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

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Abstract

Objective—In a mouse model, a second-degree burn elicits a severe inflammatory response that is mediated by circulating auto-antibody specific for a neo-antigen (non-muscle myosin). Non-muscle myosin is expressed by injured tissue, leading to amplified ulceration and scarring. We hypothesize that a synthetic peptide (N2) can mimic the neo-antigen and competitively inhibit the auto-antibody, decreasing inflammation, and reducing the extent of burn injury in a preclinical swine model of burn.

Methods—Second-degree burns were created on young swine using brass cylinders, warmed to varying temperatures prior to 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 following burn injury. Burn wound healing parameters were assessed. Immunohistochemical staining for Ki-67, IgM, and IL-8 were also performed.

Results—N2 blocking peptide administration decreased dermal injury at 4 days with increased re-epithelization, 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 reduction 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 IgM and IL-8 after thermal injury in animals treated with N2 peptide, in comparison to controls.

Conclusions—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 pre-clinical animal model.

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Keywords

wound healing; burn; natural IgM; trauma

Introduction

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 progress 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 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 IgM reproduced the wild type injury, thus identifying IgM as the pro-inflammatory antibody species (9). This finding paralleled work in models of reperfusion injury, in which circulating “natural” IgM specific to a neo-antigen exposed in injured tissue (non-muscle 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 neo-antigen, 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.* have performed an extensive comparison of results in wound healing studies in human, pigs, small mammals and in vitro studies (22). 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 re-epithelialization (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 prior to 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.

Materials and Methods

Animals

Female MGH miniature swine weighing 15–20 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 2mg/kg Telazol® (tiletamine/zolazepam, 1:1), and 2 mg/kg xylazine by intramuscular injection. Endotracheally intubated pigs were maintained under anesthesia with isoflurane 1–2% 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. Post-procedural pain was treated with 0.03mg/kg buprenorphine IV and 25mcg/h fentanyl patch transdermally.

The flank and back hair was clipped and a sterile field produced using skin prep with soap, chlorhexidine and povidone-iodine. Circular areas for burning were outlined with a marking pen. To induce a contact burn of 12 cm², 2.1 kg custom made brass blocks were preheated in different circulating water baths ranging from 54°C to 75°C (Fig. 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 4 burns per row in 2 rows for a total of eight burns (3% body surface area) 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°C 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). 6mm punch biopsies from each of the burned areas and two non-burned 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 non-adherent 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 sacrificed 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).

Random peptide control—12-amino acid synthetic peptide (New England Peptide, Gardner, MA) with the random amino acid sequence (AGCMPYVRIPA) (13).

Histopathology

Punch biopsies were fixed in 10% formalin solution and embedded in paraffin. Sections were stained by hematoxylin and eosin (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 re-epithelialization 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 ten 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 (HPF; ×40 magnifications).

Immunohistochemistry

Ki-67, IgM, and IL-8 staining was performed on formalin-fixed serial sections of skin. All slides were baked at 60°C for 1h. 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 anti-porcine IgM (Novus Biologicals, Littleton, CO), or 1:500 dilution of Rabbit monoclonal anti-Ki67 (Thermo Scientific, Fremont, Calif.), 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 ("LPF"; $\times 4$ 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 (LPF; $\times 10$ 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 $\pm$ SEM. A p value <0.05 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 in the contact burn wound at both 63°C and 68°C scald burn at 4 hour, 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 (Fig. 2A) or 68°C (Fig. 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 (Fig. 2B, 2D), 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°C and 68°C were measured ($7.73 \pm 2.47 \times 10^5$ and $1.86 \pm 0.44 \times 10^6 \mu\text{m}^2$; $n=9$ and $n=6$, respectively), and compared with treated group ($9.04 \pm 2.03 \times 10^4$ and $1.63 \pm 0.27 \times 10^5 \mu\text{m}^2$; $n=9$ and $n=6$, respectively). At both temperatures, N2 peptide treated showed significantly reduced burn area compared to untreated burns (63°C $p=0.0245$, 68°C $p=0.0033$) (Fig. 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 degrees, respectively. This was significantly decreased in treated group to $1.01 \pm 0.24\%$ ($p=0.0187$) and $1.51 \pm 0.27\%$ ($p=0.0042$) at 63 and 68 degrees, respectively (Fig. 3B). Thus, treatment with N2 reduced the depth of the wound resulting from a 2nd degree contact burn.

Cellular proliferation

The number of cells staining positive for the proliferation marker Ki-67 and the intensity of staining were significantly increased in the treated group compared to untreated (1405 ± 118.2 versus 874.3 ± 78.5 , $n=10$, $p=0.0015$) 7 days after burn at 63 degrees, suggesting greater regenerative capacity (Fig. 4B). Increased staining was noted mostly in the basal layer of the epidermis and in the hair follicle (Fig. 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 degree on day 7 after burn with a limited basal layer of the epidermis to be stained positive with Ki-67 marker. Thus, a day 7, 68C comparison of Ki67+ cells was not possible.

Reepithelialization

The rate of reepithelialization on day 4 and day 7 after burn was more rapid in the treated group compared to untreated at 63°C and 68°C, respectively. Results at 63°C were: day 4, treated $30 \pm 11.58\%$ of wound length versus untreated $5.38 \pm 3.27\%$ ($p=0.035$; $n=9$) and day 7, treated $91.33 \pm 6.6\%$ versus untreated $70 \pm 12.67\%$ at day 7, (NS; $n=9$) (Fig. 5A). Results at 68°C were: day 4, treated $5.01 \pm 3.33\%$ versus untreated $0.77 \pm 0.38\%$ (NS; $n=5$) and day 7, treated $65.54 \pm 16.42\%$ versus untreated $19 \pm 10.63\%$ at 7 days, ($p=0.044$; $n=5$) (Fig. 5B). All wounds were fully re-epithelialized 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 $102 \pm 15.12 \mu\text{m}$, $p=0.008$, respectively, $n=6$) (Fig. 6A). Treated groups exhibited greater rete ridges formation at both 63°C and 68°C compared with untreated group but only the group burned at 63 degree reached a statistically significant increase (53.11 ± 6.11 and 34.89 ± 9.82 , $p=0.016$, 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 (Fig. 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°C and 68°C. At 63°C, thickness was $97.37 \pm 13.46 \mu\text{m}$ in N2 treated animals, compared to $209.7 \pm 44.8 \mu\text{m}$ in untreated animals ($p=0.047$, $n=6$). At 68°C, thickness was $124 \pm 37.17 \mu\text{m}$ in treated animals compared to $503.9 \pm 94.62 \mu\text{m}$ in untreated animals ($p=0.004$, $n=6$) (Fig. 7A). The thickness, in both treated and untreated animals, was

greater with 68°C burns as compared to 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°C and 68°C was significantly decreased compared with untreated group (63°C: $5.68 \pm 1.36\%$ versus $13.93 \pm 2.76\%$ ($p=0.023$, $n=6$); 68°C: $5.95 \pm 1.83\%$ versus $17.62 \pm 3.09\%$ ($p=0.009$, $n=6$)) (Fig. 7B). Thus, using the development of 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) degrees was significantly less than corresponding untreated group (4334 ± 134 and 5221 ± 161.4 , $p=0.0038$, $p=0.0039$, respectively; $n=3$). 1 day after the burn, the number of IgM-positive cells at 68°C in the treated group (2168 ± 243.7) was significantly decreased compared to the untreated group (3634 ± 138 , $p=0.0009$; $n=5$), whereas at 63°C, it no longer demonstrated significance (Fig. 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 , $p=0.0002$, $p=0.005$, respectively; $n=5$). 1 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 to the untreated group (2731 ± 141.9 and 2882 ± 124.1 , $p=0.007$, $p=0.002$, respectively; $n=5$) (Fig. 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.

Neutrophil infiltration

CAE staining of burn wound biopsies was used to identify neutrophils (Fig 9A). The number of infiltrating neutrophils peaked at 4 days, consistent with the commonly observed peak at 48–96 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 to the untreated group (231.5 ± 37.67 , $p=0.017$; $n=6$), (Fig 9B). At 68°C, the data was 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 to intact mice. Reconstitution of these Ig-deficient mice with I.V. IgM, but not IgG, from normal mice, prior to scalding, resulted in the burn extent observed in a control mouse. This identified IgM as the antibody fraction that causes temporal progression from scald application to evidence of injury. Further investigation revealed that a unique murine IgM clone (IgM^{CM-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 post-injury 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-22} is directed is the hinge region of non-muscle 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 pre-clinical 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 non-muscle 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 since 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 (33). Ischemia–reperfusion (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 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 3rd 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 (Fig. 1) between 63°C and 68°C produced a reproducible and graded 2nd 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 2nd degree burn. In addition, the pigs re-epithelialized even the deeper burns within 2 weeks, giving a similar visual appearance to the 63°C and 68°C burn at that time, despite obvious histologic differences. Thus, the primary data gathered for this study was 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 (Fig 1), as well as histologic changes characteristic of a burn injury (Fig 2A). Within 4 hours, there is epidermal sloughing, progressing to an inflammatory eschar at days 4 and 7, and re-epithelialization at day 14. Increasing the temperature of the blocks to 68°C intensified the injury (Fig 2C), culminating in increased burn depth and a bizarre neo-epidermis at day 14. Quantification of these changes (Fig 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 (Fig 2B) and 68°C (Fig 2D) with less eschar formation and ultimately a more normal structural appearance at 14 days. Burn wound cross-sectional area quantified at day 4 (Fig 3) showed significantly less tissue injury in animals treated with N2, both at 63°C 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 (Fig 4). Wounds epithelialized more quickly (Fig 5) and skin attained a more normal histology (Fig 6) in N2 treated animals compared to their untreated counterparts. Thus, treatment of burned pigs with the N2 blocking peptide reduces the severity of the burn compared to 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 post-inflammatory or granulation tissue under the wound surface, demonstrated a pronounced reduction in N2 treated animals at both 63°C and 68°C (Fig 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 (36), children younger than 6 years old (18) and adults (19) have noted that there is a reduction 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 data, 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 reduction in inflammation was the decrease in IL8, a neutrophil-specific chemoattractant, observed in wounds from untreated animals (Fig 8B). Finally, as seen in Figure 9, there is less neutrophil infiltration in N2 treated animals. Studies in man (37) and animal models of burn (38–42) 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 (43). 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 (44–49). Inhibition of complement activation through the natural complement inhibitor C1-esterase 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 (50). The findings of Suber et al. similarly support the role of complement-induced inflammation in burn wound progression (9). 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. This data suggests 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 2nd degree burn to superficial 2nd degree burn would have profound consequences on the cosmetic result of a patient who survives the burn injury.

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Figure 1.

Left Panel: Photograph of temperature-controlled water bath containing two 2.1 kg brass cylinders with an area of 12cm^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.

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 to N2 peptide treated pigs after 63°C 25" contact burns (panels A and B) and 68°C 25" contact burns (panels C and D). All sections are stained with Masson's trichrome that differentiates normal collagen (blue) from denatured collagen (red). Panels A and C show disruption of the junction between the basal cells of the epithelium with complete detachment of epidermis-dermis layer at 4h and 1d postburn (arrows). The epidermis at 4d and 7d 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 14d 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 (star). Panels B and D instead show degrees of intact epithelium at 4h and 1d. There is an absence of denatured collagen, and instead an intact blue dermis with new epidermis present on 4d and 7d. By 14d 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 μ m, $\times 10$.

Figure 3. Treatment with N2 peptide decreases dermal injury compared to untreated in 25" porcine contact burns

Assessment of total burn cross-sectional area (panel A) or normalized % of dermis burned (panel B) by analysis of Masson's trichrome stained sections on d4 postburn. n=9 sections (63°C), n=6 sections (68°C). * $p < 0.05$; *** $p < 0.005$.

Figure 4. Treatment with N2 peptide in porcine 25" 63°C contact burns show more proliferative cells at 7 days compared to untreated animals

Panel 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. Panel B: Quantification of Ki-67 cells (per 4 LPF), treated versus untreated burns. n=10 sections in each group. ***p < 0.005. Scale bar and original magnification: 200 μ m, $\times 10$.

Figure 5. Treatment with N2 peptide accelerates re-epithelialization after porcine contact burn
Quantitative analysis of H&E sections of burn wound biopsies. Panel A: N2 treated group displayed accelerated re-epithelialization rate at 4d (30 percent) compared with untreated group (5.38 percent) with 25" 63°C contact burns. Panel B: Similar results were observed at 68°C, with a significant difference on 7d: 65.54 percent re-epithelialization in the N2 treated group and 19 percent in the untreated group. All wounds were fully re-epithelialized 14 days after burn. n=9 sections (63°C), n=5 sections (68°C). * $p < 0.05$.

Figure 6. Treatment of pigs with N2 peptide normalized skin histologic appearance at 14 days after 25'' contact burns at 63°C or 68°C

Panel A: Epidermal thickness was measured in 10 representative areas of neoepidermis from H&E stained sections of burn wound biopsies. The epithelium thickness was increased in the untreated burn group compared to the N2 treated group, and compared to unburned skin, n=6. Panel 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 neoepidermis. Treated group exhibited significantly greater rete ridge formation compared to 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. * $P < 0.05$, ** $p < 0.01$.

Figure 7. Treatment with N2 peptide decreased the amount of granulation tissue 14d after porcine contact burns

Panel A: Untreated pigs burned at 63°C or 68°C had a significant increase in granulation tissue thickness compared to treated animals. Panel B: The ratio of granulation tissue area to total dermal area was greater in untreated, burned pigs at both 63°C and 68°C, compared to treated group at same temperatures. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

Figure 8. Treatment with N2 peptide decreased the early inflammatory response to porcine contact burns

Panel A: Localization and intensity of both IgM deposition and IL-8 production is increased in untreated animals compared to N2 treated animals. Images are representative of 68 degree burn at 1d. Panel B: Quantitative assessment of the number of positive IgM (n=3 sections at 4h, n=5 at 1d) and IL-8 cells (n=5 sections in all groups) in the dermis of untreated and treated group burned at 63 and 68 degrees on 4h and 1d after burn injury. **p < 0.01, ***p < 0.005. Scale bar and original magnification: 200 μ m, $\times 10$.

Figure 9. N2 peptide decreases cellular inflammatory response to porcine burn

Panel 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 d1 after burn injury. Panel B: Mean number ($\pm$ SEM) of neutrophils (per 9 HPF) identified by CAE reactivity at different time points after burn. $n=6$ for each assessment. * $p < 0.05$. Scale bar and original magnification: 50 μ m, $\times 63$.