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TITLE: Targeting Lymphatics to Treat Trauma-Induced Heterotopic Ossification

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CONTRACTING ORGANIZATION: University of Texas Southwestern Medical Center

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14. ABSTRACT

Heterotopic ossification (HO) is the abnormal growth of bone in non-skeletal tissues after trauma. Patients with HO may have difficulty performing normal daily activities because they have decreased range of motion of involved joints or suffer from severe debilitating pain. HO is caused by aberrant tissue repair and is a common complication of trauma. Extremity trauma coupled with a bone fracture is a common form of injury in military personnel and confers an increased risk of HO. The prevalence of HO is further increased in patients with a combination of musculoskeletal trauma and large surface-area burns, which is frequently seen in the setting of blast-injuries. Current treatments for HO include nonsteroidal anti-inflammatory drugs (NSAIDs) and surgery. However, these treatments are inadequate for many patients. Therefore, there is an urgent need for new therapies to prevent HO formation and reverse existing HO lesions. Our project is focused on investigating the role lymphatic vessels serve in HO formation. Lymphatic vessels play a role in maintaining tissue fluid homeostasis, trafficking immune cells, and resolving inflammation after injury. There is growing evidence that the immune system serves a critical function the formation and progression of HO. However, the precise role lymphatic vessels serve in the pathophysiology of HO is poorly understood. Filling this gap in knowledge could lead to new and novel treatments for HO and other post-traumatic complications associated with aberrant wound repair.

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1. INTRODUCTION:

Heterotopic ossification (HO) is the abnormal growth of bone in non-skeletal tissues after trauma. Patients with HO may have difficulty performing normal daily activities because they have decreased range of motion of involved joints or suffer from severe debilitating pain. HO is caused by aberrant tissue repair and is a common complication of trauma. Extremity trauma coupled with a bone fracture is a common form of injury in military personnel and confers an increased risk of HO. The prevalence of HO is further increased in patients with a combination of musculoskeletal trauma and large surface-area burns, which is frequently seen in the setting of blast-injuries. Current treatments for HO include nonsteroidal anti-inflammatory drugs (NSAIDs) and surgery. However, these treatments are inadequate for many patients. Therefore, there is an urgent need for new therapies to prevent HO formation and reverse existing HO lesions. Our project is focused on investigating the role lymphatic vessels serve in HO formation. Lymphatic vessels play a role in maintaining tissue fluid homeostasis, trafficking immune cells, and resolving inflammation after injury. There is growing evidence that the immune system serves a critical function the formation and progression of HO. However, the precise role lymphatic vessels serve in the pathophysiology of HO is poorly understood. Filling this gap in knowledge could lead to new and novel treatments for HO and other post-traumatic complications associated with aberrant wound repair.

2. KEYWORDS:

Heterotopic ossification, lymphangiogenesis, VEGF-C, VEGFR3, bone

3. ACCOMPLISHMENTS:

What were the major goals of the project?

The project contains two aims as stated in the SOW:

- Aim 1: Determine the phenotypic and functional consequences of lymphatic deletion on HO formation and the effect of lymphatic augmentation on the resorption of existing HO lesions.

- Aim 2: To validate that a small molecule lymphatic antagonist (VEGFR3 inhibitor) and surgical sentinel lymph node excision strategy will prevent traumatic heterotopic ossification.

What was accomplished under these goals?

Evaluating lymphatic vessels post-B/T

Prox1-eGFP reporter mice strongly express green fluorescent protein (GFP) in lymphatic vessels. We utilized our previously validated burn/tenotomy (B/T) model of trauma-induced heterotopic ossification (HO) to characterize the effect of HO formation on the temporospatial patterning of lymphatic vessels in *Prox1-eGFP* mice. Tissue samples were collected from *Prox1-eGFP* mice 6- and 9-weeks post-B/T and immunostained for Lyve1 and podoplanin. Triple-positive (GFP^+, $Lyve1^+$, and $podoplanin^+$) structures were considered to be lymphatic vessels. For both timepoints we found that lymphatic vessels were closely associated with heterotopic bone and that lymphatic vessels had invaded the tendon (**Figures 1 & 2**). We are continuing to analyze these samples and are adding samples from uninjured *Prox1-eGFP* mice and from *Prox1-eGFP* mice 1- and 3-weeks post-B/T.

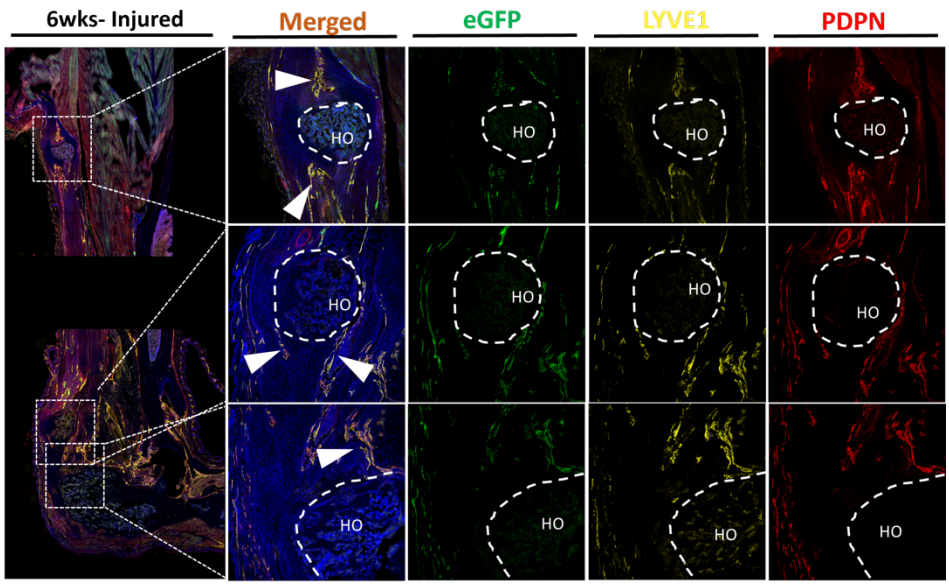

Figure 1. Triple-positive (GFP$^+$, LYVE1$^+$, and PDPN$^+$) lymphatic vessels are closely associated with heterotopic bone. Immunofluorescence imaging demonstrating co-localization of Prox1eGFP$^+$ cells (green) with LYVE1$^+$(Yellow) and PDPN$^+$(red) cells. White arrows show lymphatic vessels near the HO site (notated by dashed circle) at 6 weeks post-injury.

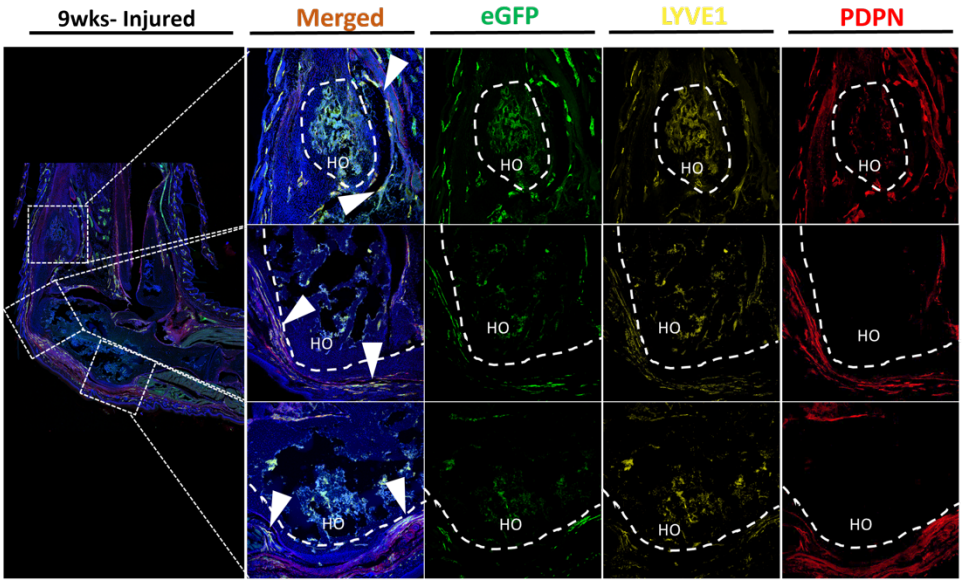

Figure 2. Triple-positive (GFP$^+$, LYVE1$^+$, and PDPN$^+$) lymphatic vessels are closely associated with heterotopic bone. Immunofluorescence imaging demonstrating co-localization of Prox1eGFP$^+$ cells (green) with LYVE1$^+$(Yellow) and PDPN$^+$(red) cells. White arrows show lymphatic vessels near the HO site (notated by dashed circle) at 9 weeks post-injury.

Single-cell transcriptomic analysis of lymphatic endothelial cells post-B/T

Next, scRNA-Seq was used to investigate the molecular crosstalk between lymphatic endothelial cells and other cell types in the injury site 7 days post-B/T. Our existing scRNA-Seq data set for the injury site had a low number of lymphatic endothelial cells. To increase the size of this population, we isolated GFP$^+$ cells from the tendon injury site in *Prox1-eGFP* mice by FACS and analyzed the cells

by scRNA-Seq. This new scRNA-Seq dataset was merged with our existing scRNA-Seq dataset (**Figure 3**) and the R package CellChat was used to look at ligand-receptor interactions between cells. This revealed an interaction between MPCs (express VEGF-C and -D) and lymphatic endothelial cells (express VEGFR3). The growth of lymphatic vessels during development and disease is driven by VEGFR3. These results suggest that MPCs could be promoting lymphangiogenesis at the injury site in our model. We are continuing to analyze our scRNA-Seq data and adding more timepoints to our data set.

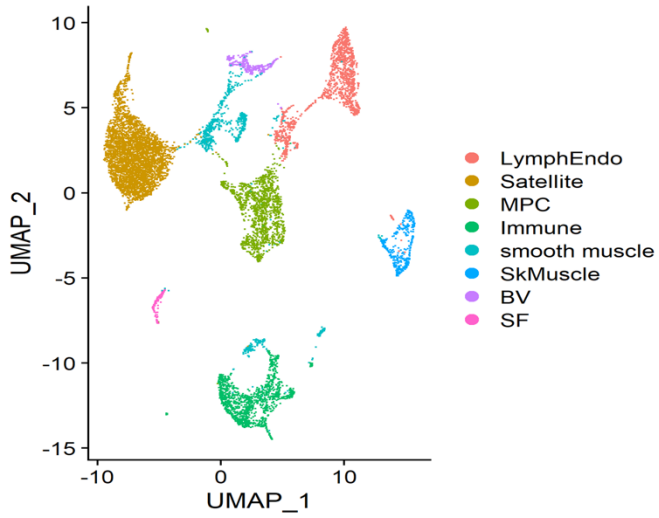

Figure 3. UMAP of merged scRNA-Seq data. scRNA-Seq data obtained from mice 7 days post-B/T was merged with scRNA-Seq data for isolated lymphatic endothelial cells isolated 7 days post-B/T.

Characterizing the effect of lymphatic insufficiency on HO formation

VEGFR3$^{wt/Chy}$ mice are heterozygous for an inactivating mutation in the kinase domain of VEGFR3 and are reported to have fewer lymphatic vessels than wildtype mice. To confirm that *VEGFR3*$^{wt/Chy}$ mice have lymphatic vessel hypoplasia, we analyzed lymphatic vessels in the skin of *Vegfr3*$^{wt/wt}$ and *Vegfr3*$^{wt/Chy}$ mice. We found that *Vegfr3*$^{wt/Chy}$ mice have significantly fewer lymphatic vessels than *Vegfr3*$^{wt/wt}$ mice (**Figure 4**). We then performed our B/T procedure on *Vegfr3*$^{wt/wt}$ and *Vegfr3*$^{wt/Chy}$ mice to characterize the effect of lymphatic insufficiency on HO formation. Samples were collected from mice 9 weeks post-B/T and heterotopic bone was measured by microcomputed tomography (μCT). This revealed that heterotopic bone volume was not significantly different between *Vegfr3*$^{wt/wt}$ and *Vegfr3*$^{wt/Chy}$ mice (**Figure 5**). We are still in the process of immunostaining samples from this experiment to evaluate lymphatic vessels around heterotopic bone in *Vegfr3*$^{wt/wt}$ and *Vegfr3*$^{wt/Chy}$ mice. To extend our observations with *Vegfr3*$^{wt/Chy}$ mice, we acquired *Vegfc*$^{wt/CreERT2}$ mice. These mice have a CreER^{T2} cassette knocked-in to the endogenous VEGF-C locus. We have found that *Vegfc*$^{wt/CreERT2}$ have significantly fewer lymphatic vessels than wildtype mice (**Figure 6**). We are using *Vegfc*$^{wt/CreERT2}$ mice to show that VEGF-C-lineage cells give rise to heterotopic bone and to investigate the effect of lymphatic insufficiency on HO formation.

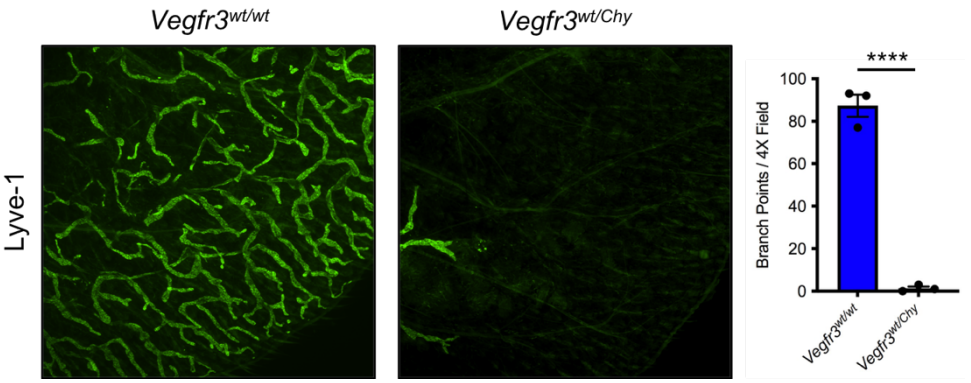

Figure 4. Whole-mount immunofluorescence staining of ear skin for Lyve-1. *Vegfr3*$^{wt/Chy}$ mice have significantly fewer lymphatic vessels than *Vegfr3*$^{wt/wt}$ mice. **** $P < 0.0001$; unpaired Student's *t*-test.

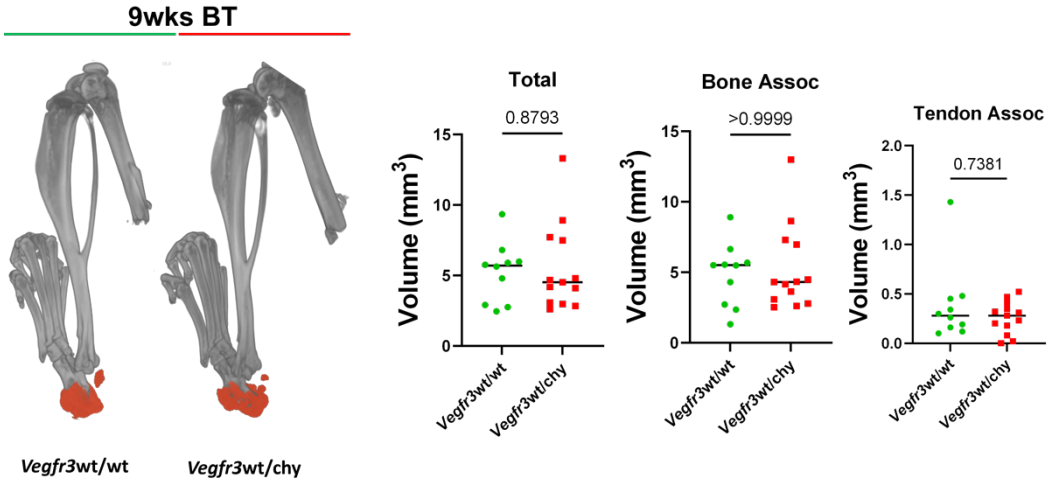

Figure 5. µCT quantification shows no significant changes in HO volume of *Vegfr3*$^{wt/Chy}$ animals compared to control animals. 3D µCT of distal hindlimb 9 weeks after surgery demonstrating HO formation (orange regions). Quantification of HO volume within *Vegfr3*$^{wt/Chy}$ animals compared to *Vegfr3*$^{wt/wt}$ animals. Unpaired Student's *t*-test

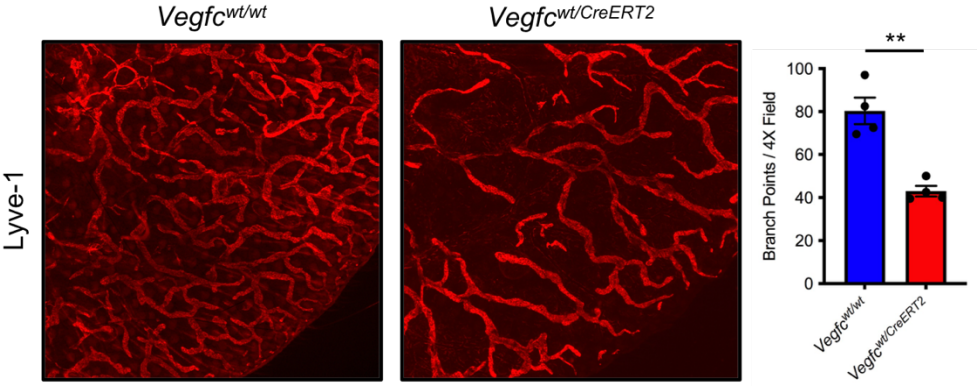

Figure 6. Whole-mount immunofluorescence staining of ear skin for Lyve-1. *Vegfc*$^{wt/CreERT2}$ mice have significantly fewer lymphatic vessels than *Vegfc*$^{wt/wt}$ mice. ** $P < 0.01$; unpaired Student's *t*-test.

Characterizing the effect of lymphangiogenesis on HO formation

Lymphatic vessels are not present in the bones of healthy individuals. However, lymphatic vessels invade bone and promote bone resorption in patients with Gorham-Stout disease. It is not known whether lymphatic vessels can invade heterotopic bone or promote heterotopic bone resorption. VEGF-D is a growth factor that promotes VEGFR3-mediated lymphangiogenesis and lymphatic vessel invasion of bone. To determine the effect of hyperactive lymphangiogenesis on HO formation, we used a genetic approach to overexpress VEGF-D in Hoxa11-lineage cells. Samples were collected from *Hoxa11-Ctrl* and *Hoxa11-Vegfd* mice 9 weeks post-B/T and heterotopic bone was measured by μCT. Importantly, we found that *Hoxa11-Vegfd* mice have significantly less proximal HO than *Hoxa11-Ctrl* mice (**Figure 7**). These preliminary results suggest that overexpressing VEGF-D for 9 weeks can affect HO formation, maintenance, or both. We are continuing to enroll mice in this experiment and plan on analyzing lymphatic vessels and other cell types by immunofluorescence staining.

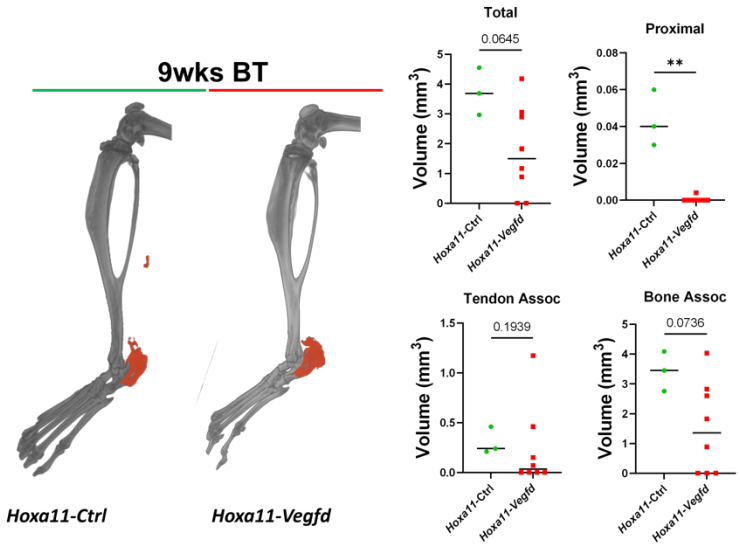

Figure 7. μCT quantification shows reduction in HO volume of VEGF-D overexpressing animals compared to control animals. 3D μCT of distal hindlimb 9 weeks after surgery demonstrating HO formation (orange regions). Quantification of HO volume within VEGF-D overexpressed animals compared to control. ** $P < 0.01$; unpaired Student's *t*-test.

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

The project has provided numerous opportunities for Ms. Griswold-Wheeler, Cori Booker, Neda Vishlaghi, Pranathi Dasari, Janna Crossley, and Sonya Ostashevskya-Gohstand, to learn surgical techniques and various histology/imaging techniques. The Dellinger and Levi laboratories have a joint meeting 1-2 times a month. This gives trainees and staff an opportunity to present their research, discuss papers, and gain in-depth knowledge about lymphatic vessels and HO. It also allows trainees and staff to receive mentorship from both Drs. Dellinger and Levi.

How were the results disseminated to communities of interest?

Dr. Levi's team presented a poster at Plastic Surgery Research Conference during the second weekend of June (8^{th}-12^{th}) in Toronto, Canada. This received best the Peter Gingrass Award for Best Medical Student Presentation.
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What do you plan to do during the next reporting period to accomplish the goals?
During the next year, we will finish analyzing samples from our *Prox1-eGFP* time course experiment. We will also finish analyzing B/T-induced HO formation in *Vegfr3*$^{wt/Chy}$ and *Vegfc*$^{wt/CreERT2}$ mice. Additionally, we will stain samples from *Hoxa11-Ctrl* and *Hoxa11-Vegfd* mice for lymphatic vessels, various immune cell populations, and cartilage/bone. Last, we will test whether lymphatic vessels induce the resorption of existing HO lesions in *Hoxa11-Vegfd* mice.

4. IMPACT:

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

This study has yielded the first scRNA-Seq data set that includes a high number of lymphatic endothelial cells at an injury site. This valuable data set will be used by our group to investigate the molecular mechanisms driving lymphangiogenesis post-B/T and will be made publicly available after we publish our results. Our study has also produced data that suggests that lymphatic vessels negatively impact HO formation. Understanding the molecular mechanisms by which lymphatic vessels impair HO formation could lead to new treatments for this disabling disease.

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.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to Report.

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.

6. PRODUCTS:

- **Publications, conference papers, and presentations**

  Journal publications.

  Nothing to Report.

  Books or other non-periodical, one-time publications.

  Nothing to Report.

Other publications, conference papers and presentations.
Dr. Levi's team presented a poster at Plastic Surgery Research Conference during the second weekend of June (8^{th}-12^{th}) in Toronto, Canada. This received best the Peter Gingrass Award for Best Medical Student Presentation

- **Website(s) or other Internet site(s)**

  Nothing to Report.

- **Technologies or techniques**

  Nothing to Report.

- **Inventions, patent applications, and/or licenses**

Nothing to Report.

- **Other Products**

  Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Michael Dellinger
Project Role:	PI
Researcher Identifier:	ORCID ID: 0000-0002-3315-4239
Nearest person month worked:	1.8
Contribution to Project:	Dr. Dellinger is responsible for overseeing the planning, performance, and interpretation of experiments. He is also responsible for preparing manuscripts, reports, and ensuring all aspects of the project are compliant with UTSW and DOD policies.
Name:	Danielle Griswold-Wheeler
Project Role:	Research Technician II (Dellinger Lab)
Researcher Identifier:	ORCID ID: 0000-0002-2382-6361
Nearest person month worked:	11
Contribution to Project:	Ms. Griswold-Wheeler is responsible for managing the animal colony, genotyping mice, and administering drugs to mice.
Name:	Lorenzo Fernandes
Project Role:	Research Scientist (Dellinger Lab)
Researcher Identifier:	ORCID ID: 0000-0001-9727-7847
Nearest person month worked:	4
Contribution to Project:	Dr. Fernandes is responsible for data acquisition and analysis.
Name:	Benjamin Levi
Project Role:	PI
Researcher Identifier:	0000-0001-8272-7139
Nearest person month worked:	1.36
Contribution to Project:	Dr. Levi is responsible for overseeing the planning, performance, and interpretation of experiments. He is also responsible for preparing manuscripts, reports, and ensuring all aspects of the project are compliant with UTSW and DOD policies.

Name:	Cori Booker
Project Role:	Postdoctoral Researcher (Levi Lab)
Researcher Identifier:	NA
Nearest person month worked:	4.0
Contribution to Project:	Dr. Booker was responsible for surgeries, histology, flow cytometry, manuscript writing, data interpretation and reporting.
Name:	Neda Vishlaghi
Project Role:	Postdoctoral Researcher (Levi Lab)
Researcher Identifier:	NA
Nearest person month worked:	1.9
Contribution to Project:	Dr. Vishlaghi
Name:	Pranathi Dasari is responsible for surgeries, histology, flow cytometry, manuscript writing, data interpretation and reporting.
Project Role:	Lab Manager (Levi Lab)
Researcher Identifier:	NA
Nearest person month worked:	3.0
Contribution to Project:	Ms. Dasari is responsible for data acquisition and analysis.
Name:	Janna Crossley
Project Role:	Research Assistant II (Levi Lab)
Researcher Identifier:	NA
Nearest person month worked:	2.5
Contribution to Project:	Ms. Crossley is responsible for data acquisition and analysis.
Name:	Sonya Ostashevskaya-Gohstand
Project Role:	Research Associate (Levi Lab)
Researcher Identifier:	NA
Nearest person month worked:	2.5
Contribution to Project:	Ms. Ostashevskaya-Gohstand is responsible for data acquisition and analysis.

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.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *N/A*

QUAD CHARTS: N/A

9. APPENDICES: *N/A*