Blockade of IgM-Mediated Inflammation Alters Wound Progression in a Swine Model of Partial-Thickness Burn

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In a mouse model, a second-degree burn elicits a severe inflammatory response that is mediated by circulating autoantibody specific for a neoantigen (nonmuscle myosin). Nonmuscle myosin is expressed by injured tissue, leading to amplified ulceration and scarring. We hypothesize that a synthetic peptide (N2) can mimic the neoantigen and competitively inhibit the autoantibody, decreasing inflammation, and reducing the extent of burn injury in a preclinical swine model of burn. Second-degree burns were created on young swine using brass cylinders, warmed to varying temperatures before skin contact. Animals were treated in double-blind fashion with normal saline, control peptide, or blocking peptide. Biopsies were taken at 2 hours, 1, 4, 7, and 14 days after burn injury. Burn wound healing parameters were assessed. Immunohistochemical staining for Ki-67, immunoglobulin (Ig)M, and interleukin (IL)-8 were also performed. N2 blocking peptide administration decreased dermal injury at 4 days with increased reepithelization, indicating more rapid healing. N2 normalized skin histology by 14 days and showed improved epidermal healing. Granulation tissue thickness was decreased, and there was an accompanying decrease in neutrophil infiltration. The basal layer of epidermis in N2-treated animals displayed more cells positive for Ki-67, suggesting a prompter regenerative capacity. Immunohistochemical staining demonstrated decreased deposition of immunoglobulin M and interleukin-8 after thermal injury in animals treated with N2 peptide, in comparison to controls. The findings of this study identify N2 blocking a specific inflammatory pathway, as a novel therapeutic approach, preventing the evolution of cutaneous burn injuries in a preclinical animal model. (J Burn Care Res 2017;38:148–160)

Burn injury causes an estimated 265,000 deaths per year, worldwide.[1] Burn injuries requiring medical treatment occur in 486,000 patients per year in the United States,[2] with far more scalds and sunburns treated by the patients themselves. Fire and burn injuries represent 1% of all hospitalized injuries and 2% of the total costs of injury, or $7.5 billion each year.[3] Burns continue to be common in combat-related trauma, with the majority in civilians rather than military personnel.[4] The development of therapies or medications that would decrease the extent or severity of the burn injury would lead to improved wound healing, possibly decreasing the need for grafting and subsequent decreased hospital stay and cost.

The area and depth of a burn wound are critical factors that influence the appearance and survival after thermal injury.[5] A superficial second-degree burn, which heals without scarring, can progresses early in its evolution, to involve the superficial dermis and the deep dermis, resulting in wound healing with severe scarring or requiring skin grafting. The transition from superficial to deep second-degree

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This project was supported by NIH SBIR 2R44GM076743 awarded to Decimmune, Inc., Cambridge, MA. Dr. Moore is a co-founder of Decimmune, Inc. and retains an equity interest.

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1559-047X/2016

DOI: 10.1097/BCR.0000000000000459

burn has been attributed to the acute inflammatory response that follows initial injury. Prior theories of the amplification of the burn included bacterial contamination of wounds; however, recent studies attribute activation and influx of neutrophils.[6–8] In our previous murine burn studies, we established a standard scalding burn condition following an initial heat titration.[9] Wounds of moderate severity in wild-type mice were created without causing skin cells necrosis; however, the wounds healed with ulceration and wound contracture. In the same setting, we found that mice deficient in immunoglobulin healed burn wounds with decreased scarring and ulceration. This finding confirmed that a thermal injury could be amplified by antibody, mediated inflammation. Reconstitution of the immunodeficient mice with pooled wild-type murine immunoglobulin (Ig)M reproduced the wild-type injury, thus identifying IgM as the proinflammatory antibody species.[9] This finding paralleled work in models of reperfusion injury, in which circulating "natural" IgM specific to a neoantigen exposed in injured tissue (nonmuscle myosin heavy chain, "nMMHC") provoked inflammation, leading to necrosis.[10–12] A clone of the murine natural IgM isolated and administered produced a severe reperfusion injury in the otherwise protected immunoglobulin-deficient mice.[13] This clone was shown to bind to a specific region of nMMHC, and a peptide that mimicked the binding region was synthesized (N2). Administration of N2 before or shortly after reperfusion blocked reperfusion injury in wild-type mice and rats.[14–17] We hypothesized that a similar inflammatory response was induced by scald burn, mediated by natural IgM directed to the same neoantigen, thereby extending the burn to ulceration and scarring. Administration of the IgM clone active in reperfusion injury, in immunoglobulin-deficient mice, led to injury similar to wild-type scald burns.[9] We then studied normal mice scalded after administration of N2 synthetic peptide and found that they were protected from injury and never developed scarring, contracture, or ulceration.[9] Studies on human burn patients[18–20] and in a rat burn model[21] have shown that the level of circulating IgM has decreased immediately after burn wounds, correlating with severity of the burn.[18] The murine combined with human studies indicate that further investigations in a large animal model of burn injury may be warranted.

Many preclinical models have attempted to evaluate the mechanism of burn wounds, with limited studies have aimed to predict how the treatment will alter a human wound under clinical conditions. Sullivan et al[22] have performed an extensive comparison of results in wound healing studies in human, pigs, small mammals, and in vitro studies. Pig and human skin share similar structure and physiology. Small mammals have a dense layer of body hair, thin epidermis and dermis, and panniculus carnosus muscle under their loose skin. Both pig and human have sparser body hair, a thick epidermis, similar dermal architecture with comparable measurements of dermal–epidermal thickness ratio and the absence of a panniculus carnosus. Both pig and human show well-developed rete ridges, dermal papillary bodies, and abundant subdermal adipose tissue.[23–26] The size, orientation, and distribution of blood vessels in the dermis of the pig are similar to blood vessels in human skin.[27] More importantly, wound healing in rodents is primarily through wound contraction, but human and swine heal partial-thickness wounds largely through reepithelialization.[22] Finally, the pig's overall physiology is close to human physiology, with most key organ systems being similar in anatomy and function.[28] The many similarities between human and pig seem to make porcine thermal burns, a good candidate for testing novel burn treatments before clinical application.

The purpose of this study is to evaluate the effect of treatment with the N2 blocking peptide on burn wound progression and inflammation in a large animal, burn model. There are no immunoglobulin-deficient pigs, and, thus, the general method of reconstituting deficient animals to reproduce wild-type injury is not available. Instead, beneficial activity of the N2 blocking peptide would be presumptive evidence of the presence of the murine pathophysiology in the pig, as the N2-pathogenic IgM interaction is specific. The sequence of the N2 region of nMMHC is conserved in all species examined.[29] Thus, therapeutic activity of N2 in pig burn would identify the same pathogenic IgM that binds to porcine nMMHC as the inciting factor in burn wound amplification.

METHODS

Animals

Female Massachusetts General Hospital (MGH) miniature swine weighing 15 to 20 kg were used in this study.[30] MGH miniature swine were bred in a pathogen-free facility and housed at the Transplantation Biology Research Center, in accordance with the Guide for the Care and Use of Laboratory Animals. All experiments were conducted with the approval of the Institutional Animal Care and Use Committee of the MGH, and in accordance with the National

Institute of Health and Public Health Service guidelines for animal care.

Burn Experimental Protocol

Animals were sedated with a combination of 2 mg/kg Telazol® (tiletamine/zolazepam, 1:1, MI), and 2 mg/kg xylazine by intramuscular injection. Endotracheally intubated pigs were maintained under anesthesia with isoflurane 1 to 2% in an operating room in a prone position for the duration of the experiment. Oxygen saturation and heart rate were measured with pulse oximeter ear sensors, and respiratory rate, mucus membrane color, and rectal temperature monitored throughout the procedure. Postprocedural pain was treated with 0.03 mg/kg buprenorphine IV and 25 mcg/hr fentanyl patch transdermally.

The flank and back hair was clipped and a sterile field was produced using skin prep with soap, chlorhexidine, and poviodone-iodine. Circular areas for burning were outlined with a marking pen. To induce a contact burn of 12 cm^2, 2.1 kg custom-made brass blocks were preheated in different circulating water baths ranging from 54 to 75°C (Figure 1). Temperatures of all blocks were monitored until equilibrated with the temperature of the water bath. The heated blocks were wiped dry just before application to prevent water droplets from creating a steam burn on skin. Burns were created with applying the blocks to the skin's surface for 25 seconds in a pattern of four burns per row in two rows for a total of eight burns (3% BSA) evenly distributed between both sides of the pigs in the thoracic paravertebral region.

After an initial temperature titration, all further experimentations were done at 63 and 68°C, representing partial-thickness burns of different severity. Animals were treated in double-blind fashion with 4.6 mg/kg of normal saline, random peptide control, or N2 peptide intravenously 10 minutes before burns followed by 4.6 mg/kg/hr for the first 4 hours after burn injuries were induced. Digital imaging was performed (Canon, Rebel T3i/EOS 600D, NY). Six millimeter punch biopsies from each of the burned areas and two nonburned control areas were harvested, and blood sampled at 4 hours, 1 day, 4 days, 7 days, and 14 days after burn. The burn area was covered with nonadherent gauze covered with polyurethane occlusive dressing (Tegaderm™, 3M, Health care, St. Paul, MN) and wrapped with a tubular net to prevent manipulation by the animal subject. Dressings were changed after biopsies on days 1 and 4, and completely removed on day 7. Burn wounds were carefully monitored by clinical observation for any sign of infection at every dressing change and daily after dressing removal. All animals were killed at the end of the experiment on day 14 by intravenous administration of 100 mg/kg pentobarbital euthanasia.

The assessment of the wounds was primarily histological, based on examination of the punch biopsies. The sample size has been reported as "n," which represents one section per wound from multiple pigs. Blistering of burned skin was not observed at any temperature, an unanticipated difference between porcine and human skin.

Peptides

N2

12-amino acid synthetic peptide (New England Peptide, Gardner, MA) with the amino acid sequence of the hinge region of nonmuscle myosin heavy chain II (LMKNMDPLNDNV).[13]

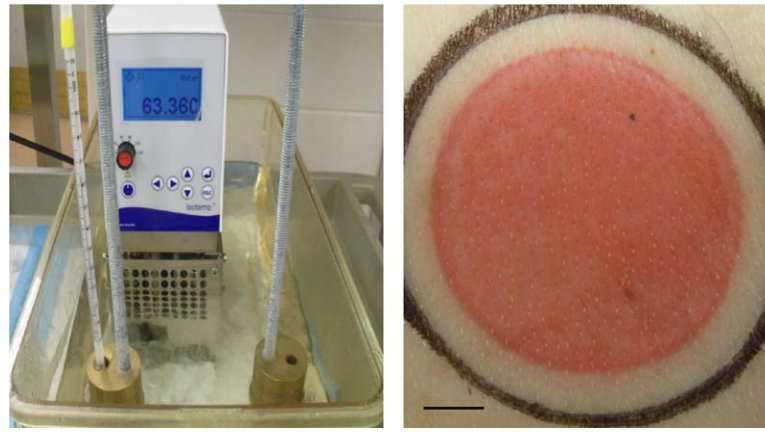

Figure 1. Left panel: Photograph of temperature-controlled water bath containing two 2.1 kg brass cylinders with an area of 12 cm^2 at each end. Each cylinder contains openings for insertion of thermometers to confirm block temperature. Right panel: Photograph of a representative contact burn produced by contact with heated brass cylinders. Scale bar: 1 cm.

Random Peptide Control. 12-amino acid synthetic peptide (New England Peptide, Gardner, MA) with the random amino acid sequence (AGCMPYVRIPTA).[13]

Histopathology

Punch biopsies were fixed in 10% formalin solution and embedded in paraffin. Sections were stained by H&E or Masson's trichrome or for chloroacetate esterase (CAE) reactivity as previously described.[31] For morphometric analysis, digital photographs of the burn sections were taken at different magnification using an ECLIPSE E400 light microscope, DIGITAL SIGHT camera, and NIS-Elements D3.0 digital image analysis system (Nikon Corporation, Kanagawa, Japan). Quantitative measurements were performed using Image J software (National Institutes of Health, Bethesda, MD).

At day 4 postburn, Masson's trichrome stain was used to differentiate denatured collagen (red staining) from viable collagen (blue staining) in the dermis of the burn wound. The cross-sectional area of the burn was determined by measuring the area of denatured collagen (expressed in square micrometers). As skin thickness varied to a small degree by anatomic location and by animal, a normalizing parameter, the percent of damaged dermis, was calculated by dividing the area of burn to total dermis area in each cross-section samples.

The percentage of reepithelialization at days 4, 7, and 14 was calculated by measuring the length of the neoepidermis in H&E-stained cross-sections and dividing it by the specimen's length, multiplied by 100.

At day 14 postburn, epidermal thickness was measured on H&E cross-sections. Values are the average of the 10 randomly selected points across each section and are expressed in micrometers. The number of rete formation per millimeter of neoepithelium was also counted in each cross-section at the same time point.

Masson's trichrome was used to demonstrate granulation tissue formation and the amount of normal collagen in the burn wound at 14 days after burn. With Masson's trichrome stain, the thickened epidermis was purple, and the granulation tissue in dermis was white in contrast with the surrounding dark-blue-stained collagen in normal dermis. The granulation tissue thickness was measured at three different locations, at the middle, left edge, and right edge of the burn. Values are the average of the three measurements and are expressed in micrometers. Skin thickness was normalized by calculating the percentage of granulation tissue in each cross-section by dividing total area of granulation tissue to total dermis area of the same sample.

At different time points after burn at 63°C, CAE was employed to highlight neutrophil infiltration. Neutrophils were quantified and expressed as the total number per 9 high-power fields (×40 magnifications).

Immunohistochemistry

Ki-67, IgM, and interleukin (IL)-8 staining was performed on formalin-fixed serial sections of skin. All slides were baked at 60°C for 1 hour. Sections were deparaffinized in xylene and rehydrated in a graded ethanol series, and antigens retrieved by microwaving in 10 mM sodium citrate (pH 6.0). Peroxidase activity was quenched with the dual endogenous enzyme block. Slides were incubated with a 1:1500 dilution of HRP-labeled rabbit antiporcine IgM (Novus Biologicals, Littleton, CO), or 1:500 dilution of Rabbit monoclonal anti-Ki67 (Thermo Scientific, Fremont, CA), or 1:250 mouse monoclonal anti-IL-8 (Abcam, Cambridge, MA) at 4°C overnight. Biotinylated second antibodies were applied at room temperature for 20 minutes. The signal was intensified using the Tyramide Signal Amplification system, detected with diaminobenzidine, and counterstained with hematoxylin.

Digital images of stained cross-sections were captured for each sample in one low-power field (×4 magnifications). IgM and IL-8 density was quantified as the number of positive cells in the entire depth of the dermis (epidermis to subcutaneous tissue) by image J software. Cellular proliferation was quantified as the number of positive Ki-67 cells present in the basal layer of the epidermis and expressed as the total number per 4 low-power fields (×10 magnifications).

Statistical Analysis

Student *t*-test for unpaired samples was used for direct comparisons of means of observations made in various animals. All results are given as mean ± SEM. A P value <.05 was used as an indicator that the results were not obtained at random.

RESULTS

Histological Characterization of the Scald Burn

Animals that received saline alone or saline with a control peptide showed no histological differences

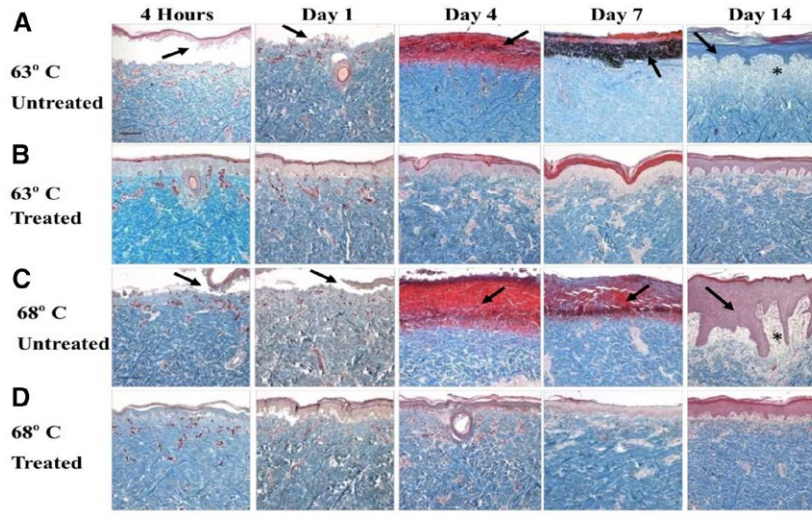

Figure 2. Treatment with N2 peptide causes relative protection from a 25 second contact burn to porcine skin. Representative histologic changes in untreated pigs are compared with N2 peptide-treated pigs after 63°C 25″ contact burns (A and B) and 68°C 25″ contact burns (C and D). All sections are stained with Masson's trichrome that differentiates normal collagen (blue) from denatured collagen (red). A, C. Disruption of the junction between the basal cells of the epithelium with complete detachment of epidermis–dermis layer at 4 hours and 1 day postburn (arrows). The epidermis at 4 and 7 days is denuded leaving an ulceration demarcated by denatured collagen (stained red, arrows). The denatured collagen extends through the dermis reflecting the breadth and depth of the burn and the loss of hair follicles. By 14-day postburn, the morphological changes include a thickened epithelium with only a few rete ridges covering the wound (arrows), and thick granulation tissue underneath the injured site (asterisk). B and D instead show degrees of intact epithelium at 4 hours and 1 day. There is an absence of denatured collagen, and instead an intact blue dermis with new epidermis present on 4 and 7 days. By 14-day postburn, there is a thin epidermis with more rete ridges and a thin layer of granulation tissue that is more similar to normal pig skin. Scale bars and original magnification: A–D, 200 μm, ×10.

in the contact burn wound at both 63 and 68° scald burn at 4 hours, 1, 4, 7, and 14 days after burn. They will be referred to as "untreated animals" in the remainder of the text. At 4-hour postburn, in the untreated animals burned at 63°C (Figure 2A) or 68°C (Figure 2C), the epidermis showed disruption of the basal cells of the epithelium with complete detachment of epidermis–dermis layer, with evident damage to dermis on days 4 and 7 and new epidermis appearing at day 14, with significant granulation tissue beneath, especially at the higher temperature. In contrast in the treated animals burned at the same temperatures (Figure 2B, D), intravenous administration of the blocking N2 peptide blunted the injury. There were modest epidermal–dermal junction changes at 4 hours with no significant damage to dermis on day 4, a new epidermis growing on day 7, and complete epithelization by day 14. This resembled the features of normal epidermis with a small amount of granulation tissue underneath. Thus, treatment with N2 blocking peptide attenuated the histologic evidence of burn injury, as well as hastened the rate of healing.

Burn Area in Cross-Section

On day 4 postburn, the mean histologically determined burn area in untreated group at both 63 and 68°C were measured ($7.73 \pm 2.47 \times 10^5$ and $1.86 \pm 0.44 \times 10^6$ μm^2; n = 9 and n = 6, respectively), and compared with treated group ($9.04 \pm 2.03 \times 10^4$ and $1.63 \pm 0.27 \times 10^5$ μm^2; n = 9 and n = 6, respectively). At both temperatures, N2 peptide-treated burns showed significantly reduced burn area compared with untreated burns (63°C, $P = .0245$; 68°C, $P = .0033$; Figure 3A). In the untreated group, the percentage of damaged dermis to total skin dermis were $8.05 \pm 2.7\%$ and $17.9 \pm 4.4\%$ at 63 and 68°, respectively. This was significantly decreased in treated group to $1.01 \pm 0.24\%$ ($P = .0187$) and $1.51 \pm 0.27\%$ ($P = .0042$) at 63 and 68°, respectively (Figure 3B). Thus, treatment with N2 reduced the depth of the wound resulting from a second-degree contact burn.

Cellular Proliferation

The number of cells staining positive for the proliferation marker Ki-67 and the intensity of staining were

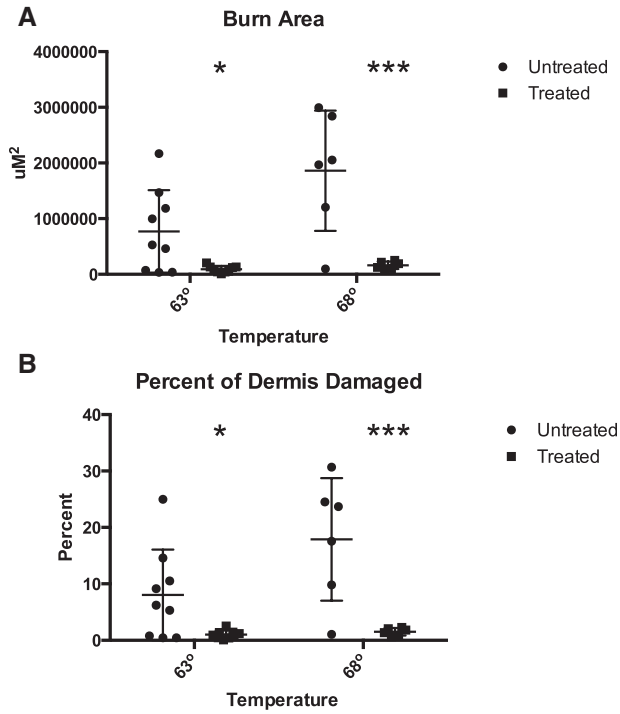

Figure 3. Treatment with N2 peptide decreases dermal injury compared with untreated in 25″ porcine contact burns. Assessment of total burn cross-sectional area (A) or normalized % of dermis burned (B) by analysis of Masson's trichrome stained sections on 4-day postburn. n = 9 sections (63°C), n = 6 sections (68°C). **P* < .05; ****P* < .005.

significantly increased in the treated group compared with untreated group (1405 ± 118.2 vs 874.3 ± 78.5, n = 10, *P* = .0015) 7 days after burn at 63°, suggesting greater regenerative capacity (Figure 4B). Increased staining was noted mostly in the basal layer of the epidermis and in the hair follicle (Figure 4A). Thus, treatment with N2 increased the number of proliferating cells in the burn wound, likely indicative of a more rapid rate of wound healing. The rate of the reepithelialization in the untreated group was very limited at 68° on day 7 after burn with a limited basal layer of the epidermis to be stained positive with Ki-67 marker. Thus, at day 7, 68°C comparison of Ki-67+ cells was not possible.

Reepithelialization

The rate of reepithelialization on days 4 and 7 after burn was more rapid in the treated group compared with the untreated group at 63 and 68°C, respectively. Results at 63°C were day 4, treated 30 ± 11.58% of wound length vs untreated 5.38 ± 3.27% (*P* = .035; n = 9); and day 7, treated 91.33 ± 6.6% vs untreated

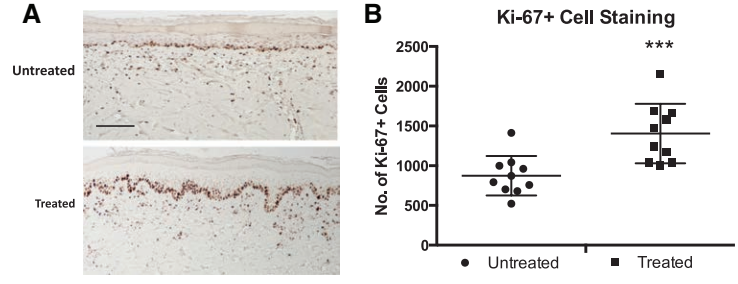

Figure 4. Treatment with N2 peptide in porcine 25″ 63°C contact burns show more proliferative cells at 7 days compared with untreated animals. A. Representative photomicrographs of immunoperoxidase stained burn wound biopsies for the Ki-67 proliferative marker, showing more proliferative cells in treated tissue than in untreated tissue. B. Quantification of Ki-67 cells (per 4 LPF), treated vs untreated burns. n = 10 sections in each group. ****P* < .005. Scale bar and original magnification: 200 μm, ×10. *LPF,* low-power field.

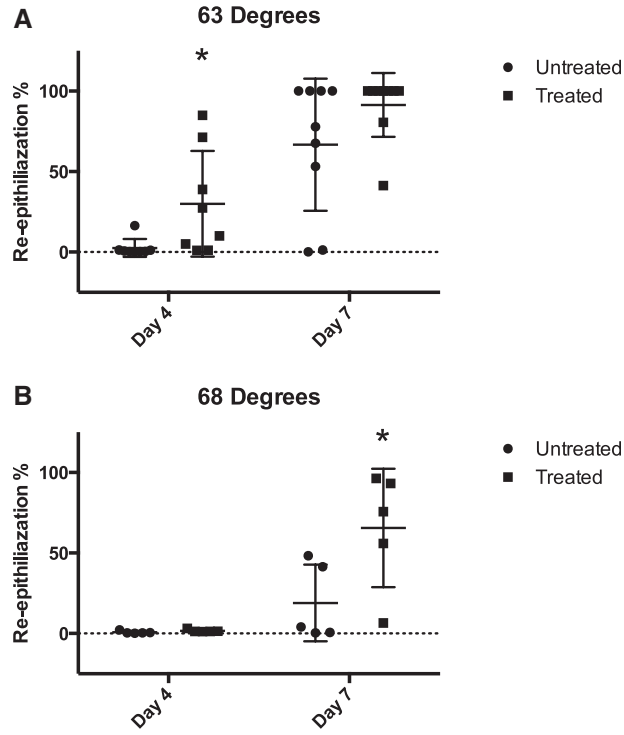

Figure 5. Treatment with N2 peptide accelerates reepithelialization after porcine contact burn. Quantitative analysis of hematoxylin and eosin sections of burn wound biopsies. A. N2-treated group displayed accelerated reepithelialization rate at 4 days (30%) compared with untreated group (5.38%) with 25″ 63°C contact burns. B. Similar results were observed at 68°C, with a significant difference on 7 days: 65.54% reepithelialization in the N2-treated group and 19% in the untreated group. All wounds were fully reepithelialized 14 days after burn. n = 9 sections (63°C), n = 5 sections (68°C). **P* < .05.

70 ± 12.67% at day 7 (NS; n = 9; Figure 5A). Results at 68°C were day 4, treated 5.01 ± 3.33% vs untreated 0.77 ± 0.38% (NS; n = 5); and day 7, treated 65.54 ± 16.42% vs untreated 19 ± 10.63% at 7 days, (*P* = .044; n = 5; Figure 5B). All wounds were fully reepithelialized by day 14 after burn. Thus, treatment with N2 accelerated the regeneration of epithelium over the burn wound.

Epidermal Morphology

Epidermal maturation was assessed 14 days after the burn. The new epidermis was thicker in all untreated groups compared with treated groups, but only burning at 68°C produced a statistically significant increase in epidermal thickness (195 ± 23.81 and 102 ± 15.12 μm, *P* = .008, respectively, n = 6; Figure 6A). Treated groups exhibited greater rete ridges formation at both 63 and 68°C compared with untreated group, but only the group burned at 63° reached a statistically significant increase (53.11 ± 6.11 and 34.89 ± 9.82, *P* = .016, respectively, n = 8). There was no significant difference between the numbers of rete ridges in treated group at 63°C with control unburned skin of the same animal (Figure 6B). Thus, treatment with N2 produced healing skin with a more normal structure than in untreated animals, similarly burned.

Granulation Tissue

On day 14 postburn, maximum granulation tissue thickness was significantly decreased in treated group at both 63 and 68°C. At 63°C, thickness was 97.37 ± 13.46 μm in N2-treated animals, compared with 209.7 ± 44.8 μm in untreated animals (*P* = .047, n = 6). At 68°C, thickness was 124 ± 37.17 μm in treated animals compared with 503.9 ± 94.62 μm in untreated animals (*P* = .004, n = 6; Figure 7A). The thickness, in both treated and untreated animals, was greater with 68°C burns as compared with 63°C burns. To assess for total wound granulation tissue, we calculated the percentage of granulation tissue area to total dermis area in each cross-section samples. In the peptide-treated group, granulation tissue percentage for both 63 and 68°C was significantly decreased compared with untreated group (63°C: 5.68 ± 1.36% vs 13.93 ± 2.76% [*P* = .023, n = 6]; 68°C: 5.95 ± 1.83% vs 17.62 ± 3.09% [*P* = .009, n = 6]; Figure 7B). Thus, using the development of

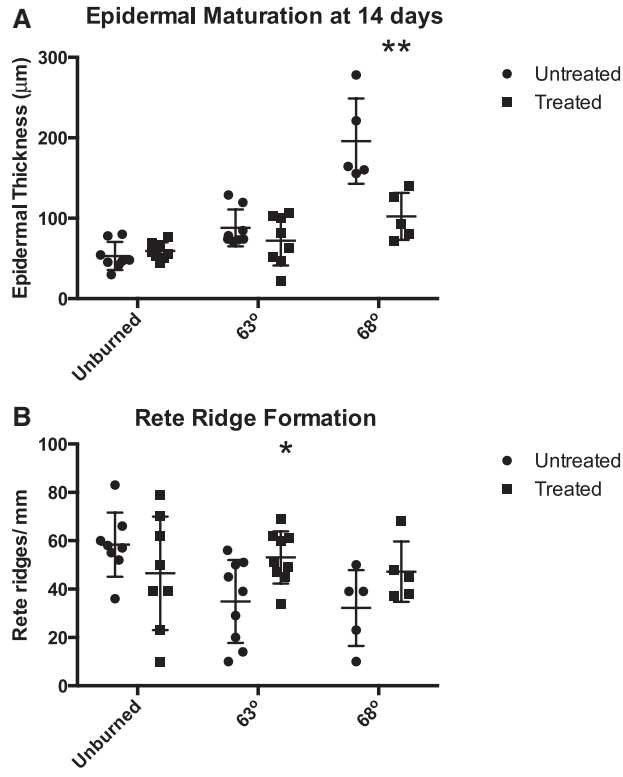

Figure 6. Treatment of pigs with N2 peptide normalized skin histologic appearance at 14 days after 25″ contact burns at 63 or 68°C. A. Epidermal thickness was measured in 10 representative areas of neoepidemis from hematoxylin and eosin-stained sections of burn wound biopsies. The epithelium thickness was increased in the untreated burn group compared with the N2-treated group, and compared with unburned skin, n = 6. B. The number of rete ridges of each wound was counted and expressed as the number of rete ridges per millimeter of cross-section of neoepidemis. Treated group exhibited significantly greater rete ridge formation compared with untreated, but fewer rete ridges seen in unburned porcine skin. Note that the appearance of the thickened neoepidermis and rete ridge changes is shown in the photomicrographs of Figure 1. $*P < .05$, $**P < .01$.

granulation tissue as a surrogate for the degree of preceding inflammation, treatment with N2 appears to have resulted in less initial inflammation after porcine contact burns.

IgM and IL-8 Immunohistochemistry Analysis

Cross-sections of biopsy specimens from untreated and treated animals at 4 hours and day 1 were examined for signs of early inflammation, using pig IgM deposition and the presence of IL-8 by immunohistochemistry. Staining was observed for both IgM and IL-8 as early as 4 hours. At 4 hours after the burn, the mean number of IgM-positive cells in wound biopsies from the treated group burned at 63°C (2128 ± 23) and 68°C (2981 ± 241) was significantly less than corresponding untreated group (4334 ± 134 and 5221 ± 161.4, $P = .0038$, $P = .0039$, respectively; n = 3). One day after the burn, the number of IgM-positive cells at 68°C in the treated group (2168 ± 243.7) was significantly decreased compared with the untreated group (3634 ± 138, $P = .0009$; n = 5), whereas at 63°C, it no longer demonstrated significance (Figure 8A). It was not possible to determine by this analysis whether the IgM-positive cells had IgM adhered to the cell surface or present in the cytoplasm. At 4 hours after the burn, the mean number of IL-8-positive cells in burn wounds of the treated group burned at 63°C (1705 ± 251.7) and 68°C (1764 ± 335.9) was also significantly less than in the corresponding untreated group (3875 ± 204.7 and 5243 ± 746.4, $P = .0002$, $P = .005$, respectively; n = 5). One day after the burn, the number of IL-8-positive cells in burn wounds of the treated group at 63°C (2046 ± 140.2) and 68°C (1686 ± 233.5) remained significantly decreased compared with the untreated group (2731 ± 141.9 and 2882 ± 124.1, $P = .007$, $P = .002$, respectively; n = 5; Figure 8B). Thus, treatment with N2 peptide led to a diminution of two elements of the initial inflammatory response of pigs to contact burns. The immunohistochemical staining appears more intense in burned tissue. However, our attempts to quantify this have been hampered by random variation in background and intravascular staining.

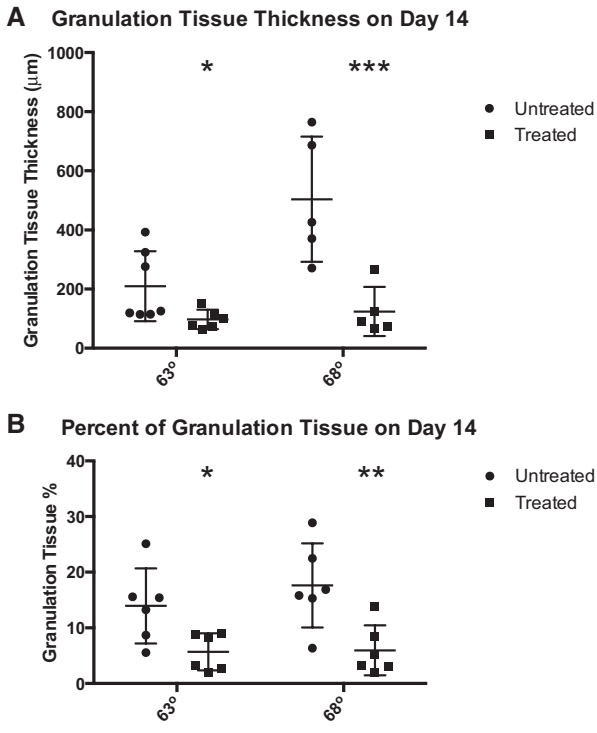

Figure 7. Treatment with N2 peptide decreased the amount of granulation tissue 14 days after porcine contact burns. A. Untreated pigs burned at 63 or 68°C had a significant increase in granulation tissue thickness compared with treated animals. B. The ratio of granulation tissue area to total dermal area was greater in untreated, burned pigs at both 63 and 68°C, compared with treated group at same temperatures. $*P < .05$, $**P < .01$, $***P < .005$.

Neutrophil Infiltration

CAE staining of burn wound biopsies was used to identify neutrophils (Figure 9A). The number of infiltrating neutrophils peaked at 4 days, consistent with the commonly observed peak at 48 to 96 hours after any surgical wound. At 63°C, 4 days after the burn, the number of CAE+ cells in the treated group (107.8 ± 9.88) was significantly decreased compared with the untreated group (231.5 ± 37.67, $P = .017$; n = 6; Figure 9B). At 68°C, the data were uninterpretable, likely due to more exaggerated expression of esterases by increasing numbers of infiltrating macrophages and monocytes, and by regeneration of resident mast cells.

Wound Infection

None of the burns became visibly infected at any time during the study period nor were any bacteria noted in the wound biopsies. We have no information from this study or past studies on the possible salutary impact on N2 on subsequent burn wound infection.

DISCUSSION

In a murine model of scald thermal injury, we reported that mice totally deficient in Ig had little evidence of the burn compared with intact mice. Reconstitution of these Ig-deficient mice with IV IgM, but not IgG, from normal mice, before scalding, resulted in the burn extent observed in a control mouse. This identified IgM as the antibody fraction causes temporal progression from scald application to evidence of injury. Further investigation revealed that a unique murine IgM clone ($IgM^{CM\text{-}22}$) restored the full extent of thermal injury in the Ig-deficient mice.[9] Together, these findings paralleled what we had observed in several types of reperfusion injury,[10–12] suggesting that there is a common pathway by which postinjury inflammation significantly amplifies the extent of an injury, after the injury has occurred. Interference with this pathway would represent a therapeutic opportunity of importance.

The antigen to which $IgM^{CM\text{-}22}$ is directed is the hinge region of nonmuscle myosin heavy chain[13] and is highly conserved in all species examined, including man. It is also a fundamental protein constituent of all cells. This suggests that the pathobiology in question could extend to other species, and already has been shown in rats.[16,17] In this study, we examined this biology in a preclinical setting using swine. We tested the presence of this pathobiology by attempting to interfere with the evolution of an injury, in this case a burn, by intravenous infusion of a peptide mimicking nonmuscle myosin antigen. The peptide mimic prevents the relevant natural IgM from binding to the injured tissue by occupying the IgM binding site. This interference would prevent amplification of injury by inflammation and indict this pathway in the pig, due to the specificity of the peptide mimic for this pathway. We have chosen burns because they are dynamic injuries that typically progress over the course of the first few days,[32] also thought by others to have components of an inflammatory response, ischemia, and/or ischemia–reperfusion (I/R).[33] I/R injury, as classically modeled, represents an acute, severe inflammatory response following an ischemic insult and subsequent restoration of blood flow. The reperfusion response is largely responsible for the final extent of myocardial infarction, cerebral ischemic events, and intestinal ischemia, as well as many detrimental effects of vascular surgery, trauma, and transplantation.[34] For I/R models, preclinical testing of this pathway is feasible. However, as opposed to inbred mice or strains of rats, in large animals, experimental I/R injuries have to be titrated one animal at a time, tremendously increasing the expense, the

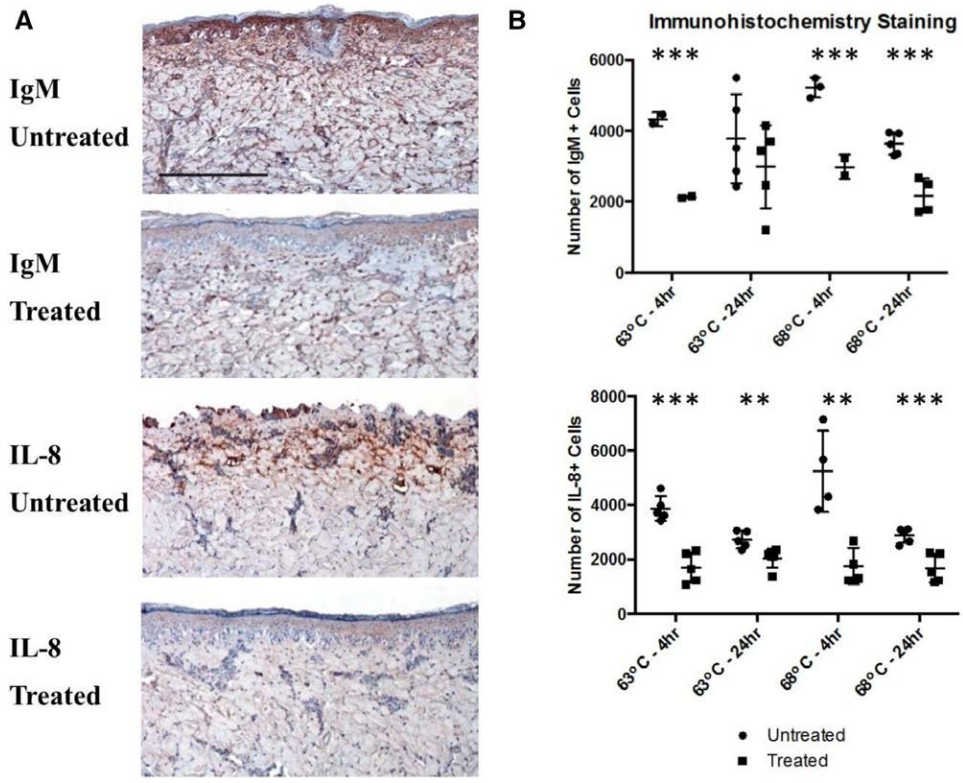

Figure 8. Treatment with N2 peptide decreased the early inflammatory response to porcine contact burns. A. Localization and intensity of both IgM deposition and IL-8 production is increased in untreated animals compared with N2-treated animals. Images are representative of 68 degree burn at 1 day. B. Quantitative assessment of the number of positive IgM (n = 3 sections at 4 hours, n = 5 at 1 day) and IL-8 cells (n = 5 sections in all groups) in the dermis of untreated and treated group burned at 63 and 68° on 4 hours and 1 day after burn injury. $^{**}P < .01$, $^{***}P < .005$. Scale bar and original magnification: 200 μm, ×10. *Ig,* immunoglobulin.

total duration of the whole experiment, and the variability caused by uncharacterized differences between each animal. On the other hand, multiple small burns with differing experimental characteristics can be placed on a single pig, thus avoiding many of the issues with experimental I/R. Thus, a porcine burn model is advantageous because of the apparent similarity to humans and for the ability to use animals as their own controls.

Most porcine models of burn injury have been designed to study the effects of a burn on the metabolic aspects of the animals' responses. For those models, surface area involved was the parameter to be varied and the depth of the wound was kept constant by inflicting generally third-degree burns. In the experiments reported here, we studied the burn wound itself, and thus needed to be able to[1] modulate the depth by contact with brass blocks of varying temperature for short intervals to avoid significantly decreased heat content in the blocks,[2] keep the total surface area burned less than the level that would cause systemic illness in the pigs, and[3] be able to use a single animal as its own control for burn intensity. Using this method, we found that application of brass blocks (Figure 1) between 63 and 68°C produced a reproducible and graded second-degree burn in the pig. Despite the perceived similarity between pig and human skin, and while there was epidermal sloughing, at no temperature did we observe the exuberant blistering that is so characteristic of human skin with a second-degree burn. In addition, the pigs reepithelized even the deeper burns within 2 weeks, giving a similar visual appearance to the 63 and 68°C burn at that time, despite obvious histologic differences. Thus, the primary data gathered for this study were histologic, rather than parameters of wound appearance.

Contact with the skin of pigs treated with intravenous saline or intravenous saline with control peptide for 25 seconds with a 63°C brass block produced a visible burn (Figure 1), as well as histologic changes characteristic of a burn injury (Figure 2A). Within 4 hours, there is epidermal sloughing, progressing to an inflammatory eschar at days 4 and 7, and

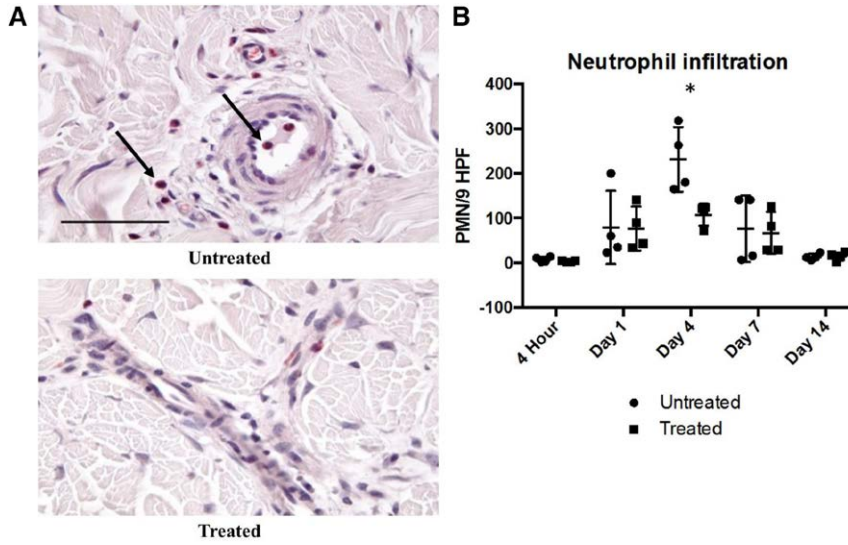

Figure 9. N2 peptide decreases cellular inflammatory response to porcine burn. A. CAE reactivity in the dermis shows neutrophils (arrows) in the blood vessels and tissue of untreated and treated samples burned at 63°C on day 1 after burn injury. B. Mean number (±SEM) of neutrophils (per 9 HPF) identified by CAE reactivity at different time points after burn. n = 6 for each assessment. **P* < .05. Scale bar and original magnification: 50 μm, ×63. *CAE,* chloroacetate esterase; *HPF,* high-power field.

reepithelialization at day 14. Increasing the temperature of the blocks to 68°C intensified the injury (Figure 2C), culminating in increased burn depth and a bizarre neoepidermis at day 14. Quantification of these changes (Figure 3) demonstrated that on day 4, more of the biopsy cross-section, by area or by percent involvement, was injured at 68°C than at 63°C. In contrast, pigs burned under identical conditions, which received intravenous N2 interfering peptide just before and after injury, demonstrated less severe histologic alterations at both 63°C (Figure 2B) and 68°C (Figure 2D) with less eschar formation and ultimately a more normal structural appearance at 14 days. Burn wound cross-sectional area quantified at day 4 (Figure 3) showed significantly less tissue injury in animals treated with N2, both at 63 and 68°C. Reflecting the diminution in the burn injury was evidence of more rapid healing. More proliferating cells were observed in skin from N2-treated animals (Figure 4). Wounds epithelialized more quickly (Figure 5) and skin attained a more normal histology (Figure 6) in N2-treated animals compared with their untreated counterparts. Thus, treatment of burned pigs with the N2 blocking peptide reduces the severity of the burn compared with untreated, and led to more rapid wound healing.

We also sought evidence that this was reflected by a decrease in wound inflammation in treated animals. One measure, the amount of postinflammatory or granulation tissue under the wound surface, demonstrated a pronounced decrease in N2-treated animals at both 63 and 68°C (Figure 7). In our mouse experiments, we were able to estimate that at least 5% of circulating, natural IgM specificity is directed to the nMM injury antigen. Thus, interference with binding of this IgM to an injury might be detectable. As shown in Figure 8A, treatment with N2 reduced the amount of IgM deposited in the burn wound at the relevant early time points. Of note, studies in mice,[35] rat,[21] children younger than 6 years old[18] and adults[19] have noted that there is a decrease in the serum concentration of IgM early after burn that correlates with the severity of the burn. Changes in IgM concentration could be the result of decreased synthesis, increased catabolism, vascular leakage to burn area, redistribution of fluid and protein between edema and intravascular space, and several other mechanisms. While we cannot exclude other possibilities based on our date, it is possible that binding of specific IgM to the injured tissue at the site of neoantigens exposed at the burn site could explain this serum reduction. The proportion of circulating IgM that would be bound in or near the site of injury is unknown. In this regard, N2 treatment cannot only prevent the inflammatory cascade activation, but possibly, by maintenance of serum IgM concentration, could improve immunocompetence and strengthen the host defense against infection. Further evidence for a decrease in inflammation was the decrease in IL-8, a neutrophil-specific chemoattractant, observed in wounds from untreated animals (Figure 8B). Finally, as seen in Figure 9, there

is less neutrophil infiltration in N2-treated animals. Studies in man[6] and animal models of burn[7,8,36–38] identify activation of neutrophils as one component of the inflammatory response, leading to amplification of burn injury depth. IL-8 is a potent chemoattractant for keratinocytes, neutrophils, as well as other leukocytes.[39] Many studies suggest a relation between both blood serum and tissue levels of the inflammatory mediators, complement and IL-8, to the progression of the burn wound in the postburn period resulting in further tissue destruction, slowly healing wounds, and finally resulting in scar formation.[33,40–44] Inhibition of complement activation through the natural complement inhibitor C1-estrase inhibitor (C1inh) did reduce capillary leakage, neutrophil activation, wound depth, and scarring in burned pigs. C1 inhibitors are known to decrease bradykinin levels and to inhibit the release of C3a and C5a. Excessive C5a functions as a critical inflammatory mediator to enhance IL-8 production.[45] The findings of Suber et al[9] similarly support the role of complement-induced inflammation in burn wound progression. In a mouse burn model, $C4^{-/-}$ complement-deficient mice healed without contracture, hair loss, or neutrophil infiltration, and that complement-sufficient mice pretreated with the complement inhibitor sCR1, that blocks cleavage of C3, likewise demonstrated reduced injury postburn. Moreover, their experiments revealed that IgM is responsible for stimulating the complement-induced injury in this mouse burn model. Thus, based on these parameters, treatment of burned pigs with N2 interfering peptide decreased wound inflammation. From these observations, it appears that pig burns are improved with N2 peptide treatment and that the anticipated mechanism of action could be similar to that seen in mice.

For these experiments, the interfering N2 peptide was administered at the same time as the burn. This is not the clinical scenario, but was done to provide proof of its principle of action. We have previously reported in mouse burns that topical N2 peptide also is effective, even if applied several hours after the burn. Based on that, we hypothesized that the most effective treatment modality would be a topical application that was kept in contact with the burn for hours, by means of a dressing. However, in the case of the pig, we could not devise a method to keep the animals from disrupting a topical application. Thus, intravenous administration was used, done in a way that would mean that N2 peptide was present for certain at the time that the circulation gained access to the wound, after the period of obligatory vasospasm. It remains our opinion that topical application will be the most efficacious, especially considering the extremely low concentrations needed to treat a mouse.[9]

In summary, pig burns appear to progress over time by the same pathway of inflammation that causes burn progression in mice, as well as the evolution of multiple forms of reperfusion injury. These data suggest that there is a mechanism for amplifying the tissue loss from injury that transcends species and type of injury. Although we have used N2 peptide for its specificity of inhibition to test this hypothesis in pig, its efficacy in reducing burn wound severity suggests that drugs based on this pathway may be useful for treating human burns. In addition, a change in wound depth from deep second-degree burn to superficial second-degree burn would have profound consequences on the cosmetic result of a patient who survives the burn injury.

REFERENCES

1. World Health Organization website. Available from http://www.who.int/violence_injury_prevention/other_injury/burns/en/ home page; accessed 10 March 2016.
2. American Burn association website. Available from http://www.ameriburn.org/resources_factsheet.php. accessed 10 March 2016.
3. Finkelstein EA, Corso PS, Miller TR. Incidence and Economic Burden of Injuries in the United States. Oxford: Oxford University Press; 2006.
4. Roeder RA, Schulman CI. An overview of war-related thermal injuries. J Craniofac Surg 2010;21:971–5.
5. Runyan CW, Casteel C, Perkis D, et al. Unintentional injuries in the home in the United States: part I: mortality. Am J Prev Med 2005;28:73–9.
6. Moore FD Jr, Davis C, Rodrick M, Mannick JA, Fearon DT. Neutrophil activation in thermal injury as assessed by increased expression of complement receptors. N Engl J Med 1986;314:948–53.
7. Horton JW, Mileski WJ, White DJ, Lipsky P. Monoclonal antibody to intercellular adhesion molecule-1 reduces cardiac contractile dysfunction after burn injury in rabbits. J Surg Res 1996;64:49–56.
8. Nwariaku FE, Mileski WJ, Lightfoot E Jr, Sikes PJ, Lipsky PE. Alterations in leukocyte adhesion molecule expression after burn injury. J Trauma 1995;39:285–8.
9. Suber F, Carroll MC, Moore FD Jr. Innate response to self-antigen significantly exacerbates burn wound depth. Proc Natl Acad Sci U S A 2007;104:3973–7.
10. Zhang M, Austen WG Jr, Chiu I, et al. Identification of a specific self-reactive IgM antibody that initiates intestinal ischemia/reperfusion injury. Proc Natl Acad Sci U S A 2004;101:3886–91.
11. Austen WG Jr, Zhang M, Chan R, et al. Murine hindlimb reperfusion injury can be initiated by a self-reactive monoclonal IgM. Surgery 2004;136:401–6.
12. Zhang M, Michael LH, Grosjean SA, Kelly RA, Carroll MC, Entman ML. The role of natural IgM in myocardial ischemia-reperfusion injury. J Mol Cell Cardiol 2006;41:62–7.
13. Zhang M, Alicot EM, Chiu I, et al. Identification of the target self-antigens in reperfusion injury. J Exp Med 2006;203:141–52.
14. Chan RK, Verna N, Afnan J, et al. Attenuation of skeletal muscle reperfusion injury with intravenous 12 amino acid peptides that bind to pathogenic IgM. Surgery 2006;139:236–43.

15. Haas MS, Alicot EM, Schuerpf F, et al. Blockade of self-reactive IgM significantly reduces injury in a murine model of acute myocardial infarction. Cardiovasc Res 2010;87:618–27.
16. Ahmadi-Yazdi C, Williams B, Oakes S, Moore FD Jr. Attenuation of the effects of rat hemorrhagic shock with a reperfusion injury-inhibiting agent specific to mice. Shock 2009;32:295–301.
17. Afnan J, Ahmadi-Yazdi C, Sheu EG, Oakes SM, Moore FD Jr. Inhibition of rat gut reperfusion injury with an agent developed for the mouse. Evidence that amplification of injury by innate immunity is conserved between two animal species. Am J Physiol Regul Integr Comp Physiol 2010;298:R1675–81.
18. Sobouti B, Fallah S, Ghavami Y, Moradi M. Serum immunoglobulin levels in pediatric burn patients. Burns 2013;39:473–6.
19. Arturson G, Högman CF, Johansson SG, Killander J. Changes in immunoglobulin levels in severely burned patients. Lancet 1969;1:546–8.
20. Bjornson AB, Altemeier WA, Bjornson HS. Changes in humoral components of host defense following burn trauma. Ann Surg 1977;186:88–96.
21. Man YC, Yee CW, Shing WK, Lai TP, Ching WK, Kei KK. The enhancing effects of a chicken-meat extract on serum Ig concentrations in normal and scalded animals. Br J Nutr 2005;94:51–5.
22. Sullivan TP, Eaglstein WH, Davis SC, Mertz P. The pig as a model for human wound healing. Wound Repair Regen 2001;9:66–76.
23. Meyer W, Schwarz R, Neurand K. The skin of domestic mammals as a model for the human skin, with special reference to the domestic pig. Curr Probl Dermatol 1978;7:39–52.
24. Vardaxis NJ, Brans TA, Boon ME, Kreis RW, Marres LM. Confocal laser scanning microscopy of porcine skin: implications for human wound healing studies. J Anat 1997;190(Pt 4):601–11.
25. Morris GM, Hopewell JW. Epidermal cell kinetics of the pig: a review. Cell Tissue Kinet 1990;23:271–82.
26. MontagnaW, Yun JS. The skin of the domestic pig. J Invest Dermatol 1964;43:11–21.
27. Forbes PD. Vascular supply of the skin and hair in swine. In: Montagna W, Dobson RL, editors. Advances in the Biology of Skin: Hair Growth. Vol. 9. Oxford: Pergamon; 1969. p. 419–32.
28. Lee KT. Swine as animal models in cardiovascular research. In: Tumbleson ME, editor. Swine in Biomedical Research. New York, NY: Plenum Press; 1986. p. 1481–98.
29. Hammer JA III, Bowers, B, Paterson BM, Kern ED. Complete nucleotide sequence and deduced polypeptide sequence of a non muscle myosin heavy chain gene from Acantomoeba: evidence of a hinge in the rodlike tail. J Cell Bio 1987; 105:913–925.
30. Lunney JK, Sachs DH. Transplantation in miniature swine. V. Characterization of Ia antigens. J Immunol 1979;122:623–7.
31. Abonia JP, Friend DS, Austen WG Jr, et al. Mast cell protease 5 mediates ischemia-reperfusion injury of mouse skeletal muscle. J Immunol 2005;174:7285–91.
32. Zawacki BE. The natural history of reversible burn injury. Surg Gynecol Obstet 1974;139:867–72.
33. Shupp JW, Nasabzadeh TJ, Rosenthal DS, Jordan MH, Fidler P, Jeng JC. A review of the local pathophysiologic bases of burn wound progression. J Burn Care Res 2010;31:849–73.
34. Cotran RS. Ischemic and hypoxic injury. In: Robbins Pathologic Basis of Disease. St. Louis, MO: WB Saunders Company; 1999. p. 7–12.
35. Kinoshita M, Shinomiya N, Ono S, et al. Restoration of natural IgM production from liver B cells by exogenous IL-18 improves the survival of burn-injured mice infected with *Pseudomonas aeruginosa*. J Immunol 2006;177:4627–35.
36. Mileski W, Borgstrom D, Lightfoot E, et al. Inhibition of leukocyte-endothelial adherence following thermal injury. J Surg Res 1992;52:334–9.
37. Mileski W, Gates B, Sigman A, et al. Inhibition of leukocyte adherence in a rabbit model of major thermal injury. J Burn Care Rehabil 1993;14:610–6.
38. Mileski WJ, Rothlien R, Lipsky P. Interference with the function of leukocyte adhesion molecules by monoclonal antibodies: a new approach to burn injury. Eur J Pediatr Surg 1994;4:225–30.
39. Kunkel SL, Lukacs N, Strieter RM. Chemokines and their role in human disease. Agents Actions Suppl 1995;46:11–22.
40. Iocono JA, Colleran KR, Remick DG, Gillespie BW, Ehrlich HP, Garner WL. Interleukin-8 levels and activity in delayed-healing human thermal wounds. Wound Repair Regen 2000;8:216–25.
41. Garner WL, Rodriguez JL, Miller CG, et al. Acute skin injury releases neutrophil chemoattractants. Surgery 1994;116:42–8.
42. Yeh FL, Lin WL, Shen HD, Fang RH. Changes in levels of serum IL-8 in burned patients. Burns 1997;23:555–9.
43. van de Goot F, Krijnen PA, Begieneman MP, Ulrich MM, Middelkoop E, Niessen HW. Acute inflammation is persistent locally in burn wounds: a pivotal role for complement and C-reactive protein. J Burn Care Res 2009;30:274–80.
44. Kang HJ, Kim JH, Lee EH, Lee YK, Hur M, Lee KM. Change of complement system predicts the outcome of patients with severe thermal injury. J Burn Care Rehabil 2003;24:148–53.
45. Henze U, Lennartz A, Hafemann B, Goldmann C, Kirkpatrick CJ, Klosterhalfen B. The influence of the C1-inhibitor BERINERT and the protein-free haemodialysate ACTIHAEMYL20% on the evolution of the depth of scald burns in a porcine model. Burns 1997;23:473–7.