

**Animal Studies Subcommittee (IACUC)  
Department of Veterans Affairs Medical Center  
Research and Development Service**

University & Woodland Avenue • Philadelphia, PA 19104 • 215-823-6024 • Fax: 215-823-5171

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**IACUC APPROVAL - Amendment**

Date: February 20, 2015

From: Scott R. Akers, M.D., Ph.D., Vice Chairperson



Investigator: Victoria P. Werth, M.D.

Protocol: The Role of TNF-Alpha in Cutaneous Integrity

ID: 01384 Prom#: 0042 Protocol#: N/A

The following items were reviewed and approved at the 02/12/2015 meeting:

- Amendment - Add 80 mice to the study/protocol change (01/15/2015)

The protocol was determined to have the following level of risk:  
Minimal

The following other committee reviews are scheduled:  
Research & Development Committee [03/03/2015]

MODIFICATION OF THE ANIMAL COMPONENT OF RESEARCH PROTOCOL  
(ACORP)

Date: 01/15/2015

Principal Investigator: Victoria P. Werth

Proposal Title: "The role of TNF-alpha in cutaneous integrity"

Department: Dermatology VA Address: Woodland Ave, Philadelphia, PA 19104  
Telephone #: 1-215-823-6024

Project Number (R&D Office): # 0042

Date of Initial VA R&D Approval: October 18, 2012

Funding source for this project (if not VA funded): VA

Beginning date: 04/01/13 Ending date: 08/01/15

Type of study:  Surgical  
 Non-Surgical  
 Survival  
 Terminal  
 Blood/Tissue  
 Behavioral

*USE A SEPARATE FORM FOR EACH SPECIES*

Animal Species: mouse

           Number of animals originally approved.  
           Number of additional animals approved by previous modifications, if any.  
           Number of animals used to date.  
  80   Number of additional animals requested, if any.

1. If increasing the number to be used, list the species, strain, and source and include the statistical basis for the number of animals requested (i.e., # of groups, # of animals per group, #of doses, etc.):

We will add 8 groups of mice with 10 mice/each group. Groups are listed below:

- 1) ROCK1<sup>+/-</sup> (Rock1<sup>tm1Liao</sup>, ROCK1<sup>-/-</sup> mice die embryonically, Jackson Lab),
- 2) ROCK2<sup>+/-</sup> (Rock2<sup>tm2Liao</sup>, ROCK2<sup>-/-</sup> mice die embryonically, Jackson Lab),

- 3) C57BL/6J wildtype (WT, Jackson Lab)
- 4) Lupus prone MRL-*Fas*<sup>lpr</sup> mice with NS (stocking #: 000482, Jackson Lab)
- 5) Lupus prone MRL-*Fas*<sup>lpr</sup> mice with ROCK inhibitor HA-1077 peritoneally
- 6) C57BL/6J WT mice with NS
- 7) C57BL/6J WT mice with ROCK inhibitor HA-1077 peritoneally

All mice in the groups 1-7 will be irradiated by UVB.

- 8) C57BL/6J WT mice with no UVB exposure and no peritoneal injection.

ROCK inhibitor HA-1077 dissolved in normal saline (NS), and will be injected for intraperitoneally in a dose, 30 mg/kg/day. It has been used in published studies.

2. Indicate the maximum distress/pain that the animals will experience (complete explanation and justification must be attached to the protocol for pain level "E"):

- USDA Category B - Animals that will be bred but not used for research
- USDA Category C - Short-term discomfort/distress/pain
- USDA Category D - Pain avoided by appropriate drug use
- USDA Category E - Long-term distress or pain not avoided by appropriate drug use.

If USDA Category E,

3. Have there been any procedural changes from the originally approved proposal for the use of animals?  Yes  No. If yes, provide a complete description of any changes.

To explore the novel role of ROCK, we will use either use genetically manipulated knockout mice or use chemical inhibitors in the study. Otherwise, there is no changes on the procedures.

4. If any procedural changes(s) cause more than momentary or slight pain or distress to the animal(s),

NA

(a) Describe the methods and sources used to determine that alternatives to the painful methods are not feasible or appropriate:

(b) list pain level and what methods (analgesic, anesthetic, tranquilizing drug etc.) with dose/route) will be used to minimize discomfort and pain:

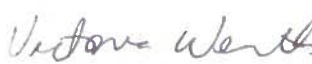
5. Are biohazards or radioactive agents being added to the protocol? Yes  No  If yes, indicate substance(s) and attach appropriate approval form.

6. List any additions/deletions in personnel assigned to the study. For additions, list name, degree, experience, status (VA/WOC), phone number, role/responsibility for project. For deletions, list name only.

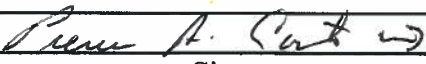
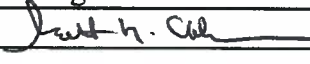
Ming-Lin Liu, MD, PhD, Research Assistant Professor at Penn Dermatology, WOC at VA. Design and conduct the project with Dr. Meena Sharma, under the supervision of the PI, Dr. Werth

Assurance by Principal Investigator. [PI's signature is mandatory.]  
*The information given in response to the questions above is accurate and I understand that prior to initiating changes in my research protocol affecting the use and care of laboratory research animals, the approval of the Animal Studies Subcommittee will be obtained. In signing this form, I am also certifying that the approved/proposed activities do not unnecessarily duplicate previous experiments.*

SIGNATURES:

Name of Principal Investigator(s)	Signature	Date
Victoria Werth		1/15/15

(To be completed after VA IACUC Approval)

Name of Attending Veterinarian	Signature	Date
Pierre A. Conti, VMD		3/12/2015
Name of IACUC Chair	Signature	Date
Laurence Buxbaum M.D., Ph.D.		02/23/2015

This is an amendment to our established protocol entitled  
“The role of TNF-alpha in cutaneous integrity”.

PI: Victoria Werth, MD

We are including the scientific justification and experimental details for depletion of inflammatory cells using antibody and knockout mice.

**Objective:**

Photodamage and photoaging are major medical problems in armed forces personnel and Veterans. Health consequences include short-term damage (sunburn); long-term problems include photosensitive autoimmune diseases and skin cancer. TNF- $\alpha$  (Tumor Necrosis Factor-alpha) and IL-1 $\alpha$  are the key mediators of UVB (Ultraviolet B) damage. UVB-induced TNF $\alpha$  alters the expression of genes of structural components in the extracellular matrix in skin such as glycosaminoglycans, collagen and elastin, causing photodamage.

UVB-induced TNF $\alpha$ , release from skin keratinocytes, stimulates the release of many pro-inflammatory cytokines, chemokines, and adhesion molecules in the skin. The TNF-induced pro-inflammatory mediators may increase the permeability of capillaries leading to infiltration and activation of neutrophils and other phagocytic cells into the skin. These inflammatory cells secrete MMPs and other proteolytic enzymes that alter collagen and glycosaminoglycans (GAGs), leading to cutaneous damage. In addition, UVB-induced TNF $\alpha$  release could trigger the infiltrated neutrophils and result in formation of neutrophil extracellular traps (NETs), a novel type of neutrophil cell death. The neutrophil NETs are proinflammatory and could further enhance autoimmune skin inflammation and cutaneous disintegration.

Our preliminary studies showed that Rho Kinase (ROCK) activation mediate NET formation. We hypothesize that ROCK inhibition may attenuate NET formation, and block the consequent autoimmune skin inflammation, therefore preventing UVB-induced skin damage.

**Changes in Research design/methodology:**

We will follow same procedure for our experiments as described in the main protocol except for the addition of ROCK1 or ROCK2 knockout mice, or using ROCK chemical inhibitors in lupus prone MRL-*Fas*<sup>lpr</sup> mice vs C57BL/6J wild-type mice by peritoneal administration of ROCK inhibitor(s) to study the role of ROCK inhibition in neutrophil NETosis and their role in UVB-induced skin inflammation and disintegration.

### **UVB-irradiation and group of animals-**

We proposed to examine the role of ROCK in neutrophil NETosis, autoimmune skin inflammation, and their role in photoaging. We will use both ROCK KO mice or use ROCK chemical inhibitors.

With ROCK KO mice, 1) ROCK1 KO mice ROCK1<sup>+/-</sup> (Jackson Laboratory), and 2) ROCK2 KO mice ROCK2<sup>+/-</sup> (Jackson Laboratory), 3) C57BL/6J wildtype (WT) mice will be irradiated with UVB for 6 consecutive days. Each group will have 10 mice, total 30 mice.

With ROCK chemical inhibitors, 4) female lupus prone MRL-*Fas*<sup>lpr</sup> mice, and 5) C57BL/6J WT mice will be treated without (control, normal saline) or with intraperitoneal administration of ROCK inhibitor HA-1077 (dissolved in normal saline, 30 mg/kg/day) during each of 6 consecutive days 1h prior to UVB irradiation. Therefore, there will be 2 groups for MRL-*Fas*<sup>lpr</sup> and WT mice and each group will have 10 mice, for a total of 40 mice.

Mice will be irradiated with one dose of 500 mJ/cm<sup>2</sup> daily for 6 consecutive days. In addition to the above discussed, ten WT mice without UVB-irradiation will be used as naïve negative control group. A day after the 6th day of UVB exposure, mice will be sacrificed, then the skin, bone marrow (from hind leg bones femur and tibia), and blood samples will be harvested.

### **Detection of the effects of ROCK on the changes in different group of mice**

To define the role of ROCK, ROCK1<sup>+/-</sup> and ROCK2<sup>+/-</sup> (Rock1<sup>tm1Liao</sup>, Rock1<sup>tm2Liao</sup>, Homozygous mice die embryonically, Jackson Lab), Lupus prone MRL-*Fas*<sup>lpr</sup> mice (stocking number, 000482), and C57BL/6J wildtype mice (Stock number 000664) will be used.

We will detect for the neutrophil NET formation and their colocalization with pDCs in mouse skin by immunohistochemistry. In addition, the Bone marrow (BM) neutrophils will be isolated from hind leg bones of each mouse for detection of ROCK activity and NETosis. Pooled serum will also be used for measurement of NET component, extracellular DNA and LL-37, as well as serum IFN $\alpha$ .

### **Findings:**

We will assay the neutrophil NET formation and its effects on skin inflammation and cutaneous integrity after UVB irradiation, as well as the role of ROCK signaling pathway in the NET formation that can affect skin inflammation and integrity.

### **Clinical relationship/Significance:**

UVB irradiation has been convincingly linked to photodamage of skin which leads to cancer development. The development of skin cancer can be suppressed by the immune system and skin cancer has a higher incidence in the population that shows increased UV and IL-1 induced TNF $\alpha$ . These studies will clarify the role of UVB-induced TNF $\alpha$  and neutrophil NETosis in skin inflammation that can affect cutaneous integration.