

AWARD NUMBER: W81XWH-20-1-0911

TITLE: Immunological Contributions to the Chronification of Pain

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14. ABSTRACT The chronification of pain after surgery and other forms of trauma is common, results in suffering, poor functional recovery, and delays return to normal activities. In addition, chronic pain contributes to mood disorders and cognitive decline. Pain after surgery/trauma to the extremities is particularly common and is of high impact in military, Veteran and civilian populations. Emerging data suggest that posttraumatic immune system activation mediated by the formation of autoantibodies contributes to the chronification of pain. Critically, we may have the ability to control posttraumatic immune activation using existing mechanistically distinct, clinically available well-tolerated pharmacological agents. Drugs potentially regulating oxidative stress and the activation of B lymphocytes in germinal centers are key in this regard. Our studies make extensive use of a limb fracture model in mice. In work performed to-date we have demonstrated that the activator of nrf2, a transcription element responsive to oxidate stress with the agent dimethyl fumarate (DMF) leads to a reduction in nociceptive and functional changes after limb fracture, a reduction in pain-supporting autoantibodies, reduce activation of germinal centers in B lymphocytes and moderate reductions in the production of pain-related cytokines in the skin of fractured animals as well as reduced levels of oxidized protein derivatives. While only the beginning of a 4-year project, we are now positioned to refine our understanding of the roles of oxidative stress and the transition from acute to chronic pain.						
15. SUBJECT TERMS Pain, Autoimmunity, Oxidative stress						
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1. INTRODUCTION: *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

The chronification of pain after trauma including military and civilian injuries is common, results in suffering, poor functional recovery, and delays return to normal activities. The main objective of this research is to explore three independent methods of reducing immune system activation occurring at the time of injury to prevent the chronification of acute pain and downstream adverse outcomes using a mouse limb fracture model. The approaches used may be applicable to soldiers with battlefield injuries, Veterans and civilians suffering limb traumas and all patients undergoing surgeries on their extremities. The specific treatments under study are highly diverse allowing for the selection of the most appropriate treatment under specific circumstances. For example, approaches less profoundly affecting immune system function (antioxidant regulators) might be used for battlefield and other low resource environments while more aggressive approaches (B lymphocyte-directed immunosuppressives) might be selected in well-supported hospital settings. Some approaches use inexpensive drugs, and all would be relatively straightforward to implement without special equipment.

2. KEYWORDS:

Pain, Oxidative Stress, Fracture, Mice, Disability, Autoimmunity, Autoantibodies, Biomarkers, Germinal Center, Antioxidant

3. ACCOMPLISHMENTS:

What were the major goals of the project?

This preclinical project has two major goals:

1. To determine whether oxidative stress initiates B cell-mediated autoimmunity resulting in adverse nociceptive, functional and neuropsychiatric outcomes after limb fracture.
2. To determine whether T_h cell activation and germinal center formation are responsible for B cell-mediated autoimmunity and adverse outcomes after limb fracture.

Milestones from SOW

1. (Major Task 1, Approvals and Authorizations):
 - a. Local IACUC and ACURO approvals have been obtained and are current. Completed prior to 1/1/2021
2. (Major Task 2, Aim 1):
 - a. Controlled DMF experiments reflecting effects of DMF on biological, nociceptive and functional consequences of fracture and immune system activation. Completed 8/1/2021.
 - b. Controlled DMF experiments measuring more complex functional and neuropsychiatric outcomes as well as autoantibody production. 50% complete 10/1/2021.

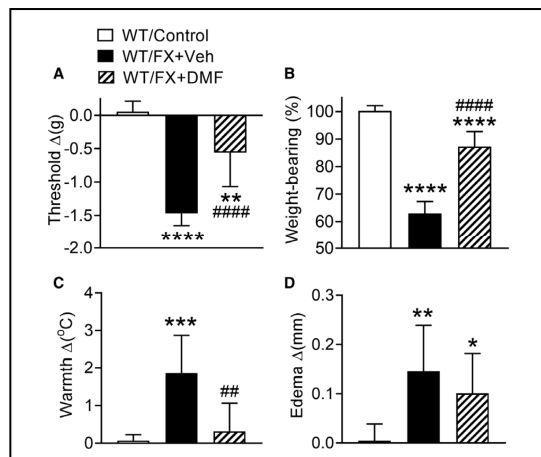
What was accomplished under these goals?

Summary of findings:

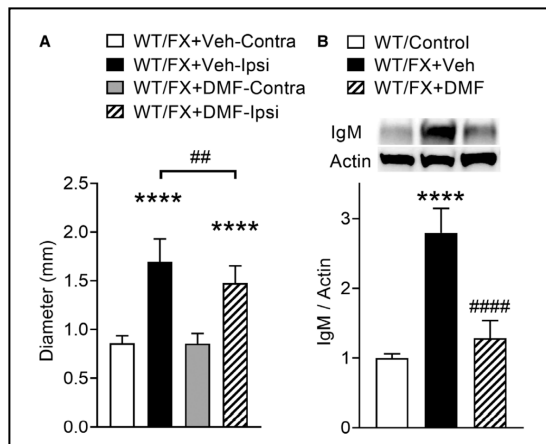
Male C57BL/6 mice were used in the well-characterized tibial fracture model of limb injury featuring the transition of acute to chronic pain. Some groups of mice received DMF 25 mg/kg/d orally, per os for 3 weeks after fracture versus vehicle alone. Homozygous Nrf2 null mutant mice were used as test subjects to address the need for this transcription factor for DMF activity. Allodynia was assessed using von Frey filaments and hindlimb weight-bearing data were collected. The markers of oxidative stress malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) were quantified in the skin of the fractured mice using immunoassays along with the innate immune system cytokines IL-1 β and IL-6. The accumulation of IgM in the fractured limbs and lymph node hypertrophy were used as indexes of adaptive immune system activation, and the passive transfer of serum from wildtype fractured mice to B cell-deficient fractured muMT mice (mice lacking B cells and immunoglobulin) helped to assess the pronociceptive activity of humoral factors. We observed that oral DMF administration strongly prevented nociceptive sensitization and reduced uneven hindlimb weight bearing after fracture. DMF was also very effective in reducing the accumulation of markers of oxidative stress, activation of innate immune mediator production, lymph node hypertrophy, and the accumulation of IgM in fractured limbs. The sera of fractured vehicle-treated but not DMF-treated mice conferred pronociceptive activity to recipient mice. Unexpectedly, the effects of DMF were largely unchanged in the Nrf2 null mutant mice.

Key data from these experiments are provided below:

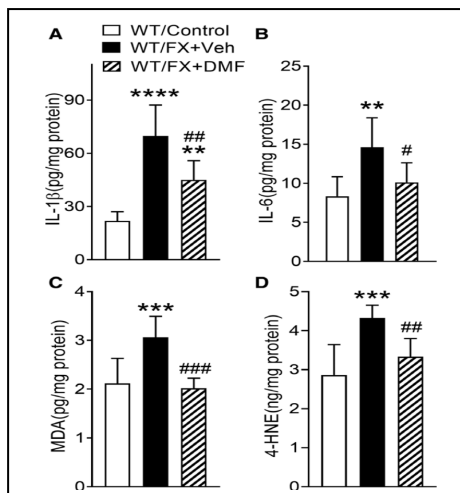
DMF treatment inhibited the post-FX development of ipsilateral hindpaw nociceptive sensitization and warmth in WT mice. At 3 wk after tibia FX and casting the Veh-treated WT C57BL6 mice exhibited unilateral hindpaw von Frey allodynia (A), unweighting (B), warmth (C), and edema (D). Daily treatment with DMF (25mg/kg/d, orally, per os) for 3 wk reduced hindpaw allodynia, unweighting, and warmth in the FX mice, but had no effect on hindpaw edema. Measurements for (A), (C), and (D) represent the difference between the FX side and contralateral paw, thus a negative von Frey threshold value represents a decrease in mechanical withdrawal thresholds on the affected side and positive temperature and thickness values represent increased hindpaw warmth and edema on the FX side. Measurements for (B) represent weight bearing on the FX hind limb as a ratio to half of the total bilateral hind limb loading, thus, a percentage < .001, and #####P < .0001 for differences between the vehicle and DMF treatment groups, *P < .05, **P < .01, ***P < .001, ****P < .0001 for differences from control values. Control, no fracture; DMF, dimethyl fumarate; FX, fracture; SD, standard deviation; Veh, vehicle; WT, wildtype.



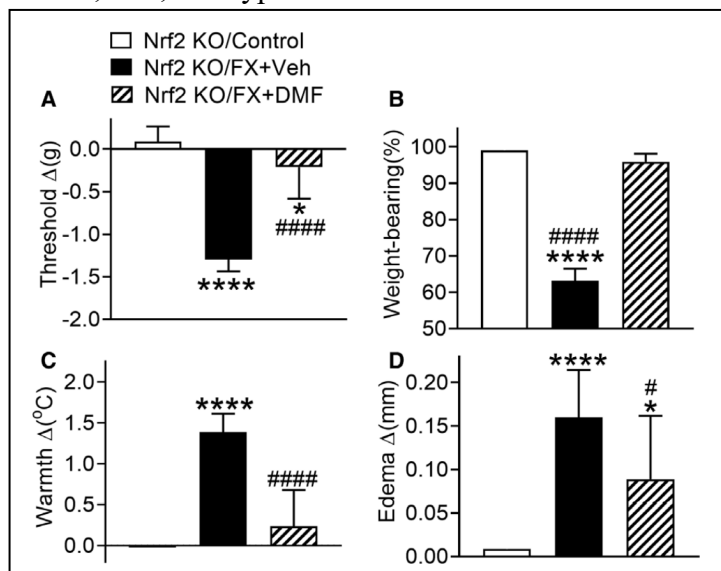
DMF treatment slightly inhibited fracture-induced popliteal lymphadenopathy and blocked IgM immune complex deposition in the hindpaw skin. A, At 3 wk post fracture, the diameters of the popliteal lymph nodes in the fracture limb were increased (in comparison to the contralateral side) in the vehicle-treated WT mice (n = 12 per cohort). Daily treatment with DMF for 3 wk after fracture slightly inhibited the postfracture increase in ipsilateral popliteal lymph node diameter. B, At 3 wk postfracture, there was increased IgM immune complex deposition in the ipsilateral hindpaw skin WT mice, compared to control nonfracture mouse values, as measured by Western blot analysis. Daily treatment with DMF for 3 wk after fracture blocked the post fracture increase in IgM immune complex deposition in skin and cord (n = 4 per cohort). Statistical analysis was performed using a 1-way ANOVA and a Holm-Sidak correction test for post hoc contrasts. Data are expressed as mean values \pm SD. ###P < .01, #####P < .0001 for differences between the vehicle and DMF treatment groups, ****P < .0001 for differences from contralateral or control values.



DMF treatment prevented the postfracture upregulation of inflammatory cytokines and oxidative stress markers in WT mice. At 3 wk post fracture, vehicle-treated WT mice developed increased levels of the inflammatory cytokines (A) IL-1 β and (B) IL-6, and the lipid peroxidation products (C) MDA, and (D) 4-HNE in the fracture limb hindpaw skin, relative to control nonfracture mouse values, as measured by enzyme immunoassay. Daily treatment with DMF for 3 wk after fracture blocked the post fracture increase in IL-1, IL-6, MDA, and 4-HNE. Statistical analysis was performed using a 1-way ANOVA and a Sidak correction test for post hoc contrasts. Data are expressed as mean values \pm SD, n = 8 per cohort. #P < .05, ###P < .01, #####P < .001 for differences between the vehicle and DMF treatment groups, **P < .01, ***P < .001, ****P < .0001 for differences from control nonfracture values. Control, no fracture; DMF, dimethyl fumarate; FX, fracture; IL, interleukin; MDA, malondialdehyde; muMT, mice lacking B cells and immunoglobulin; SD, standard deviation; Veh, vehicle; WT, wildtype.



DMF treatment blocked the postfracture development of nociceptive sensitization in Nrf2-deficient mice. At 3 wk post fracture, vehicle-treated Nrf2 KO mice developed allodynia (A), unweighting (B), warmth (C), and edema (D) to a similar extent as WT fracture mice (Figure 1). Daily treatment with DMF for 3 wk blocked the development of hindpaw allodynia, unweighting, and warmth in the fracture Nrf2 knockout mice, but had no effect on hindpaw edema. Statistical analysis was performed using a 1-way ANOVA, using a Sidak correction test for post hoc contrasts. Data are expressed as mean values \pm SD, $n = 7$ per cohort. # $P < .05$, ##### $P < .0001$ for differences between the vehicle and DMF treatment groups, * $P < .05$, **** $P < .0001$ for differences from control nonfracture values. Control, no fracture; DMF, dimethyl fumarate; FX, fracture; Nrf2, nuclear factor erythroid 2-related factor 2; Nrf2 KO, nuclear factor erythroid 2-related factor 2 knockout; SD, standard deviation; Veh, vehicle; WT, wildtype.



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

Nothing to Report

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 next year's plans will follow the set SOW. The major activities will be as listed below.

1. Conduct controlled DMF experiments measuring more complex functional and neuropsychiatric outcomes as well as autoantibody production.
2. Conduct controlled Vit C experiments reflecting effects of Vit C on biological, nociceptive and functional consequences of fracture and immune system activation.
3. As time allows, we will conduct controlled tacrolimus experiments reflecting effects of tacrolimus on biological, nociceptive and functional consequences of fracture and immune system activation.

4. IMPACT:

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

Our experiments have impacted the fields of pain medicine and pain management by providing a demonstration and mechanism for the clinically available agent DMF in reducing the transition from acute to chronic pain in an animal model. We believe that this could form the basis for a translational trial.

What was the impact on other disciplines?

While our laboratory is most closely associated with the field of pain management, the demonstration of the effects of DMF on pain, function, autoantibody production, cytokine generation and the formation of oxidized proteins has importance in other fields. Those include the study of oxidative stress and rehabilitation.

The results to-date of this project support considerations for trials using the same test agent used in these preclinical studies.

What was the impact on society beyond science and technology?

Nothing to report

5. CHANGES/PROBLEMS:

Nothing to report.

Actual or anticipated problems or delays and actions or plans to resolve them

Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Because of the opening of a new animal care facility and the closing of our older location, it is possible that laboratory experiments will be delayed a few weeks. However, the efficiencies intrinsic to the design of the new structure should allow the more rapid acquisition of data thus avoiding overall project delays.

Changes that had a significant impact on expenditures

There was some delay in appointing staff to this project as residual funds from preceding efforts were being expended. This had some cost saving effects on this project in the initial phases. Still, overall costs of the project while remaining within the scope of the approved work are likely to be approximately equal.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

No human subjects are involved in this project.

Significant changes in use or care of vertebrate animals

There have been no changes to the animal components of this project.
IACUC approval date: CLA1955, Approved 10/02/2020, expires 10/7/2023.

Significant changes in use of biohazards and/or select agents

None used.

6. **PRODUCTS:** *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state “Nothing to Report.”*

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

Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

1. Dimethyl Fumarate Reduces Oxidative Stress and Pronociceptive Immune Responses in a Murine Model of Complex Regional Pain Syndrome. Guo TZ, Shi X, Li W, Wei T, Kingery WS, Clark JD. *Anesth Analg.* 2021;132(5):1475-1485.

Published
DoD support listed

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

None

Other publications, conference papers and presentations.

None

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

None

- **Technologies or techniques**

None

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

None

- **Other Products**

None

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: David Clark
Project Role: PI
Researcher Identifier: Clark.David
Nearest person month worked: 0.9

Contribution to Project: Principal investigator. Supervises project design, data interpretation, manuscript writing and administrative issues.

Funding Support: Salary from the Department of Veterans Affairs

Name: Peyman Sahbaie
Project Role: Research Assistant
Researcher Identifier:
Nearest person month worked: 2

Contribution to Project: This person is responsible for coordinating animal use and testing on the project including ordering, ACORP maintenance, some measurement, data processing and the writing of reports.

Funding Support: Salary from the Department of Veterans Affairs.

Name: Tian-Zhi Guo
Project Role: Research Assistant
Researcher Identifier:
Nearest person month worked: 2.5

Contribution to Project: This individual performed most of the fracture surgeries and much of the drug and autoantibody administration.

Name: Xiaoyou Shi
Project Role: Research Assistant
Researcher Identifier:
Nearest person month worked: 2.5

Contribution to Project: This individual provides laboratory organizational approach for all other investigators, manages reagent inventories, conducts tissue harvests and performs all biochemical analyses.

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

None

What other organizations were involved as partners?

None

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to <https://ebrap.org/eBRAP/public/index.htm> for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on <https://www.usamraa.army.mil/Pages/Resources.aspx>) should be updated and submitted with attachments.*

9. APPENDICES: *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*

Immunological Contributions to the Chronification of Pain

CP190028

W81XWH-20-1-0911



PI: David J. Clark

Org: PAVIR

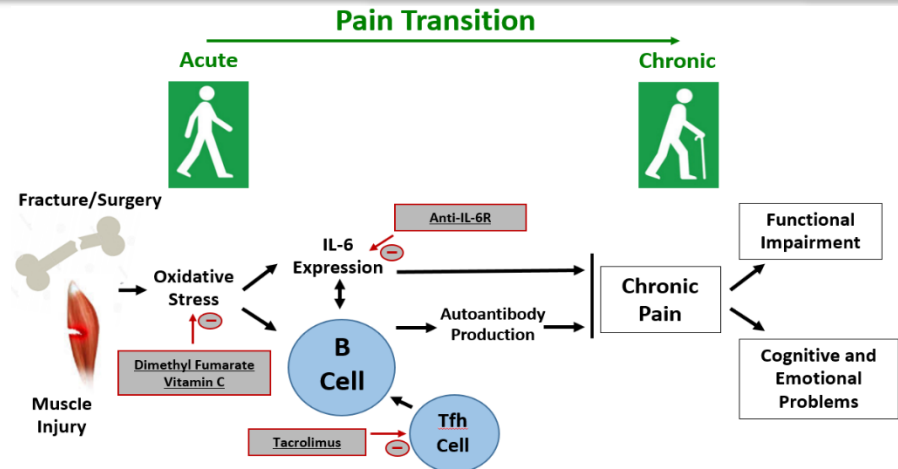
Award Amount: \$455,799

Study/Product Aims

- To determine whether oxidative stress initiates B cell-mediated autoimmunity resulting in adverse nociceptive, functional and neuropsychiatric outcomes after limb fracture.
- To determine whether Tfh cell activation and germinal center formation are responsible for B cell-mediated autoimmunity and adverse outcomes after limb fracture.

Approach

This project makes extensive use of a murine tibial fracture injury model displaying a transition from acute to chronic pain. In the first aim pharmacological antioxidants and nrf2 agonists will be used to study oxidative stress and pain-related autoimmunity. Nociception, limb function, activity and cognitive abilities will be measured. In the second aim experiments focus on the formation of germinal center structures in lymph nodes and the roles of Tfh cells in generating pain-supporting autoantibodies.



Our central hypothesis is that oxidative stress occurring after injury activates B cell maturation and autoantibody production. These autoantibodies then support many of the adverse outcomes of injury including the transition to chronic pain.

Timeline and Cost

Activities	CY	20/21	22	23	24
Approvals and Authorizations		█			
Ox. Stress Studies, Drugs		█	█		
Ox. Stress Studies, Behaviors		█	█	█	
Murine Tfh Functional Studies				█	█
Estimated Budget (\$K)		\$100	\$115	\$121	\$120

Goals/Milestones

CY20/21 Goal – Project Initiation and first experimentation

- Write, submit and attain local and ACURO approvals for project.
- Complete initial experiments using anti-oxidants.

CY22 Goals – Pharmacological studies on oxidative stress

- Use both antioxidants and nrf2 agonists to prevent acute and chronic pain and limb dysfunction.

CY23 Goal – Oxidative stress and complex behaviors

- Conduct studies using complex behavioral outcomes and complete assessments of effects on autoantibody production.

CY24 Goal – Examine role of oxidative stress on Tfh function

- Use Tfh inhibitors to identify effects on autoantibody production.

Comments/Challenges/Issues/Concerns

- Project initiated and experimentation started. No delays anticipated at this point.

Budget Expenditure to Date

Projected Expenditure: \$108,968

Actual Expenditure: \$65,112

Updated: 10/26/2021