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TITLE: T-Cell Trafficking into the Cold Tumor Immune Microenvironment

PRINCIPAL INVESTIGATOR: Dr. Seth Pollack, MD

CONTRACTING ORGANIZATION: Fred Hutchinson Cancer Research Center

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Dr. Seth Pollack, MD

E-Mail:

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14. ABSTRACT: Our goal is to improve the efficacy of anti-cancer immunotherapy, a 2018 PRCRP Topic Area. These studies focus on the immune-inhibiting tumor microenvironment (TME) in a subset of soft-tissue sarcoma (STS) tumors called Synovial Sarcoma (SS) and Myxoid/ Round Cell Liposarcoma (MRCL) that affect all ages, including children, adolescents and young adults, another FY18 Topic Area. The prevalence of admission for STS in the military health system has been estimated at 1.7 cases per 100,000 per year, and some STS are presumed to be related to Veterans' exposure to Agent Orange or other herbicides during military service, one of the FY18 Military Relevance Focus Areas.1,2 Long-term, our findings are likely to help advance effective immunotherapies for patients with many types of solid malignancies in which the TME commonly inhibits effective immune responses. Successful immunotherapies have the potential to address significant gaps in cancer treatment within the U.S. population, including military Service members and their beneficiaries, and perhaps especially Veterans, our other FY18 Focus Area.
In this project, we will examine T cell trafficking and function in the context of tumor, stroma, tumor associated macrophages (MΦ) and endothelium within an ex-vivo 3-dimensional organoid culture system3,4 that includes microfluidics to model real-time trafficking from the vasculature into the tumor's interstitium. Our findings will likely support new, even more effective therapeutic strategies to manipulate the tumor immune microenvironment to increase delivery of tumor-specific T cells into immunologically cold tumors.

15. SUBJECT TERMS

NONE LISTED

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1. INTRODUCTION

Our goal is to improve the efficacy of anti-cancer immunotherapy, a 2018 PRCRP Topic Area. These studies focus on the immune-inhibiting tumor microenvironment (TME) in a subset of soft-tissue sarcoma (STS) tumors called Synovial Sarcoma (SS) and Myxoid/ Round Cell Liposarcoma (MRCL) that affect all ages, including children, adolescents and young adults, another FY18 Topic Area. The prevalence of admission for STS in the military health system has been estimated at 1.7 cases per 100,000 per year, and some STS are presumed to be related to Veterans' exposure to Agent Orange or other herbicides during military service, one of the FY18 Military Relevance Focus Areas.[1,2] Long-term, our findings are likely to help advance effective immunotherapies for patients with many types of solid malignancies in which the TME commonly inhibits effective immune responses. Successful immunotherapies have the potential to address significant gaps in cancer treatment within the U.S. population, including military Service members and their beneficiaries, and perhaps especially Veterans, our other FY18 Focus Area.

In this project, we will examine T cell trafficking and function in the context of tumor, stroma, tumor associated macrophages (MΦ) and endothelium within an *ex-vivo* 3-dimensional organoid culture system[3,4] that includes microfluidics to model real-time trafficking from the vasculature into the tumor's interstitium. Our findings will likely support new, even more effective therapeutic strategies to manipulate the tumor immune microenvironment to increase delivery of tumor-specific T cells into immunologically cold tumors.

2. KEYWORDS:

Synovial sarcoma, myxoid liposarcoma, immunotherapy, organoids, macrophages, NY-ESO-1, tumor immune microenvironment, T cells

3. ACCOMPLISHMENTS:

3a. What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

The most significant results over the past year have related to Major Tasks 2 and 5 and are discussed under **accomplishments** below.

Major task 1: Processing of fresh tumor samples
IRB/HRPO approval of secondary use tissue analysis has been obtained. We have collected 5 tumors that have been cryopreserved in single cell suspension for the specific aims in this proposal. Tissue collection is on-going but we currently have adequate tissue to conduct our experiments.

Major task 2: Treat freshly procured patient samples with engineered HLA-matched NY-ESO-1 specific T cell in the organoid model.
We have made significant progress in our use of HLA-A*0201 specific T cells in the context of our organoid models and used FFPE tissue to analyze expression of MHC and other key factors of the tumor immune microenvironment resulting in the two manuscripts described below (see

accomplishments below). Work incorporating CD4 cells remains a critical piece that will be a focus of the coming year.

Major Task 3: Analyze changes in the phenotype of tumor cells, macrophages and T cells in sarcoma samples following treatment with IFNγ.
Because scRNAseq is expensive and labor intensive, we have not yet used this methodology for analysis of our chip data, but we plan to do this over the coming year. We have performed experiments using IFNγ and T cells using flow cytometry and mIHC to look at cell death, T cell markers and macrophage markers. This work is on-going. All relevant IRB approvals have been obtained.

Major Task 4: Analyze relation between tumor, stroma and macrophages in the absence of T cells.
We have performed experiments using macrophage, stroma and tumor using macrophages in the extracellular matrix. We have also conducted experiments using FUJI and Huvec in transwell plate, but more experiments and analysis are necessary for both of these goals. Work on these goals will continue in year 2.

Major Task 5. Assess CD8+ T cell function in a precisely defined organoid model.
We have performed experiments combining IFNγ and NY-ESO-1 specific T cells in precisely defined organoid models. More experiments and analysis are necessary. We have not yet looked at T cells derived from different phenotypic states in the system. Work on these goals will continue in year 2.

Major Task 6. Introduce CD4+ T cells into organoid model
Work on the goals under Major Task 6 has not yet begun and was not supposed to start until year 2 according the SOW schedule. Over the coming year we will begin assessing the impact of CD4 cells on macrophage state or along with CD8 cells to measure the impact on the tumor microenvironment. Work on this will be done in year 2.

3b. What was accomplished under these goals?

1) Major Activities: The overarching goal of the grant is to overcome barriers in the SS and MRCL tumor microenvironment. The proposal utilizes *ex-vivo* organoid culture systems to accomplish these goals. As a result of our works most significant activities over the past year, we have found that IL-15 may allow transferred T cells function better in the SS and MRCL tumor microenvironments. Deeper exploration of these findings (within the context of the grant objectives) has been a major focus over the previous year.

2) Specific Objectives: Under “Major Goals” we describe accomplishments with respect to the SOW timeline that we initially provided. We have completed experiments related to each Aim, with significant findings that we are preparing for publication

3) Specific Results and Other Achievements

Experiments using a chip-based model systems to explore mechanisms of resistance to NY-ESO-1 specific adoptive cellular therapy (ACT) required preparation of NY-ESO-1 specific CD8 T cells for infusion into the organoid systems (these experiments are most prominently sub-aims ***E.1.a*** and ***E.2.b*** from the grant document). To more accurately model this resistance within the organoid system, we began exploring more whether it might be possible to isolate and expand *ex-vivo* NY-ESO-1 specific T cells from patients who had progressed following cellular therapy for infusion into the organoid because changes occurring in the post-ACT T cell could be tightly linked to those occurring in the tumor microenvironment. We found that these rare persisting cells lacked markers of activation despite expressing markers of memory and we hypothesized that these cells might be stimulated with IL-15, which can stimulate other cell phenotypes that are difficult to stimulate and also have stem-like properties.[1]

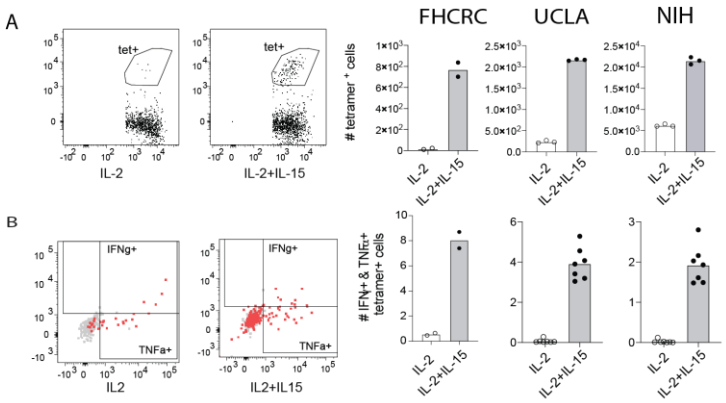

Fig. 1: On 3 different ACT trials, NY-ESO-1-specific T cells were sorted from PBMC 4-6 weeks after transfer in patients who ultimately progressed, and were cultured with NY-ESO-1 peptide plus IL-2 or IL-2/IL-15. IL-15 dramatically expanded tet+ cells, but not tet- cells (not shown). B) IL-2/IL-15-treated cells produced increased IFNγ and TNFα.

To test this, we cultured PBMCs from post-infusion samples with NY-ESO-$1_{157\text{-}165}$ peptide and low dose IL-2 with and without IL-15. The addition of low dose IL-15 led to a significant increase in expansion of tet^+ cells compared to control group by day 9 (**Fig. 1A**). Although IL-15 also induced expansion of $CD8^+$ tet- cells, the expansion of tet^+ cells was significantly higher (**Fig. 1B**). These *ex-vivo* expanded cells expressed CD39 and Ki67 at the end of the 9-day which indicates that they had recognized their cognate antigen. These cells were from a patient treated on a protocol performed at our center but we also confirmed this effect in PBMCs from patients treated with TCR engineered products at UCLA and the NIH who also had low number of tet^+ cells following tumor progression.[2,3]

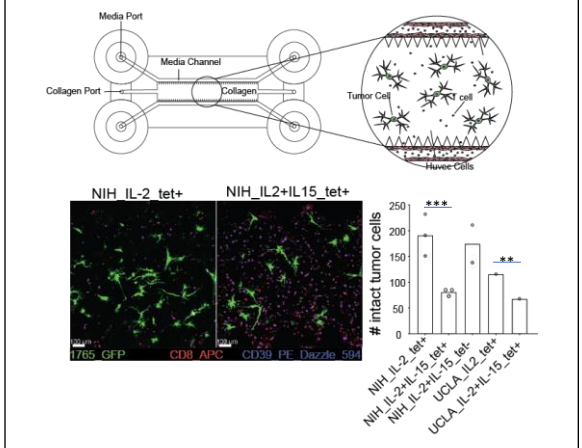

Fig. 2: Organoid model demonstrating increased trafficking and tumor killing of tet+ and tet- cells following culture with IL-2/IL-15. Few T cells entered the organoid tumor chamber without IL-15 (left), but T cells (pink) filled the chamber with IL-15 (right), and fewer tumor cells were present (green). Quantitative killing data are shown (far right).

Moreover, we also observed the same effect of IL-15 in expanding tet^+ cells from TILs isolated from a post-treatment biopsy from one of these patients approximately 2 months after their ACT. Neither anti-PD-1 or higher doses of IL-15 further enhanced this effect. In some other patients, the effect of IL-15 in inducing T cell expansion was higher in samples from intermediate time points compared with early or late time points suggesting that effect of IL-15 may be time dependent for some patients (**Fig. 1A and B**). To test if IL-15 increased the effector function of these T cells, we stimulated the cells with NY-ESO-$1_{157\text{-}165}$ peptide following 9-day culture with or without IL-15 and performed intracellular cytokine staining

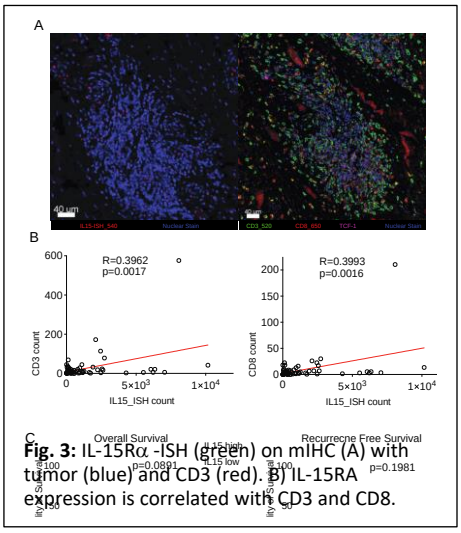

Fig. 3: IL-15Rα -ISH (green) on mIHC (A) with tumor (blue) and CD3 (red). (B) IL-15RA expression is correlated with CD3 and CD8.

for IFNγ and TNFα. The number of cells positive for both IFNγ and TNFα were significantly higher in IL-15 supplemented cultures and these results were confirmed in samples from patients treated with TCR engineered products at UCLA and the NIH as well as TIL from Cy1.

In order to simulate T cell migration from blood through endothelial cells into tumor parenchyma, we performed T cell-tumor killing assays using a 3D organoid chip. Although we initially used the Nortis chip, we ultimately found that this assay worked better in AIM Biotech chips which function similarly to the Nortis chips in many respects as both infuse microfluids through channels that pass through collagen matrices however we saw better T cell migration through the AIM Biotech chip's fluid channels. In this system, we were able to perform robust assays using sorted tet$^+$ cells. Tet$^+$ cells cultured with IL-15 had greater than two-fold increase in their killing efficiency as compared to the control group. By 48 hours, the number of IL-15 treated tet$^+$ cells were significantly higher than the control group, despite starting with the same number of cells suggesting that T cells continued to expand post transfer into the chips (**Fig. 2**). These results were observed in both products from patients treated at UCLA and the NIH.

IL-15 signaling often occurs through trans-presentation. [4] Although exogenous IL-15 can be highly stimulator toward TIL, it requires the presence IL-15R in situ to activate T cells. [5,6] In order to determine whether SS and MRCL tumors will have sufficient IL-15Rα in their TME to induce proliferation of RPC's, we established an *in situ* hybridization assay staining IL-15Rα in conjunction with mIHC. Using a tissue microarray (TMA) constructed from 58 untreated SS and MRCL tumors we confirmed a consistent presence of IL-15Rα. (**Fig. 3A**). Through quantitative image analysis, we confirmed that IL-15Rα was highly correlated CD3 and CD8 staining (**Fig. 3B**).

We sought to test whether IL-15 could expand TIL from tumor samples in ex-vivo culture (**Fig. 4**). SS and MRCL specimens were digested into single cell suspension were supported in *ex-vivo* culture for 9 days in low dose IL-2 and with or without IL-15. In cells supplemented with IL-15 there was a higher number of CD8+ T cells at the end of 9-day culture resulting in better cytokine release and tumor killing..

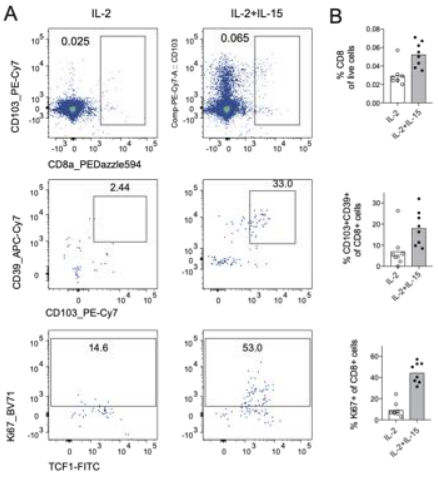

Fig 4: An MRCL tumor was made into a single cell suspension and cultured *ex-vivo* in either IL-2 alone or along with IL-15. IL-15 induced marked expansion of activated T cells as evidenced by the large numbers of CD8+, CD39+, CD103+ T cells. These cells express Ki67 demonstrating proliferation. Also seen in SS tumors (not shown).

Stated Goals Not Met: This project has resulted in significant achievement that we expect to result in two publications over the coming year. Not all objectives have been met but we will continue to work on these over the coming year.

1. Chen Z, Ji Z, Ngiow SF, et al: TCF-1-Centered Transcriptional Network Drives an Effector versus Exhausted CD8 T Cell-Fate Decision. Immunity 51:840-855 e5, 2019
2. Nowicki TS, Berent-Maoz B, Cheung-Lau G, et al: A Pilot Trial of the

Combination of Transgenic NY-ESO-1-reactive Adoptive Cellular Therapy with Dendritic Cell Vaccination with or without Ipilimumab. Clin Cancer Res 25:2096-2108, 2019
3. Robbins PF, Morgan RA, Feldman SA, et al: Tumor regression in patients with metastatic synovial cell sarcoma and melanoma using genetically engineered lymphocytes reactive with NY-ESO-1. J Clin Oncol 29:917-24, 2011
4. Burkett PR, Koka R, Chien M, et al: Coordinate expression and trans presentation of interleukin (IL)-15Ralpha and IL-15 supports natural killer cell and memory CD8+ T cell homeostasis. J Exp Med 200:825-34, 2004
5. Colpitts SL, Stonier SW, Stoklasek TA, et al: Transcriptional regulation of IL-15 expression during hematopoiesis. J Immunol 191:3017-24, 2013
6. Sosinowski T, White JT, Cross EW, et al: CD8alpha+ dendritic cell trans presentation of IL-15 to naive CD8+ T cells produces antigen-inexperienced T cells in the periphery with memory phenotype and function. J Immunol 190:1936-47, 2013

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

Although this project was not intended to support professional development, it has surreptitiously aided the career development of Dr. Karan Kholi, a post-doctoral fellow who has contributed significantly to the work to date and is the first author of both manuscripts currently in progress. In order to carry out the aims of this work, Dr. Kholi learned new skills with respect to microscopy and image analysis. Dr. Kholi has now left my lab but is still using these skills in his new lab. Furthermore, a different post-doctoral fellow Dr. Shihong Zhang has been assisting in the organoid work in this project and is now applying her skills at creating organoids to help her execute her own project for my lab that has aims outside this award. Lab technician Graeme Black has also developed skills at flow cytometry, microscopy and 3D organoid cell culture.

How were the results disseminated to communities of interest?

The work is not yet published so there is currently nothing to report. However, we expect to disseminate these results over the coming year.

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

I believe we have been successful over the past year given that we have significant results that are serving as the basis for two separate manuscripts that we expect to submit over the coming months. Now that we are getting ready to publish our interesting findings related to IL-15, I plan to emphasize the studies outlined in the grant regarding macrophages and CD4 cells.

4. IMPACT

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

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Over the past year we gained a key insight through our work on this project. We learned that following cellular therapy, rare persistent cells can be expanded from the peripheral blood of patients who have progressed after cellular therapy using IL-15 and in organoid systems we demonstrated that these cells can be effective at eliminating tumor even in a cold tumor immune microenvironment. We also demonstrated that these cold tumors express the IL-15 receptor so they may be sensitive to therapy using exogenous IL-15. We are writing these results up now and plan to submit them for publication shortly. Although our work on this proposal in now shifting to other proposed aims, we submitted an R01 grant to the NIH to help us explore that finding in more depth.

What was the impact on society beyond science and technology?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as: improving public knowledge, attitudes, skills, and abilities; changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or improving social, economic, civic, or environmental conditions.

Nothing to report.

5. CHANGES/PROBLEMS: *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

Nothing to report.

6. Products:

Manuscripts in preparation mainly arising from CA180380:

Kholi K, Zhang S, Black FG, Spadinger S, Jones RL, Riddell SR, Yee C, and **Pollack SM**. IL-15 Mediated Expansion of Rare Durable Memory T Cells Following Adoptive Cellular Therapy. *Submission planned shortly.*

Kholi K, Meusner A, Zhang S, Black FG, Spadinger S, Jones RL, Pierce R, and **Pollack SM**. IL-15 augments endogenous anti-tumor T cell immunity in the Sarcoma Tumor Immune Microenvironment. *Submission planned shortly.*

Other manuscripts:

Pollack SM, Redman MW, Baker K, Wagner MJ, Schroeder BA, Loggers ET, Kholi K, Zhang S, Black RG, McDonnell S, Gregory J, Johnson R, Moore R, Jones RL, Cranmer LD. A Phase I/II Trial of Doxorubicin and Pembrolizumab in Patients with Metastatic/ Unresectable Anthracycline Naïve Sarcomas. *JAMA Oncology*. Accepted and awaiting publication.
Federal support is acknowledged.

Somaiah N, Chawla SP, Block MS, Morris JC, Do K, Kim JW, Druta M, Sankala KK, Hwu P, Jones RL, Gnjatic S, Kim-Schulze S, Lu H, Yakovich A, ter Meulen J, Chen M, Kenney RT, Bohac C, **Pollack SM**. A Phase 1b Study Evaluating the Safety, Tolerability, and Immunogenicity of CMB305, a Lentiviral-Based Prime-Boost Vaccine Regimen, in Patients with Locally Advanced, Relapsed, or Metastatic Cancer Expressing NY-ESO-1. *Oncoimmunology*. Accepted with revisions.
Federal support is acknowledged.

Books or other non-periodical, one-time publications.
Nothing to report

Other publications, conference papers and presentations.

Brett A. Schroeder, Natalie LaFranzo, Bonnie LaFleur, Rachel Gittelman, **Seth M. Pollack**. A Single-Center Retrospective Study Of the Immune Landscape in Patients with Untreated Liposarcoma (LS) with Long-Term Follow Up. Presented at the annual meeting of the American Association for Cancer Research. 2020. Virtual Meeting.

Website(s) or other Internet site(s)

List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to report

Technologies or techniques

Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to report

Inventions, patent applications, and/or licenses

Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to report

Other Products

Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include: data or databases; physical collections; audio or video products; software; models; educational aids or curricula; instruments or equipment; research material (e.g., Germplasm; cell lines, DNA probes, animal models); clinical interventions; new business creation; and other.

Nothing to report

6. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Seth Pollack, M.D. – Principal Investigator (1.2 calendar months)
Beverly Torok-Storb, M.Ed, Ph.D. – co-Principal Investigator (0.6 calendar months)
Brian Hayes, Ph.D. – Post-doctoral research fellow (3.0 calendar months)
Shihong Zhang, Ph.D. – Post-doctoral research fellow (4.2 calendar months)
Graeme Black, Project Manager (6.0 calendar months)
Andrew Meuser, Lab Technician 1 (6.0 calendar months)
Rylee Johnson, Regulatory Lead (0.12 calendar months)

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

Nothing to report

What other organizations were involved as partners?

Nothing to report

7. SPECIAL REPORTING REQUIREMENTS

Nothing to report