approaches to phage selection.

5. Conclusion: To date we have validated the hypothesis that phage-display approaches can be used to identify molecules that are selectively expressed on the surface of CD8+ TIL as opposed to other CD8+ T cell populations. This has considerable significance as we may be able to use these phage as a non-invasive imaging tool to determine whether cancer patients have CD8+ TIL within their tumors. This is highly pertinent as the majority of patients that are demonstrating responses to checkpoint blockade (e.g. Ipilimumab, Nivolimumab) have pre-existing CD8+ TIL available to respond to immunotherapy. These phage could be used to select patients who are suitable for checkpoint inhibitors, while also selecting patients who need further intervention prior to checkpoint inhibitor therapy. One phage we have identified to date appears to have agonistic activity, which supports the proof-of-principle that phage could be used to influence T cell function. We have yet to establish this principle in TIL, and therefore may need to modify the way we deliver the phage sequences in order to block inhibitory molecule function.

6. Publications, Abstracts and Presentations:

Nothing to report

7. Inventions, Patents and Licenses

Nothing to report

8. Reportable outcomes

The practical use of phage-display libraries to selectively bind to tumor-infiltrating T cells.

9. Other Achievements

We have refined technical approaches for applying phage display with activated lymphocytes that exist in very fragile states. Mr Dustin Bauknight, a graduate student supported on this award, passed his qualifying exams. Mr Andrew Buckner, a laboratory technician supported on this award, scored excellent GRE results and has applied to Graduate School.