AWARD NUMBER: W81XWH-15-1-0606

TITLE: Mechanisms and Treatments of Heterotopic Ossification Following Spinal Cord Injuries

PRINCIPAL INVESTIGATOR: Jean-Pierre Levesque

CONTRACTING ORGANIZATION: The University of Queensland
Brisbane 4072
Australia

REPORT DATE: October 2016

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.
Mechanisms and Treatments of Heterotopic Ossification Following Spinal Cord Injuries

Neurological heterotopic ossification (NHO) is a frequent complication of spinal cord injuries. NHO manifests as abnormal ossification of soft tissues near joints. NHO is debilitating, causing pain, joint deformation, ankylosis and vascular and nerve compression. The mechanisms leading to NHO are unknown and the only effective treatment is surgical resection. To elucidate NHO pathophysiology we have developed the first animal model of NHO in genetically unmodified mice. This model shows that formation of NHO requires the combined insult of SCI and soft tissue damage and inflammation via macrophages. To further elucidate the mechanisms driving NHO we treated SCI mice with the TNF-α antagonist etanercept or the CSF-1 receptor kinase inhibitor GW2580 (preventative modality). NHO volumes were measured by µCT 10 days after surgery and confirmed that treatment of mice with either etanercept or GW2580 results in a significant reduction in NHO bone volume (30% and 37% reduction respectively). Finally we confirmed that conditional deletion of the Hif1a gene in myeloid cells (which impairs M1 macrophage polarization) significantly enhanced NHO formation by 47% after SCI and muscle injury. Overall our data suggests that targeting macrophages and/or macrophage mediated inflammation may serve as viable therapies for the treatment of NHO.

Mechanisms and Treatments of Heterotopic Ossification Following Spinal Cord Injuries

Neurological heterotopic ossification (NHO) is a frequent complication of spinal cord injuries. NHO manifests as abnormal ossification of soft tissues near joints. NHO is debilitating, causing pain, joint deformation, ankylosis and vascular and nerve compression. The mechanisms leading to NHO are unknown and the only effective treatment is surgical resection. To elucidate NHO pathophysiology we have developed the first animal model of NHO in genetically unmodified mice. This model shows that formation of NHO requires the combined insult of SCI and soft tissue damage and inflammation via macrophages. To further elucidate the mechanisms driving NHO we treated SCI mice with the TNF-α antagonist etanercept or the CSF-1 receptor kinase inhibitor GW2580 (preventative modality). NHO volumes were measured by µCT 10 days after surgery and confirmed that treatment of mice with either etanercept or GW2580 results in a significant reduction in NHO bone volume (30% and 37% reduction respectively). Finally we confirmed that conditional deletion of the Hif1a gene in myeloid cells (which impairs M1 macrophage polarization) significantly enhanced NHO formation by 47% after SCI and muscle injury. Overall our data suggests that targeting macrophages and/or macrophage mediated inflammation may serve as viable therapies for the treatment of NHO.

Mechanisms and Treatments of Heterotopic Ossification Following Spinal Cord Injuries

Neurological heterotopic ossification (NHO) is a frequent complication of spinal cord injuries. NHO manifests as abnormal ossification of soft tissues near joints. NHO is debilitating, causing pain, joint deformation, ankylosis and vascular and nerve compression. The mechanisms leading to NHO are unknown and the only effective treatment is surgical resection. To elucidate NHO pathophysiology we have developed the first animal model of NHO in genetically unmodified mice. This model shows that formation of NHO requires the combined insult of SCI and soft tissue damage and inflammation via macrophages. To further elucidate the mechanisms driving NHO we treated SCI mice with the TNF-α antagonist etanercept or the CSF-1 receptor kinase inhibitor GW2580 (preventative modality). NHO volumes were measured by µCT 10 days after surgery and confirmed that treatment of mice with either etanercept or GW2580 results in a significant reduction in NHO bone volume (30% and 37% reduction respectively). Finally we confirmed that conditional deletion of the Hif1a gene in myeloid cells (which impairs M1 macrophage polarization) significantly enhanced NHO formation by 47% after SCI and muscle injury. Overall our data suggests that targeting macrophages and/or macrophage mediated inflammation may serve as viable therapies for the treatment of NHO.

Mechanisms and Treatments of Heterotopic Ossification Following Spinal Cord Injuries

Neurological heterotopic ossification (NHO) is a frequent complication of spinal cord injuries. NHO manifests as abnormal ossification of soft tissues near joints. NHO is debilitating, causing pain, joint deformation, ankylosis and vascular and nerve compression. The mechanisms leading to NHO are unknown and the only effective treatment is surgical resection. To elucidate NHO pathophysiology we have developed the first animal model of NHO in genetically unmodified mice. This model shows that formation of NHO requires the combined insult of SCI and soft tissue damage and inflammation via macrophages. To further elucidate the mechanisms driving NHO we treated SCI mice with the TNF-α antagonist etanercept or the CSF-1 receptor kinase inhibitor GW2580 (preventative modality). NHO volumes were measured by µCT 10 days after surgery and confirmed that treatment of mice with either etanercept or GW2580 results in a significant reduction in NHO bone volume (30% and 37% reduction respectively). Finally we confirmed that conditional deletion of the Hif1a gene in myeloid cells (which impairs M1 macrophage polarization) significantly enhanced NHO formation by 47% after SCI and muscle injury. Overall our data suggests that targeting macrophages and/or macrophage mediated inflammation may serve as viable therapies for the treatment of NHO.
# Table of Contents

1. Introduction .........................................................................................4  
2. Keywords ............................................................................................4  
3. Accomplishments ...............................................................................4  
4. Impact .................................................................................................12  
5. Changes/Problems ...........................................................................13  
6. Products ..............................................................................................14  
7. Participants & Other Collaborating Organizations .......................15  
8. Special Reporting Requirements .....................................................17  
9. Appendices .........................................................................................17
1. INTRODUCTION
This project is to better understand the mechanisms that lead to neurological heterotopic ossifications (NHO) in patients suffering spinal cord injuries (SCI) and to identify potential treatments. NHO is the abnormal formation of ectopic bones in muscles following SCI. NHO occurs in 15-29% of patients suffering SCI, often young men in car/sport accidents. In the case of US soldiers requiring limb amputation following blast injuries sustained during combat, incidence of NHO has been reported to increase to 82% when there is a concomitant SCI. NHO are painful, decrease range of limb motion leading to complete ankyloses of affected joints, consequently increasing patients’ functional disabilities and dependency; NHO also compromises neurological recovery thereby exacerbating disability and morbidity. Treatment is currently limited to surgical resection after NHO have matured, a procedure that is challenging, particularly when large ossifications affect joints and entrap large blood vessels and nerves as these can be damaged during NHO formation or surgical resection. Furthermore, even after resection, NHO recurrence is observed in at least 6% of patients after surgery. Thus there is an unmet need for alternative treatment strategies. Development of improved treatments for NHO has been slow and trials of pharmacological interventions continue to show limited efficacy, reflecting the current limited knowledge on NHO pathophysiology. A better understanding of this pathology and the molecular mechanisms involved could help design rational pharmacological treatment to prevent or reduce NHO development in patients at risk. In this project, we use our novel model, which is the first pre-clinical SCI model of NHO that can be induced in laboratory mice without genetic modification. Our model (that we recently published in the Journal of Pathology 2015;236:229-240) shows that formation of NHO requires the combined insult of SCI and soft tissue inflammation via macrophages. Furthermore, when both SCI and inflammation are co-existent, released systemic factor(s) present in serum are sufficient to drive osteogenic differentiation of muscle-derived progenitor cells in vitro. The objectives of this project are to:

1. Determine whether rapidly translatable macrophage-targeted therapies have efficacy as either a preventative or therapeutic approach for NHO.
2. Elucidate the specific SCI-driven changes in macrophage inflammatory profile that may promote NHO progression.
3. Determine the SCI-induced factors that contribute to NHO development.

2. KEYWORDS
Spinal cord injury, heterotopic ossification, neurological heterotopic ossification, macrophage, inflammation, osteoblast, muscle, joint, tumor necrosis factor, macrophage-colony-stimulating factor, markers, adrenergic signaling.

3. ACCOMPLISHMENTS

What were the major goals of the project?
The major goals of the SOW are as follows:

1. Determine whether etanercept and the CSF-1 receptor kinase inhibitor GW2580 can prevent or decrease neurological heterotopic ossification (NHO) development following spinal cord injury. October 2016.
4. Test if selective depletion of macrophage subsets prevents NHO development following SCI. October 2017.

What was accomplished under these goals?
Our animal protocol was approved by the ACURO in January 2016 (beginning of the 2nd quarter of the award).

Major activities/accomplishments for this reporting period

Major task 1: To determine whether etanercept and CSF1 receptor kinase inhibitor GW2580 can prevent or decrease NHO development following SCI.

1.1 Results and key outcomes
Subtask 1
IACUC and ACURO approval were obtained to perform experiments in mice.

Subtask 2
We have performed two experiments in which the TNF-α antagonist etanercept or the CSF-1 receptor kinase inhibitor GW2580 were administered from the time of surgery onward (preventative modality). NHO volumes were measured by µCT 10 days after surgery. The NHO volumes from both of these experiments were imaged and measured on two different instruments: a microcomputerized tomography scanner µCT40 (SCANCO Medical) for the first experiment, and an Inveon positron emission tomography–computed tomography (PET-CT) multimodality system (Siemens Medical Solutions) for the second experiment. To account for this, the results of these two experiments were normalized to the average NHO volume obtained in the control group treated with vehicle. Results confirmed that treatment of mice with either etanercept or GW2580 results in a significant reduction in NHO bone volume (30% and 37% reduction respectively) (Figure 1).

![Graphs showing Etanercept and GW2580 treatment effects](image)

Figure 1. Etanercept and GW2580 treatment post SCI significantly reduced NHO development. C57BL/6 mice underwent SCI and received intramuscular injection of 5 μg CDTX. Mice were treated with vehicle or
etanercept (20mg/kg/day), or GW2580 (160mg/kg/day) from the day of surgery and continued daily for ten days. Data are represented as box and whisker plots of 7-8 mice per group in each experiment. Statistics were calculated by Mann-Whitney test.

These experiments confirm our hypothesis that TNF-α and CSF-1 are important cytokines for the development of NHO after SCI.

1.2 Stated goals not yet met
Experiments to measure the effect of etanercept and GW2580 administration in the curative modality (5 days post-surgery onwards) will be performed in the next 4 months. Additionally, tissues from the completed etanercept and GW2580 experiments are being processed for immunohistology to stain for markers of M1 and M2 polarized macrophages.

**Major task 2: To perform longitudinal analysis of macrophage polarization markers in NHO.**

**Subtask 1**
This experiment has been delayed due to Sigma-Aldrich stopping the production and distribution of the cardiotoxin we inject in the muscle to trigger muscle damage. We therefore had to source cardiotoxin from a different supplier (Latoxan in France). Although this cardiotoxin causes muscle damage, in our experiments, it induces much smaller HO compared to the cardiotoxin from Sigma Aldrich and in an inconsistent manner (Figure 2).

![Figure 2: Comparative effect of cardiotoxin from Sigma-Aldrich versus Latoxan on initiating NHO in SCI mice. Both sources of cardiotoxin were injected at the same dose (0.32mg/kg) into the muscle after SCI. NHO volume was measured by µCT on an Inveon PET-CT 7 days post-surgery. Each dot represents a result from an individual mouse. Data are represented as box and whisker plots. Statistics were calculated by Mann-Whitney test.](image-url)
Prior to this award, we performed experiments where we induced mechanical muscle damage (crush) immediately after SCI, instead of cardiotoxin induced muscle damage. Mechanical muscle damage was carried out with an impactor in which a defined weight is dropped from a specific height on a defined area of the muscle. We observed that HO developed only if the mechanical damage was combined with injection of Gram-negative bacterial wall product lipopolysaccharide (LPS) purified from E. coli (Figure 3).

![Figure 3: Mechanical muscular damage requires bacterial LPS to generate NHO in SCI mice. C57BL/6 mice underwent SCI, followed by muscular injury via mechanical impact at impulse 0.29 m*kg/s followed by an intramuscular injection of saline or LPS (2.5 mg/kg) at day 0, 2, 4 and 6 after procedure. NHO volume was measured by µCT 10 days post-surgery. Each dot represents a result from an individual mouse. Data are represented as box and whisker plots of 7 mice per group. Statistics were calculated by Mann-Whitney test.](image)

From this data and in accord with the clinical observation that presence of an infection is significantly associated with higher incidence of NHO in traumatic brain spinal cord injured patients (Citak MD et al, Spine 2012;37:1953-57; Dizdar D et al, Brain Inj 2013;27:807-11), we hypothesize that the cardiotoxin from Sigma-Aldrich was not as well purified as the one from Latoxan and might have contained some bacterial products explaining why the cardiotoxin from Sigma caused larger and more reproducible HO. We have obtained approval for our IACUC to perform titration of intramuscular injection of LPS in addition to CDTX to measure effect on NHO volume and identify a dose of LPS that promotes NHO development when co-administered with cardiotoxin. We are applying to our IACUC to include a protocol modification in order to be able to co-administer this dose of LPS with cardiotoxin from Latoxan in our future experiments.

**Subtask 2**

IRB approval has been obtained to obtain access stored blood samples from 40 NHO patients and 12 healthy volunteers, and stored NHO biopsies from 40 NHO patients stored in the BANKHO and ParaOs tissue banks in France. Application for approval has been submitted to the HRPO.

**Subtask 3**
We have optimised immunohistochemical (IHC) staining on archival sections of mouse hind limb muscles generated prior to this award. As illustrated in Figure 4, we are able to successfully stain for the following M1 polarisation markers MHC Class II (Figure 4A, arrows). Very few IRF5 positive cells were noted in the injured muscle, however robust staining was noted in the adjacent bone marrow cavity (Figure 4C arrows), confirming we successfully optimised the antibody but overall IRF5 expression levels are low at this time point post-surgery. We are yet to successfully optimise IHC staining for iNOS and further optimisations are currently being carried out. We have also optimised all of our M2 polarisation markers (Figure 5). Robust staining was noted in injured muscle for both Arginase-1 (Figure 5A, arrows) and YM-1 (Figure 5B, arrows). Low level expression was noted for IRF4 (Figure 5C, arrows). Therefore we now have a panel of both M1 and M2 polarisation markers we can use for analysis of macrophage subsets in our injured muscle samples. Once we have IACUC and ACURO approval to co-administer LPS with the cardiotoxin from Latoxan, we will be able to generate this longitudinal series of mouse tissues to examine kinetics of expression of M1 and M2 markers following SCI and muscle injury.
Figure 4. Representative images of positive IHC staining for M1 macrophage markers MHC Class II and IRF5 in injured muscle. Images were taken from archival sections of mouse hind limb in which mice received an injection of CDTX after SCI. Images confirm positive staining for MHCII (A). Minimal staining was noted for IRF5 in injured muscle (B) but confirmation of positive staining was noted in the adjacent BM (C). Minimal staining was noted in matched isotype controls. Original magnification A x20, B-C x40.
Figure 5. Representative images of positive staining for M2 macrophage markers Arginase-1, YM-1 and IRF-4 in injured muscle. Images were taken from archival sections of mouse hind limb in which mice received an injection of CDTX after SCI. Images confirm positive staining for Arginase-1 (A), YM-1 (B) and IRF-4 (C), arrows. Minimal staining was noted in matched isotype controls. Original magnification A-B x20, C x40.
Major Task 4: To test if selective depletion of macrophage subsets prevent NHO development following SCI.

4.1 Results and key outcomes

We are ahead of schedule in this task, and have performed preliminary experiments with LysMCre Hif1α^floxflox mice, LysMCre Il4ra^floxflox and matching LysMcre mice using our last stocks of cardtoxin from Sigma.

![Figure 6](image)

*Figure 6. Conditional deletion of Hif1a gene in myeloid cells significantly increases HO volume. LysM^{Cre/WT} x hif1a^^{floxflox} and LysM^{Cre/WT} x Hif1a^^{wt/wt} mice underwent SCI and intramuscular injection of 5 μg CDTX. HO volume was measured by μCT 10 days post-surgery. Data are represented as box and whisker plots of 6 mice per group. Statistics were calculated by Mann-Whitney test.*

Our first experiment using the LysM^{Cre/WT} x Hif1α^{floxflox} mice confirmed that inactivation of ‘M1’ macrophage polarization (conditional deletion of the Hif1a gene in myeloid cells impairs M1 macrophage polarization) significantly enhanced NHO formation (Figure 6). As mentioned previously our issues with changing cardtoxin suppliers has meant we could not repeat this experiment, but once IACUC animal ethics has been approved for the use of LPS in conjunction with CDTX this experiment will be repeated again.

When carrying out experiments using the LysMCre x Il4ra^floxflox mice we encountered unexpected complications. We have since discovered that the floxed exon of Il4ra gene in these mice contains a mutation that impairs signalling in response to IL-4 which results in cardiac insufficiency. Therefore mice containing this floxed allele are not suitable for Aim 2.3 of our project, as cardiac insufficiency may confound the effect of blocking IL-4 signalling in macrophages in these mice. These complications and necessity to use a neutralizing anti-mouse IL-4 antibody in wild-type mice to address the question of IL-4 and M2 polarization are discussed further below in Section 5 (CHANGES/PROBLEMS).

4.2 Stated goals not yet met
We are ahead of schedule in this task and continuation of the task using a different model of IL-4 blockage and M2 polarization inhibition will be resumed when we receive approval from the ACURO.

**What opportunities for training and professional development has the project provided?**
Nominated Research Associate Dr Hsu-Wen Tseng who has been recruited to work on the project underwent full training during the first 3-4 months of the project to learn how to perform spinal cord transections under general anesthesia on mice and to monitor and care for the paraplegic mice after surgery. She also learnt to perform intramuscular injections in the hind limb. Dr Tseng also obtained a radiation license and learnt how to use the Inveon positron emission tomography–computed tomography (PET-CT) multimodality system from Siemens Medical Solutions to measure heterotopic bone volumes by micro-computerize tomography on mice.

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

**What do you plan to do during the next reporting period?**
We will stain muscle sections from mice treated with etanercept and GW2580 in the preventative modality to measure expression of M1 and M2 markers as defined in Specific Aim 1. This experiment was performed with cardiotoxin from Sigma Chemicals and tissues are being currently processed for embedding and sectioning.

For the other experiments, we have first to identify a dose of bacterial LPS that when added to cardiotoxin from Latoxan promotes heterotopic ossification (HO) formation in animal that underwent spinal cord injury (SCI) but does not promote HO in sham-operated animals. This preliminary experiment will be performed as part of an award from the National Health and Medical Research Council (NHMRC). Once this dose is identified, we will obtain approval from our IACUC and from the ACURO to add this optimal dose of LPS to the cardiotoxin from Latoxan in all experiments using cardiotoxin in the project. Once we have received approval for using LPS in our models we aim to perform the following experiments with the optimal dose of LPS added to cardiotoxin from Latoxan.

- Longitudinal analysis to investigate M1 and M2 markers in macrophages as defined in Specific Aim 2, subaim 2.1.
- Effect of etanercept and GW2580 in the curative modality in Specific Aim 1.
- Effect of specific deletion of CD169+ macrophages in Specific Aim 2, subaim 2.3.

**4. IMPACT**

**What was the impact on the development of the principal disciplines of the project?**
If we can confirm that bacterial products from bacteria that infect wounds are key drivers of HO following SCI and muscle cell damage, this will have major impact on the early management of spinal cord injury patients. Indeed, although the incidence of heterotopic ossification is known to be higher in patients with infections, it has never been proven that infections are directly correlated to HO in spinal injury patients. Indeed all studies SCI patients with HO are retrospective and many other confounding factors also occur in these patients at the same time. Therefore, it is not possible to isolate the role of infections and prove from these retrospective studies that bacterial infections are a direct trigger causing HO. If our mouse model can clearly demonstrates this direct link, then treatment of wounds and infections associated with the accident that caused the spinal cord injury, and prevention and treatment of nosocomial (hospital-acquired) infections may be essential to reduce the incidence of HO in these patients.
In terms of basic biology, these findings would confirm the immune cells called macrophages (which are the first defense against bacteria) become dysregulated following spinal cord injury and concomitant bacterial infection by promoting bone formation from the injured muscle instead of supporting normal muscle repair.

**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**
We are seeking two changes in approaches as described below.

a) As reported in previous quarterly reports, we have discovered that the floxed exon of *Il4ra* gene in mice contains a mutation that impairs signaling in response to IL-4. Therefore mice containing this floxed allele are not suitable for Aim 2.3 of our project as cardiac insufficiency may confound the effect of blocking IL-4 signaling in macrophages in these mice. However, we can still test the effect of IL-4 receptor inactivation by injecting a neutralizing anti-mouse IL-4 monoclonal antibody in wild-type mice. This should not impact expenditure as money saved on generating and maintaining LysMCre *Il4ra*^fl/fl^ and *Il4ra*^fl/fl^ mice will be used to purchase the anti-IL-4 antibody in sufficiently large quantity (25mg) to inject into mice.

b) As Sigma-Aldrich (USA) ceased commercializing cardiotoxin from Naja mossaibica snake venom at the end of 2015, we had to find an alternative source. Cardiotoxin sourced from Latoxan (France) is purified from the venom of a related but different snake species (Naja pallida). We tested the activity of this alternative cardiotoxin in our model of SCI-NHO. While performing experiments for Aim 2.1, we realized that the volume of the NHO obtained by injection the Latoxan cardiotoxin are on average much smaller than those obtained with the Sigma cardiotoxin and not all SCI mice injected with Latoxan cardiotoxin developed NHO, which makes it very difficult to establish a series of mice with consistent NHO consistent within each group.

We hypothesize this variability in HO development may be due to some contamination by bacterial products such as LPS in the Sigma-Aldrich preparation. Previously we have demonstrated that LPS administration increases in a different model of NHO, where muscle is damaged by a physical impact instead of cardiotoxin administration, leads to increased development of NHO. This hypothesis is consistent with the clinical observation that presence of an infection is significantly associated with higher incidence of NHO in traumatic brain and spinal cord injured patients (Citak MD et al, Spine 2012;37:1953-57; Dizdar D et al, Brain Inj 2013;27:807-11). As part of our current NHMRC Project Grant, we are planning experiments to test the effect of different doses of LPS from gram-negative bacteria *E. coli*, heat-killed Gram-positive *Staphylococcus aureus* and Lipoteichoic acid (LTA) on size and incidence of NHO in our mouse model. If conclusive, we will seek approval by the ACURO to administer LPS or
heat-killed bacteria or and Lipoteichoic acid (LTA) together with cardiotoxin in the rest of the experiments to obtain NHO consistently.

**Actual or anticipated problems or delays and actions or plans to resolve them.**
The problems and actions/plans to resolve them are explained in detail above. This will cause delay of the project by approximately 4 months as we need to perform experiments to optimize the dose of LPS in order to obtain NHO in mice undergoing spinal cord injury together with cardiotoxin injection but not in mice with SHAM-operation without cardiotoxin injection. We will also need to obtain ACURO approval for a) the use of anti-IL4 antibody in wild-type mice, b) injection of LPS together with cardiotoxin instead of cardiotoxin alone once we have optimized the dose of LPS to be co-administered with cardiotoxin.

**Changes that had a significant impact on expenditures**
We discovered the poor effect of cardiotoxin from Latoxan while performing experiment in Aim2.1 when we realized that mice at time points 4, 7, and 14 days displayed no or very small heterotopic ossification by micro-computerized tomography. Therefore this experiment will have to be repeated with associated costs (mice, housing, reagents, µCT time approximately $1500 USD) once we have established the optimal dose of LPS.

**Significant changes in use or care of human subjects**
Nothing to report.

**Significant changes in use or care of vertebrate animals**
In Aim 2.3, the experiment with LysMCre Il4ra flox/flox mice will have to be repeated with a different model of in vivo blockage of IL-4 as these mice are unsuitable for our studies as explained above. In the new model, wild-type mice will be injected with a neutralizing rat anti-mouse IL-4 monoclonal antibody or an isotype-matched control monoclonal antibody. This new protocol was approved by our IACUC on June 16th 2016. We have submitted a request to the ACURO to approve this modification.
As cardiotoxin from Latoxan generates small heterotopic ossifications in an inconsistent manner in mice with spinal cord injury, we are testing the effect of co-administration of different doses of bacterial lipopolysaccharides (LPS) together with cardiotoxin on HO development. This experiment was approved by our IACUC on August 18th 2016. Once we have determined the optimal dose of LPS to co-administer with cardiotoxin to obtain consistent HO formation in mice with spinal cord injury, we will apply to our IACUC for approval to co-administer this optimal dose of LPS together with cardiotoxin from Latoxan. Once this will be approved by our IACUC, we seek approval from the ACURO.

6. PRODUCTS

**Publications, conference papers, and presentations**
Nothing to report.

**Website or other internet site**
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 AND OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

<table>
<thead>
<tr>
<th>Name</th>
<th>Jean-Pierre Levesque</th>
</tr>
</thead>
<tbody>
<tr>
<td>Project Role</td>
<td>PD/PI</td>
</tr>
<tr>
<td>Researcher Identifier</td>
<td>ORCID number: 0000-0002-7299-6025</td>
</tr>
<tr>
<td>Nearest person month worked</td>
<td>3</td>
</tr>
<tr>
<td>Contribution to project</td>
<td>Supervised the project.</td>
</tr>
<tr>
<td>Funding support</td>
<td>NHMRC Research Fellowship</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name</th>
<th>Allison Pettit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Project Role</td>
<td>PD/PI</td>
</tr>
<tr>
<td>Researcher Identifier</td>
<td>ORCID number: 0000-0003-4707-7892</td>
</tr>
<tr>
<td>Nearest person month worked</td>
<td>1</td>
</tr>
<tr>
<td>Contribution to project</td>
<td>Helped in design of experiments and immunohistochemistry.</td>
</tr>
<tr>
<td>Funding support</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name</th>
<th>Hsu-Wen Tseng</th>
</tr>
</thead>
<tbody>
<tr>
<td>Project Role</td>
<td>Nominated Research Associate</td>
</tr>
<tr>
<td>Researcher Identifier</td>
<td>ORCID number: 0000-0002-9547-2718</td>
</tr>
<tr>
<td>Nearest person month worked</td>
<td>12</td>
</tr>
<tr>
<td>Contribution to project</td>
<td>Performed all animal surgeries and care of mice. Analyzed results by PET-CT.</td>
</tr>
<tr>
<td>Funding support</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name</th>
<th>Susan Millard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Project Role</td>
<td>Nominated Research Associate</td>
</tr>
<tr>
<td>Researcher Identifier</td>
<td>ResearcherID: C-8115-2011</td>
</tr>
<tr>
<td>Nearest person month worked</td>
<td>4</td>
</tr>
<tr>
<td>Contribution to project</td>
<td>Continued optimization of immunohistochemistry staining, helped Dr Tseng. Worked on identifying the unreported mutation in the IL4ra floxed allele and its effect of IL4R signaling.</td>
</tr>
<tr>
<td>Funding support</td>
<td>Mater Research Institute – University of Queensland</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name</th>
<th>Kylie Alexander</th>
</tr>
</thead>
<tbody>
<tr>
<td>Project Role</td>
<td>Research Associate</td>
</tr>
<tr>
<td>Researcher Identifier</td>
<td>ResearcherID number: P-2666-2016</td>
</tr>
<tr>
<td>Nearest person month worked</td>
<td>2</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>---</td>
</tr>
<tr>
<td>Contribution to project</td>
<td>Helped with optimization of immunohistochemistry staining, helped Dr Tseng</td>
</tr>
<tr>
<td>Funding support</td>
<td>NHMRC Project Grant</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name</th>
<th>Dietmar Hutmacher</th>
</tr>
</thead>
<tbody>
<tr>
<td>Project Role</td>
<td>Co-Investigator</td>
</tr>
<tr>
<td>Researcher Identifier</td>
<td></td>
</tr>
<tr>
<td>Nearest person month worked</td>
<td>1</td>
</tr>
<tr>
<td>Contribution to project</td>
<td>Helped in the quantification of HO volumes by CT</td>
</tr>
<tr>
<td>Funding support</td>
<td>Queensland University of Technology</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name</th>
<th>Francois Genet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Project Role</td>
<td>Co-Investigator</td>
</tr>
<tr>
<td>Researcher Identifier</td>
<td></td>
</tr>
<tr>
<td>Nearest person month worked</td>
<td>1</td>
</tr>
<tr>
<td>Contribution to project</td>
<td>Worked on human ethics clearance in France and Australia and from HRPO.</td>
</tr>
<tr>
<td>Funding support</td>
<td>Hopitaux de Paris - Assistance Publique (France)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name</th>
<th>Jean-Jacque Lataillade</th>
</tr>
</thead>
<tbody>
<tr>
<td>Project Role</td>
<td>Co-Investigator</td>
</tr>
<tr>
<td>Researcher Identifier</td>
<td></td>
</tr>
<tr>
<td>Nearest person month worked</td>
<td>1</td>
</tr>
<tr>
<td>Contribution to project</td>
<td>Worked on human ethics clearance in France and Australia and from HRPO.</td>
</tr>
<tr>
<td>Funding support</td>
<td>Ministere de la Defense (France)</td>
</tr>
</tbody>
</table>

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

PI Jean-Pierre Levesque

Previously reported active grant from the Cancer Council Queensland to Allisson Pettit, Liza Raggatt, Jean-Pierre Levesque entitled “The role of macrophages in facilitating hematopoietic stem cell engraftment and reconstitution” ended on 12/31/2015.

Previously reported pending grant from the NHMRC to Allison Pettit, Jean-Pierre Levesque, Liza Raggatt, David Hume and Andrew Perkins entitled “Recipient bone marrow macrophages contribute to hematopoietic stem cell transplantation” was successful and started on 1/1/2016.

Previously reported pending grant from the NHMRC to Jean-Pierre Levesque, Allison Pettit, Francois Genet, Dietmar Hutmacher, Natalie Sims and Marc Ruitenberg entitled “Why do macrophages promote heterotopic ossification following spinal cord injuries” was successful and started on 1/1/2016.

These do not significantly impact the project report.
PI Allison Pettit

Previously reported active grant from the Cancer Council Queensland to Allison Pettit, Liza Raggatt, Jean-Pierre Levesque entitled “The role of macrophages in facilitating hematopoietic stem cell engraftment and reconstitution” ended on 12/31/2015.

Previously reported pending grant from the NHMRC to Allison Pettit, Jean-Pierre Levesque, Liza Raggatt, David Hume and Andrew Perkins entitled “Recipient bone marrow macrophages contribute to hematopoietic stem cell transplantation” was successful and started on 1/1/2016.

Previously reported pending grant from the NHMRC to Jean-Pierre Levesque, Allison Pettit, Francois Genet, Dietmar Hutmacher, Natalie Sims and Marc Ruitenberge entitled “Why do macrophages promote heterotopic ossification following spinal cord injuries” was successful and started on 1/1/2016.

Previously reported pending grant from the NHMRC to Allison Pettit entitled “Improving fracture repair by promoting reparative inflammation” was unsuccessful.

Previously reported pending grant from the NHMRC to Allison Pettit entitled “Pro-anabolic mechanisms during bone repair are promoted by tissue-resident macrophages” was unsuccessful.

These do not significantly impact the project report.

What other organizations were involved as partners?

Organization Name: Institut National de la Sante et de la Recherche Medicale (INSERM) Unit 972
Location of Organization: Clamart, France
Partner’s contribution to the project:
  • Financial Support: Salaries of staff involved in the project (Lataillade)
  • In-kind support: Partner makes equipment and laboratory space available to project staff.
  • Facilities: Project staff (Lataillade, Genet, Torossian) use partner’s facilities for project activities.
  • Collaboration: Partner’s staff work with project staff on the project.

8. SPECIAL REPORTING REQUIREMENT
None

9. APPENDICES
None