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14. ABSTRACT

We recently found that a new agent (anti-RANKL antibody) rescues melanoma-fighting T cells from thymus elimination. Anti-RANKL antibody is different from other cancer immunotherapies because of this unique mode of action. By itself, anti-RANKL antibody improves the survival of mice injected with melanoma cells. **Because anti-RANKL antibody and checkpoint inhibitors work in distinct, non-redundant ways, we hypothesize that anti-RANKL antibody will increase the effectiveness of checkpoint inhibitors in rejecting melanoma tumors in mice and humans.** This grant proposal will provide critical information needed to bring anti-RANKL antibody to the clinic for treating advanced melanoma patients. To date, our findings include: RANKL is expressed at high levels on human thymocytes; RANK is expressed at higher levels in medullary thymic epithelial cells (mTECs) than in cortical thymic epithelial cells (cTECs). These finding lend preclinical evidence for using anti-RANKL antibody to block central tolerance.

15. SUBJECT TERMS
Melanoma, checkpoint inhibition, anti-RANKL antibody, RANKL, anti-CTLA-4 antibody, anti-PD1 antibody, thymus, central tolerance, Aire

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INTRODUCTION: Our preliminary data demonstrate that central tolerance blockade 1) expands the anti-melanoma immune response and 2) enhances the anti-melanoma effects of immune checkpoint inhibition. Furthermore, we have identified anti-RANKL antibody as a pharmacologic agent that blocks central tolerance. Therefore, RANKL blockade is a promising therapy for enhancing checkpoint inhibitor efficacy in advanced melanoma. This observation has immediate clinical relevance given the FDA approval of anti-RANKL antibody for other indications, including bone metastases from cancer. In order to develop anti-RANKL antibody as a combination therapy with checkpoint inhibitors for advanced melanoma patients, several critical issues remain to be clarified and are the objectives of this grant proposal. These objectives are 1) to determine whether anti-RANKL-antibody similarly depletes Aire-expressing mTECs in the human thymus and 2) to determine whether central tolerance blockade with anti-RANKL and checkpoint inhibition will have additive effects in immune rejection of melanoma in mice. Based on our preliminary data, **we hypothesize that combining anti-RANKL antibody and checkpoint inhibition will have additive effects on increasing the intratumoral ratio of Teff:Treg cells and rejecting melanoma cells in mice and humans.**

KEYWORDS: Melanoma, checkpoint inhibition, anti-RANKL antibody, RANKL, anti-CTLA-4 antibody, anti-PD1 antibody, thymus, central tolerance, Aire

ACCOMPLISHMENTS:

What were the major goals of the project?

Tasks	Months	Completion?
HRPO approval	1-2	100%
Major Task 1. Localization of RANK and RANKL in human thymus.		
Subtask 1. Flow cytometric analysis of RANK expression in human thymus cell subsets with anti-RANK antibody.	3-6	100%
Subtask 2. Flow cytometric analysis of RANKL expression in human thymus cell subsets with anti-RANKL antibody.	3-6	100%
Subtask 3. Flow cytometric analysis of RANKL expression in human thymus cell subsets with OPG-Fc.	3-6	100%
Milestone(s) Achieved: Local IRB approval	2	100%
Major Task 2. Effects of soluble RANKL on human mTEC cellularity.		
Subtask 1. Isolation of $CD45^-$ stromal cells from human thymus by magnetic bead separation, and verification that isolation procedure enriches for mTEC population.	6-8	100%
Subtask 2. Incubation of human $CD45^-$ thymic stromal cells with 100, 500, and 1000 ng/ml of recombinant human soluble RANKL or vehicle control. After 24 hour incubation, mTEC frequency within $CD45^-$ stromal cells will be determined by flow cytometry for each culture condition.	8-12	75%
Subtask 3. Determine relative Aire expression levels by quantitative RT-PCR in $CD45^-$ stromal cells after incubation with 100, 500, and 1000 ng/ml of recombinant human soluble RANKL or vehicle control.	8-12	75%

What was accomplished under these goals?

1) Major activities: We have obtained HRPO approval and local IRB approval. To date, we have collected 49 human thymus samples in collaboration with Dr. Jennifer Nelson, MD at UNC Chapel Hill. As outlined in the SOW, we have been focused on determining whether RANK and RANKL are expressed on the mTECs and thymocytes within human thymus, respectively. Additionally, we have been focused on determining whether addition of RANKL protein increases the expression of Aire and the proportion of mTECs in vitro. Our major activities have been isolating human thymic epithelia, culturing them in tissue culture conditions, and performing flow cytometry to compare gene expression differences between thymic cell populations.

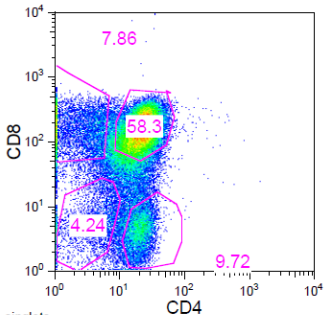

Figure 1. Representative flow cytometry plot of thymocyte subpopulations from human thymus. DP= CD4+ CD8+; DN= CD4-CD8-; CD4SP= CD4+ CD8-; CD8SP= CD4-CD8+

2) Specific objectives: We have obtained HRPO approval and local IRB approval. As outlined in the SOW, we have been focused on determining whether RANK and RANKL are expressed on the mTECs and thymocytes within human thymus, respectively. Additionally, we have been focused on determining whether addition of RANK increases the expression of Aire and the proportion of mTECs in vitro.

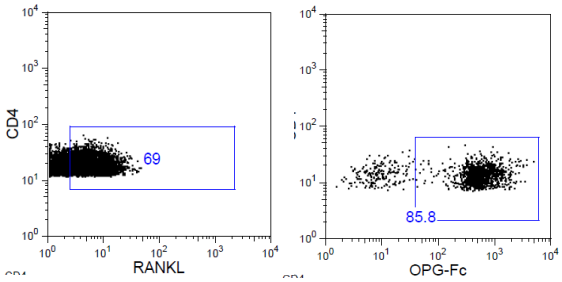

Figure 2. Representative flow cytometry plot of RANKL among CD4SP thymocyte subpopulations from human thymus. Thymocytes were stained with anti-RANKL antibody (left) or OPG-Fc.

3) Significant results: Significant progress has been made in delineating the human thymocyte populations that express RANKL. Four thymocyte subpopulations (Double positive or DP; Double negative or DN; CD4+ single positive or CD4SP and CD8+ single positive or CD8SP) were delineated, as shown in Figure 1. Using anti-RANKL antibody to detect RANKL expression, our data indicate that RANKL is expressed by the majority of human CD4 single positive (CD4SP) cells, and by approximately 40% of CD8 single positive (CD8SP), double positive (DP), and double negative (DN) thymocytes (Figures 2 and 3). These findings are significant because they a) recapitulate what is seen in mice, and b) suggest that RANKL is present on thymocytes and therefore can be targeted for therapy by anti-RANKL antibody.

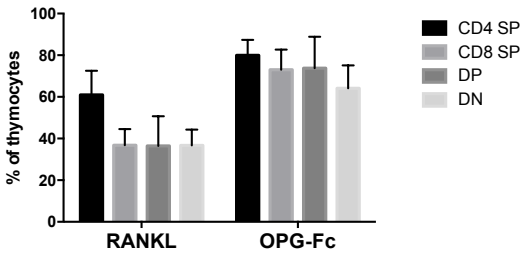

Figure 3. Average frequency of RANKL expression in the 4 subpopulations of human thymocytes. Cells were stained for RANKL using either anti-RANKL antibody (left) or OPG-Fc (right).

Signficant progress has also been made in determining RANK expression in mTECs. Approximately 21% of CD45-, EpCAM+, CDR2low mTEC cells express RANK (Figures 4 and 5). In contrast, <5% of CD45-, EpCAM+, CDR2high cTECs express RANK. This is consistent with reports of RANK expression in mouse thymic epithelial cells, which suggests that blocking RANKL signaling will have similar effects in human and mouse mTECs.

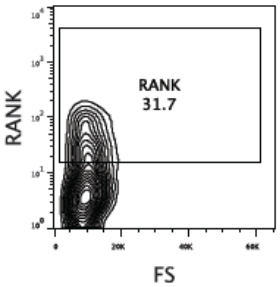

Figure 4. Representative flow cytometry plot of RANK expression in mTECs. Cells were stained with anti-RANK antibody.

Thymic epithelial cells can be identified by low expression of CD45 and expression of EpCAM (Figure 6). We have made considerable efforts in troubleshooting CD45 negative isolation of thymic epithelial

cells using CD45 magnetic bead separation. Because of the large amount of dead cells in the thymus, many cells were nonspecifically sticking to the beads. We believe we have circumvented this issue by first spinning down the cells in Ficoll graident to separate and remove dead cells. We now see approximately 75% CD45 negative cells in the flow through, and 77% CD45 positive cells on the CD45 magnetic beads. We have successfully detected mTECs by flow cytometry and Aire by qRT-PCR, so are poised to complete Major Task 2 in the next few weeks.

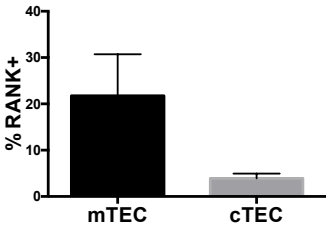

Figure 5. Average frequency of RANK expression mTECs and cTECs. Cells were stained with anti-RANK antibody.

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

I have benefited from an active mentoring relationship with Dr. Norman Sharpless and Dr. Nancy Thomas. We meet on a one-to-one basis to review progress and obstacles in this project. A highlight of my training and professional development this year has been attending the Melanoma Research Alliance (MRA) Scientific Meeting in Washington DC. This provided me an opportunity to network with colleagues and hear what other investigators are exploring. Another highlight was our participation in the Southeast Immunology Symposium in Durham, NC, where we presented our work in a poster session. Pearl Bakhru, postdoctoral fellow in my lab, was first author on this project and I was senior author.

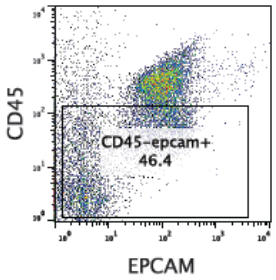

Figure 6. Representative flow plot of thymus cells stained with EpCAM and CD45.

How were the results disseminated to communities of interest?
Nothing to Report.

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

The plan for the next reporting period is outlined below:

	Months
Major Task 3. Effects of in vitro blockade of RANK-RANKL interactions on human mTECs.	
Subtask 1. Culture human thymus sections with anti-RANKL antibody or isotype control at 10, 20 and 50 mcg/mL. After two week incubation, we will determine the frequency of mTECs by flow cytometry for each culture condition.	12-15
Subtask 2. Culture human thymus sections with anti-RANKL antibody or isotype control at 10, 20 and 50 mcg/mL. After two week incubation, we will determine relative Aire expression by quantitative RT-PCR in cultured thymic tissue.	12-15
Subtask 3. Culture human thymus sections with OPG-Fc or vehicle control. After two week incubation, we will determine the frequency of mTECs by flow cytometry for each culture condition.	15-18
Subtask 4. Culture human thymus sections with OPG-Fc or vehicle control. After two week incubation, we will determine relative Aire expression by quantitative RT-PCR in cultured thymic tissue.	15-18
ACURO approval	13-14
Major Task 1. Effect of concurrent anti-RANKL and	

checkpoint inhibitor antibody administration on intratumoral Teff:Treg ratios in melanoma-bearing mice.

Subtask 1. Flow cytometric analysis of tumor infiltrating Teff:Treg cells in C57BL/6 mice injected with B16 melanoma cells and treated with 1) anti-RANKL + isotype control 2) isotype control + anti-CTLA-4 3) anti-RANKL + anti-CTLA-4 or 4) isotype control + isotype control.	15-24
Subtask 2. Flow cytometric analysis of tumor infiltrating Teff:Treg cells in Tyr-CRE-ER^{T2}; Braf$^{CA/WT}$; Pten$^{F/F}$ mice treated with 1) anti-RANKL + isotype control 2) isotype control + anti-CTLA-4 3) anti-RANKL + anti-CTLA-4 or 4) isotype control + isotype control.	15-24
Subtask 3. Flow cytometric analysis of tumor infiltrating Teff:Treg cells in Tyr-CRE-ER^{T2}; LSL-Kras^{G12D}; Lkb1$^{L/L}$; P53$^{L/L}$ treated with 1) anti-RANKL + isotype control 2) isotype control + anti-CTLA-4 3) anti-RANKL + anti-CTLA-4 or 4) isotype control + isotype control.	15-24
Total number of mice	

Major Task 2. Effect of concurrent anti-RANKL and checkpoint inhibitor antibody administration on melanoma growth and host survival.

Subtask 1. Measure tumor growth in and host survival of C57BL/6 mice injected with B16 melanoma cells and treated with 1) anti-RANKL + isotype control 2) isotype control + anti-CTLA-4 3) anti-RANKL + anti-CTLA-4 or 4) isotype control + isotype control.	13-24
Subtask 2. Measure tumor growth in and host survival of Tyr-CRE-ER^{T2}; Braf$^{CA/WT}$; Pten$^{F/F}$ mice treated with 1) anti-RANKL + isotype control 2) isotype control + anti-CTLA-4 3) anti-RANKL + anti-CTLA-4 or 4) isotype control + isotype control.	13-24
Subtask 3. Measure tumor growth in and host survival of Tyr-CRE-ER^{T2}; LSL-Kras^{G12D}; Lkb1$^{L/L}$; P53$^{L/L}$ treated with 1) anti-RANKL + isotype control 2) isotype control + anti-CTLA-4 3) anti-RANKL + anti-CTLA-4 or 4) isotype control + isotype control.	13-24

IMPACT:

What was the impact on the development of the principal discipline(s) of the project?
Based on our findings, we are planning a Phase 2 clinical trial in human melanoma patients in which anti-RANKL antibody and checkpoint inhibitors are used in combination. If anti-RANKL antibody increases the effectiveness of checkpoint inhibitors, this could potentially have a major impact on how melanoma patients with advanced disease are treated in the clinic. These plans will be informed by our findings from this project proposal.

What was the impact on other disciplines?
Nothing to report.
What was the impact on technology transfer?
Nothing to report.
What was the impact on society beyond science and technology?
Nothing to report.

CHANGES/PROBLEMS:

Changes in approach and reasons for change

As mentioned above, we have had to include an extra step to remove dead cells from our thymus cell preparation. This has slightly delayed our finishing our analysis of CD45 negative thymus cells. This change in approach has resolved the problem, and we anticipate that Major Task 2 will be completed in the next few weeks.

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
Nothing to report.

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.

PRODUCTS:

Nothing to report.

- ***Publications, conference papers, and presentations***
  Nothing to report.
- ***Journal publications***
  Nothing to report.
- ***Books or other non-periodical, one-time publications***
  Nothing to report.
- ***Other publications, conference papers, and presentations***
  Nothing to report.
- ***Website(s) or other Internet site(s)***
  Nothing to report.
- ***Technologies or techniques***
  Nothing to report.
- ***Inventions, patent applications, and/or licenses***
  Other Products
  Nothing to report.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Maureen Su
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	4
Contribution to Project:	Dr. Su oversees this project and designs experiments.
Funding Support:	N/A
Name:	Pearl Bakhru

Project Role:	Postdoctoral Fellow
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	12
Contribution to Project:	Dr. Bakhru performs experiments on a day to day basis.
Funding Support:	N/A

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.

SPECIAL REPORTING REQUIREMENTS

- ***COLLABORATIVE AWARDS:*** Nothing to report.
- ***QUAD CHARTS:*** Nothing to report.

APPENDICES: Nothing to report.