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TITLE: Central Tolerance Blockade to Augment Checkpoint Immunotherapy
in Melanoma

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13. SUPPLEMENTARY NOTES					
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), addition of anti-RANKL antibody to human thymus cell culture decreases mTEC frequency, and addition of recombinant RANKL increases expression of mTEC-specific Autoimmune Regulator (Aire) gene. In mice, antiRANKL antibody and checkpoint inhibition have additive effects in increasing survival in response to melanoma challenge. These findings lend preclinical evidence for using anti-RANKL antibody to block central tolerance in the clinical setting.					
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?
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	90%
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	90%
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	90%
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	90%
ACURO approval	13-14	100%
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	15-24	100%

Teff:Treg cells in 4-8 week old 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 4) isotype control + isotype control 5) isotype control + anti-CTLA-4 + anti-PD1 and 6) anti-RANKL + anti-CTLA-4 + anti-PD1.

Subtask 3. 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 4) isotype control + isotype control 5) isotype control + anti-CTLA-4 + anti-PD1 and 6) anti-RANKL + anti-CTLA-4 + anti-PD1.

15-24 100%

Subtask 4. 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 4) isotype control + isotype control 5) isotype control + anti-CTLA-4 + anti-PD1 and 6) anti-RANKL + anti-CTLA-4 + anti-PD1.

15-24 60%

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 4-8 week old 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 4) isotype control + isotype control 5) isotype control + anti-CTLA-4 + anti-PD1 and 6) anti-RANKL + anti-CTLA-4 + anti-PD1.

13-24 100%

Subtask 3. 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 4) isotype control + isotype control 5) isotype control + anti-CTLA-4 + anti-PD1 and 6) anti-RANKL + anti-CTLA-4 + anti-PD1.

13-24 100%

Subtask 4. Measure tumor growth in and host survival of Tyr-CRE-ER^{T2}; LSL-Kras^{G12D}; Lkb1^{L/L}; P53^{L/L} mice treated with 1) anti-RANKL + isotype control 2) isotype control + anti-CTLA-4 3) anti-RANKL + anti-CTLA-4 4) isotype control + isotype control 5) isotype control + anti-CTLA-4 + anti-PD1 and 6) anti-RANKL + anti-CTLA-4 + anti-PD1.

13-24 60%

What was accomplished under these goals?

1) Major activities: In this reporting period, we have obtained ACURO approval and local IACUC approval. To date, we have collected 66 human thymus samples in collaboration with Dr. Jennifer Nelson, MD. As outlined in the SOW, we have been focused on performing tissue culture experiments to determine the effects of blocking RANK/RANKL interactions on human mTECs. Additionally, we have focused on mouse studies to determine the effects of anti-RANKL and checkpoint inhibitory antibody administration on intracellular T cells, melanoma

mouse studies to determine the effects of anti-RANKL and checkpoint inhibitory antibody administration on intracellular T cells, melanoma growth and host survival. We have performed our studies in both B16 melanoma model and in spontaneous models.

2) Specific objectives: We have obtained ACURO approval and local IACUC approval. As outlined in the SOW, we have been focused on determining the effects of blocking RANK/RANKL interactions on human mTECs; effects of anti-RANKL and checkpoint inhibitory antibody administration on intratumoral T cells; and effects of anti-RANKL and checkpoint inhibitory antibody administration on melanoma growth and host survival.

3) Significant results: Significant progress has been made in delineating the effect of blocking RANK/RANKL interactions. As predicted by our hypothesis, addition of aRANKL antibody to in vitro culture of human thymus cells appears to decrease the frequency of mTECs (Figure 1). In paired samples that received either isotype control antibody or anti-RANKL antibody, mTEC frequency was decreased in the majority of pairs tested. This finding suggests that RANK/RANKL interaction is also important for mTEC development in humans. Our result is on the cusp of reaching statistical significance ($p=0.05$ by ratio paired t-test), and we currently collecting our last few data points. As further predicted by our hypothesis, adding recombinant RANKL increases Aire expression in human mTECs (Figure 2). Together, these findings provide strong evidence that RANK/RANKL interactions also control development of Aire-expressing mTECs.

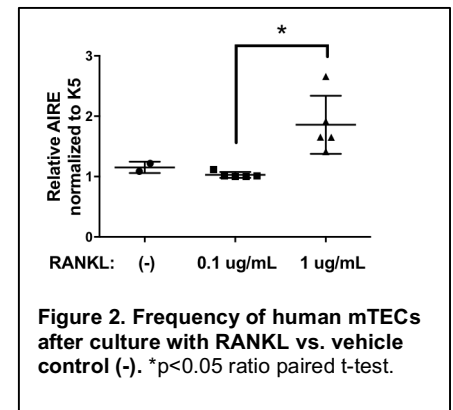


Figure 2. Frequency of human mTECs after culture with RANKL vs. vehicle control (-). * $p < 0.05$ ratio paired t-test.

Significant progress has been made in determining the in vivo effects of combining anti-RANKL and anti-CTLA-4 antibody. We have been testing the effects of anti-RANKL antibody and/or anti-CTLA-4 antibodies on melanoma growth and survival in both B16 melanoma model and spontaneous models of melanoma. Intratumoral T cells isolated from both B16 melanoma and spontaneous

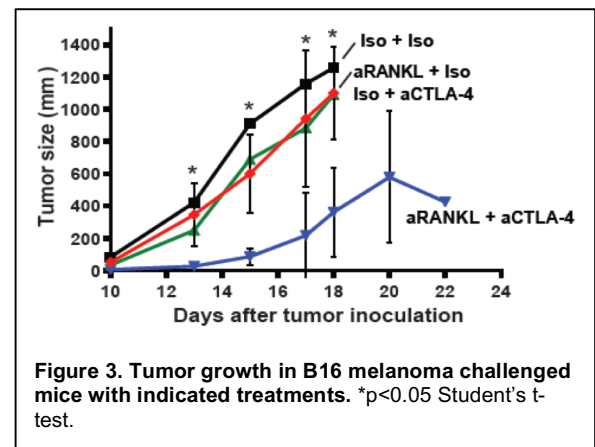


Figure 3. Tumor growth in B16 melanoma challenged mice with indicated treatments. * $p < 0.05$ Student's t-test.

melanoma have increased frequencies of T cells expressing markers of activation (data not shown). In the B16 melanoma model, tumor growth is decreased (Figure 3) and survival is increased (Figure 4) in mice receiving both anti-RANKL and anti-CTLA-4 antibodies in combination, compared to mice receiving anti-RANKL or anti-CTLA-4 antibodies as single agents. Significant differences were noted at Day 13, 15, 17, and 18 in the size of tumors between anti-RANKL/CTLA-4 antibody treatment and either anti-RANKL antibody or anti-CTLA-4 antibody alone. Furthermore, survival of mice treated with both anti-RANKL and anti-CTLA-4 antibodies in combination was significantly improved compared to either anti-RANKL antibody or anti-CTLA-4 antibody alone. This finding suggests that blockade of RANKL and CTLA-4 have synergistic effects in enhancing anti-melanoma immunity.

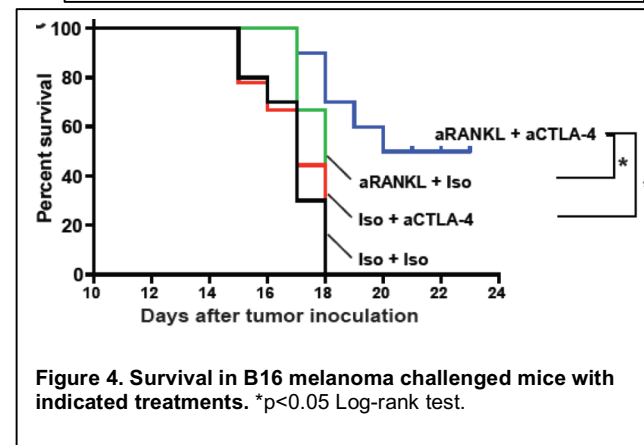
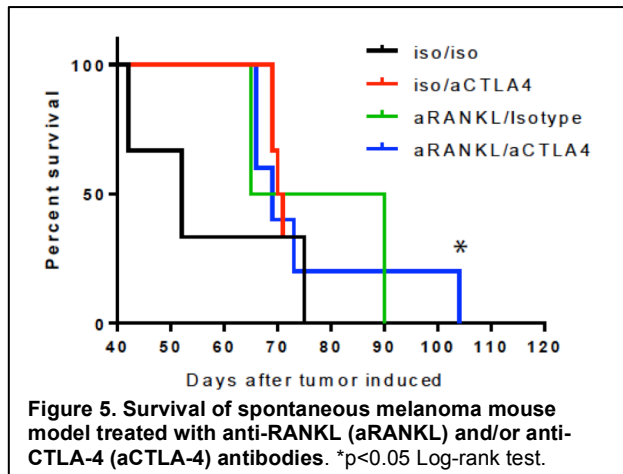
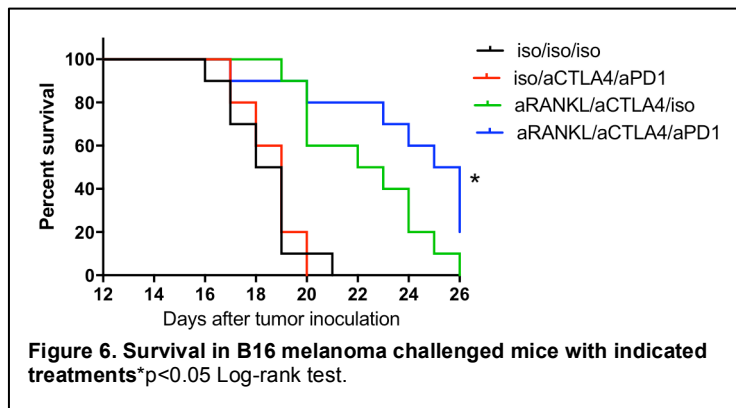


Figure 4. Survival in B16 melanoma challenged mice with indicated treatments. * $p < 0.05$ Log-rank test.

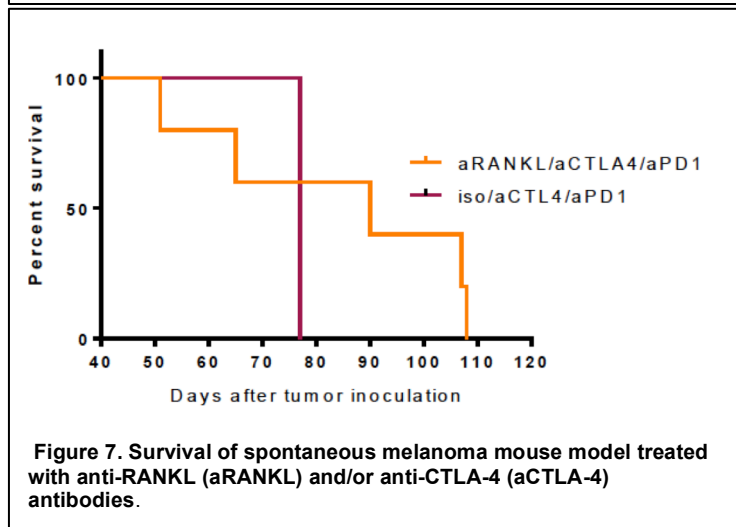


enhancing anti-melanoma immunity.

In the spontaneous Tyr-CRE-ER^{T2}; Braf^{CA/WT}; Pten^{F/F} mouse model of melanoma, anti-RANKL and anti-CTLA-4 antibodies in combination had additive effects in improving survival (Figure 5). Survival of mice treated with both anti-RANKL and anti-CTLA-4 antibodies in combination was significantly improved compared to either anti-RANKL antibody or anti-CTLA-4 antibody alone. These findings are of potential clinical importance because it may pave the way to testing the novel combination of anti-CTLA antibody and anti-RANKL antibody for



metastatic melanoma patients. Of note, these results have been written up in a manuscript that is currently under revision at the journal JCI Insight.



We have also made substantial progress in determining the effects of anti-RANKL antibodies and anti-CTLA-4/PD1 as a triple combination. As shown in Figure 6, triple combination (blue line) significantly improved survival in the B16 melanoma model. Furthermore, as shown in Figure 7, triple combination (orange line) also significantly improved survival in the spontaneous Tyr-CRE-ER^{T2}; Braf^{CA/WT}; Pten^{F/F} mouse model of melanoma. Together, these findings suggest that combining anti-RANKL/CTLA-4/PD-1 is an additive therapeutic combination in multiple mouse models of melanoma.

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 and to review progress and obstacles in this project. Pearl Bakhru, postdoctoral fellow in my lab, presented a poster at the UNC Postdoc Scholars Symposium and was awarded first place for her poster presentation. As stated above, this work is currently in revision at JCI Insight.

How were the results disseminated to communities of interest?

Nothing to Report. As stated above, this work is currently in revision at JCI Insight.

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:

Major Task 2. Effects of soluble RANKL on human mTEC cellularity.	Months
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Subtask 1. Isolation of CD45- stromal cells from human thymus by magnetic bead separation, and verification that isolation procedure enriches for mTEC population.	24-30
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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.	24-30
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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.	24-30
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Major Task 3. Effects of in vitro blockade of RANK-RANKL interactions on human mTECs.	
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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.	30-36
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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.	30-36
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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.	30-36
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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.	
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Major Task 1. Effect of concurrent anti-RANKL and checkpoint inhibitor antibody administration on intratumoral Teff:Treg ratios in melanoma-bearing mice.	
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Subtask 2. Flow cytometric analysis of tumor infiltrating Teff:Treg cells in 18 month old 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 4) isotype control + isotype control 5) isotype control + anti-CTLA-4 + anti-PD1 and 6) anti-RANKL + anti-CTLA-4 + anti-PD1.	24-36
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Subtask 5. Flow cytometric analysis of tumor infiltrating Teff:Treg	24-36
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cells in C57BL/6 mice injected with B16 melanoma cells and treated with 1) anti-RANKL + isotype control 2) isotype control + anti-PD-1 3) anti-RANKL + anti-PD-1 4) isotype control + isotype control 5) isotype control + anti-CTLA-4 + anti-PD1 and 6) anti-RANKL + anti-CTLA-4 + anti-PD1.

Subtask 6. 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-PD-1 3) anti-RANKL + PD-1 4) isotype control + isotype control 5) isotype control + anti-CTLA-4 + anti-PD1 and 6) anti-RANKL + anti-CTLA-4 + anti-PD1.

24-36

Subtask 7. 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-PD-1 3) anti-RANKL + anti-PD-1 4) isotype control + isotype control 5) isotype control + anti-CTLA-4 + anti-PD1 and 6) anti-RANKL + anti-CTLA-4 + anti-PD1.

24-36

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

Subtask 2. Measure tumor growth in and host survival of 18 week old 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 4) isotype control + isotype control 5) isotype control + anti-CTLA-4 + anti-PD1 and 6) anti-RANKL + anti-CTLA-4 + anti-PD1.

24-36

Subtask 5. 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-PD-1 3) anti-RANKL + anti-PD-1 4) isotype control + isotype control 5) isotype control + anti-CTLA-4 + anti-PD1 and 6) anti-RANKL + anti-CTLA-4 + anti-PD1.

24-36

Subtask 6. 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-PD-1 3) anti-RANKL + anti-PD-1 4) isotype control + isotype control 5) isotype control + anti-CTLA-4 + anti-PD1 and 6) anti-RANKL + anti-CTLA-4 + anti-PD1.

24-36

Subtask 7. 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-PD-1 3) anti-RANKL + anti-PD-1 4) isotype control + isotype control 5) isotype control + anti-CTLA-4 + anti-PD1 and 6) anti-RANKL + anti-CTLA-4 + anti-PD1.

24-36

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

Dr. Jennifer Nelson, MD, who was providing human thymus samples to us, changed institutions as is currently at Nemours Hospital in Orlando, FL. She has continued to ship samples to us from Nemours for this project, with approval from her institution's IRB. There has been a slight delay as a result of her move, and we anticipate Major Task 3 to be complete in the next few weeks.

Also, breeding of Tyr-CRE-ER^{T2}; LSL-Kras^{G12D}; Lkb1^{L/L}; P53^{L/L} mice has been slower than expected. These mice have not been born in adequate numbers for us to complete Subtask 3 (Major Task 2) or Subtask 3 (Major Task 3) but we anticipate that our breeding cages now set up should be adequate to complete these tasks in the next few months.

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:	Maureen 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:	2.8
Contribution to Project:	Pearl Bakhru performs experiments on a day to day basis.
Funding Support:	N/A
Name:	David Sailer
Project Role:	Research Assistant
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	6
Contribution to Project:	David Sailer 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.