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Resolvin D2 prevents secondary thrombosis and necrosis in a mouse burn wound model

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Abstract

Deep partial thickness burns are subject to delayed necrosis of initially viable tissues surrounding the primary zone of thermally induced coagulation, which results in an expansion of the burn wound, both in area and depth, within 48 hours postburn. Neutrophil sequestration and activation leading to microvascular damage is thought to mediate this secondary tissue damage. Resolvins, a class of endogenous mediators derived from omega-3 polyunsaturated fatty acids, have been shown to regulate the resolution of inflammation. We hypothesized that exogenous resolvins could mitigate the deleterious impact of the inflammatory response in burn wounds. Using two different mouse burn injury models involving significant partial thickness injuries, we found that a systemically administered single dose of resolvin D2 (RvD2) as low as 25 pg/g bw given within an interval of up to 4 hours postburn effectively prevented thrombosis of the deep dermal vascular network and subsequent dermal necrosis. By preserving the microvascular network, RvD2 enhanced neutrophil access to the dermis, but prevented neutrophil-mediated damage through other anti-inflammatory actions, including inhibition of tumor necrosis factor- α , interleukin-1 β , and neutrophil platelet–endothelial cell adhesion molecule-1. In a clinical context, RvD2 may be therapeutically useful by reducing the need for surgical debridement and the area requiring skin grafting.

Cutaneous burns trigger dynamic local and systemic responses that strongly affect clinical outcome.¹ A hallmark of the local response is that initial tissue damage is followed by progressive secondary expansion of necrosis into neighboring, initially viable tissues, both in width and depth.² Thus, repeated surgical debridement is often required.³ The mechanisms leading to secondary tissue damage in the early aseptic phase of burn injury likely involve exuberant local inflammatory responses together with protracted ischemia.⁴ There is evidence for dysfunctional microvasculature and limited perfusion.^{5,6} There is also infiltration of neutrophils which upon activation can induce significant collateral damage by releasing pro-inflammatory cytokines and reactive oxygen species.⁷ In this context, the interaction between the endothelium and neutrophils is critical.^{8–10}

Immune cells release endogenous lipid mediators that control important events during inflammation, such as neutrophil migration, adhesion, activation, and clearance. In particular, omega-3 polyunsaturated fatty acid-derived lipid mediators, known as resolvins,

regulate critical cellular events in the resolution of inflammation. Resolvins derived from docosahexaenoic acid are synthesized by neutrophils during the resolution phase of inflammation, blocking the secretion of interleukin-1 beta (IL-1 β) and tumor necrosis factor α (TNF- α), reducing neutrophil adhesion to the endothelium, and inhibiting neutrophil infiltration into the tissue.^{11,12} In this study, we specifically focus on resolvin D2 (RvD2), which is endogenously produced by the sequential actions of 15-lipoxygenase and 5-lipoxygenase on docosahexaenoic acid, and whose stereochemistry (7S,16R,17S-trihydroxy-4Z,8E,10Z,12E,14E,19Z-docosahexaenoic acid) has been confirmed by total organic synthesis.^{13,14} In addition, the highly potent and specific inhibitory action of RvD2 on neutrophil chemotaxis is well defined. In general, resolvins have been shown to be beneficial in animal models of inflammatory bowel disease, colitis, sepsis, asthmatic airway inflammation, conjunctivitis, and myocardial ischemia-reperfusion injury.^{15–18} RvD2 in particular proved protective in murine models of sepsis via its ability to regulate neutrophil infiltration, cytokine levels including TNF- α , the adhesive properties of vascular endothelial cells, and production of vasoactive mediators such as nitric oxide and prostacyclin.¹⁴

In this study, we investigated therapeutic effects of exogenously applied RvD2 during the early phase of burn injury in a mouse model. We show that wound infiltration by neutrophils correlates with microvascular thrombosis of the burn affected skin areas and beyond, the induction of ischemic damage markers, and secondary tissue necrosis within 48 hours postburn. RvD2 dampened the neutrophil-driven inflammatory response and mitigated secondary tissue damage in burn wounds.

Materials and Methods

Mouse models of burn injury and treatment

Experiments were performed using wild-type adult (6 weeks, >20 g) male C57BL/6 mice purchased from The Jackson Laboratory (Bar Harbor, ME; <http://www.jax.org>). All animal procedures were performed in accordance with National Research Council guidelines and were approved by the Subcommittee on Research Animal Care at Massachusetts General Hospital, Boston, MA. The animal's back was shaved and *NAIR*TMMed 24 hours before burn injury. To induce the burn, animals were anesthetized using intraperitoneal ketamine/xylazine (80/6 mg/kg). Two different burn models were used. In the first one, similar to a previously described comb burn (model 1),^{19,20} a serrated rectangular brass block preheated in boiling water was applied onto the animal's back parallel to the midline for 10 seconds. The brass block shape was such that five 4 $\times$ 8 mm full-thickness burns were induced separated by variable uninjured interspaces (2–2.5–3–3.5 mm). The total area of burn corresponded to 12% of the body surface area. After burn, the animals received intraperitoneal saline resuscitation (40 mL/kg/24 hours) and subcutaneous analgesia (0.05–0.1 mg/kg buprenorphine). For the second burn model (model 2), the animal was positioned in a mold and the shaved back of the animal contacted with 90 °C water for 10 seconds, thus yielding a scald burn over 20% of the total body surface area. Treatment with RvD2 (Cayman Chemical, Ann Arbor, MI) was given i.v. at the time points indicated in the figures. For neutrophilic depletion, rat antimouse Gr1 (Ly6) monoclonal antibody (Beckman Coulter, Brea, CA) was given i.v. at a dose of 0.2 μ g/g bw 24 hours prior to experiments.

Gross analysis of wounds

Macroscopic pictures of the wounds, including a background scale, were taken from a standard distance using a digital camera (Coolpix, Nikon, Tokyo, Japan). Wound surface area was quantified using ImageJ 1.43u (<http://rsb.info.nih.gov/ij>) software. To assess vascularity, the underside of the burn wound was surgically exposed, taking special care to avoid damaging the deep dermal vascular plane. The prepared skin was rinsed with saline,

pinned to a custom-made stage, and pictures taken at $7.5 \times$ magnification using a macroscope with polarizing filters (Olympus SZX12, Tokyo, Japan) mounted with a digital camera (Olympus DP10). Vascularity of the basal dermal layer was calculated as the area covered by the microvessels normalized to the total viewing area. Raw images were thresholded to highlight microvessels and analyzed using ImageJ.

Histology

Skin and wound histology was performed by taking 6 mm-diameter biopsy punches from areas of interest. Samples were fixed in 10% buffered formalin overnight at 4 °C, dehydrated with increasing concentrations of ethanol, embedded in paraffin, cut into 5- μ m sections, and stained with hematoxylin and eosin. The stained sections were examined under inverse light microscopy.

Myeloperoxidase activity assay

Biopsy punches of 6-mm diameter were taken from center areas (>5 mm distance from rim of wound) as well as rim areas (within the burn wound) and the margin areas (skin >5 mm distance from wound) at different time points. These were crushed in liquid nitrogen and further processed in order to obtain supernates of tissue homogenates. Additionally, serum samples of treated animals were taken. A modified colorimetric myeloperoxidase (MPO) assay was performed as previously described.²¹ In short, samples were exposed to three cycles of freeze-thawing in nondenaturing lysis buffer (TRIZMA, 10% glycerol, 0.2% Tween) followed by three freeze-thaw cycles and centrifugation. MPO reaction was carried out by first mixing 20 μ L skin lysate with 20 μ L assay buffer (0.167 mg/ml o-dianisidine-HCL, 50 mM Na_2HPO_4 , pH 5.4) in a 96-well plate and, after adding 200 μ L development solution (0.1% H_2O_2 , 50 mM Na_2HPO_4 , pH 5.4), measuring absorbance at 450 nm every 15 seconds. MPO activity is expressed as the change in absorbance per minute per milligram of skin lysate protein ($\Delta\text{mOD}/\text{min}/\text{mg}$ protein).

TNF- α wound content

TNF- α levels in wound tissue homogenates were measured using a mouse TNF- α enzyme-linked immunosorbent assay (BD Pharmingen, San Jose, CA).

Cell culture

Human skin microvascular endothelial cells (HSMECs, Lot#:7F3109, LONZA, CH) were cultured using MCDB 131 media (Caisson Laboratories, North Logan, UT) supplemented with 10% fetal bovine serum (FBS), penicillin (10,000 U/L), streptomycin (10 mg/mL), hydrocortisol (1 μ g/mL), dibutyryl cAMP (80 μ M), heparin (2 U/mL), endothelial cell growth supplement (25 μ g/mL) (Biomedical Technologies Inc., Stoughton, MA), and ascorbic acid (0.2 mM). Experiments were performed using standard phenol red free Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated FBS and L-glutamine (200 mM).

Isolation of neutrophils and neutrophil adhesion assay

Polymorphonuclear granulocytes (PMNs) were isolated from human peripheral blood by gradient separation using Lympholyte®-poly (CEDARLANE Labs, Burlington, NC) according to the recommended protocol. To assay adherence behavior of PMNs to endothelium in vitro, PMNs were co-cultured for 1 hour with HSMECs grown on cover slips, followed by three washing steps. Experimental pretreatments prior to the adherence assay included immersion heat shock of HSMECs for 20 seconds at 50 °C as well as stimulation of PMNs with RvD2 (150 pmol) in buffered saline solution prior to addition of neutrophils. For image analysis, HSMEC nuclei were labeled with Hoechst 33342, PMNs

with Calcein-AM™ stain (both Invitrogen, Grand Island, NY). Images were acquired using an inverted microscope equipped with an incubation chamber (Axiovert, Zeiss, Oberkochen, Germany) and analyzed using ImageJ 1.43u. To account for partial detachment of heat-shocked HSMECs, the number of adherent PMNs was normalized to the density of HSMECs. Washing steps effectively eliminated nonspecific PMN adherence to HSMEC-free areas.

Gene expression analysis

RNA was extracted from cell cultures or tissue samples using either TRIzol Reagent extraction process (<http://www.invitrogen.com>) or AllPrep DNA/RNA/Protein Mini Kit® (Qiagen, Germantown, MD). Total RNA quality was assessed by spectroscopy (NanoDropND-1000, ThermoScientific, Surrey, United Kingdom) and microfluidic gel analysis (Agilent RNA 6000 Nano Chip/Agilent2100 Bioanalyzer, Agilent, Santa Clara, CA) followed by cDNA conversion using the SuperScriptII Platinum® *Two-Step* qRT-PCR Kit (Invitrogen) or a RT² First Stand Kit *C-03* (SABiosciences) together with a Perkin Etus Thermal Cycler 480 (Perkin-Elmer, Waltham, MA). The gene expression patterns were then assessed by quantitative polymerase chain reaction (qPCR) with a RT² SYBR Green/Rox qPCR Master Mix (SABiosciences, Germantown, MD) using a Stratagene mx3005P instrument (Stratagene, La Jolla, CA). A melting curve was used to confirm the specificity of each primer pair. Each sample was run in triplicate to exclude outliers. Gene expression was analyzed using the commonly used $\Delta\Delta CT$ -method (RT²qPCR Primer Assay User Manual, Version2.17, SABiosciences) with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the normalizer gene. The average gene expression was computed for each experimental condition ($n = 3$) relative to the control ($n = 3$).

Primers for following genes were commercially acquired (SABiosciences or Sigma, St. Louis, MO): GAPDH, chemokine C-C motif ligand 5 (CCL-5), IL-10, glutathione peroxidase 1 (Gpx-1), Superoxide dismutase 1 (SOD1), NAD(P)H quinonic dehydrogenase 1 (Nqo-1), interferon gamma-induced protein 10 kDa (IP-10), IL-1(β, nuclear factor (erythroid-derived 2)-like 2 (Nfe2l-2), TNF-α, inhibitor of NF-κB alpha (IκB-α). The following primers were designed using Primer3 v. 0.4.0 (<http://frodo.wi.mit.edu/primer3/>) software based on coding DNA sequences (<http://www.ncbi.nlm.nih.gov/nucleotide>): Fas, forward 5'-GTCAAGATTGATGAA GAAT-3', reverse 5'-GCTGCAGACATGCTGTGGATC-3'; NFκappaB-1, forward 5'-CTGACCTGAGCCTTCTGGAC-3', reverse 5'-GCAGGCTATTGCTCATCACA-3'; hypoxia-inducible factor 1, alpha subunit (HIF1-α), forward 5'-CCTACCATCACTGCCACTGC-3', reverse 5'-GAGGCTGT CCGACTGTGAGT-3'; HIF3-α, forward 5'-CCCTGACAC TCTGGACTTGG-3', reverse 5'-GGTAGTAGGGAGGGC TCAGG-3'; platelet-endothelial cell adhesion molecule-1 (PECAM-1), forward 5'-GGAGTTTACAGAAATTATCA-3', reverse 5'-GAAACAGCTCTGTTATGTT-3'; vascular cell adhesion molecule 1 (VCAM-1), forward 5'-CCAGACAGA CAGTCCCCTCA-3', reverse 5'-GACCTCCACCTGGGTTC TCT-3'.

Statistics

The results are presented as mean ± standard deviation and/or standard error of the mean. Pairwise comparisons were performed by a two-tailed Student's *t*-test, whereas multiple comparisons were tested by two-way analysis of variance. Statistical analysis was performed using Prism4.0 (GraphPad, La Jolla, CA) software. In all the tests, $p < 0.05$ was considered statistically significant.

Results

RvD2 treatment delays wound contraction and increases scar area

Neutrophil infiltration of areas bordering coagulated tissue occurs very rapidly postburn. We hypothesized that early treatment with RvD2 would dampen neutrophil-mediated damage during this early phase of inflammation. To test this hypothesis, we first deployed a comb burn model in mice, where a preheated brass block is applied onto the skin to induce full-thickness injury in a pattern where burn areas are separated by increasingly distant interspaces where no direct thermal injury occurred (Figure 1A–C). In this model, secondary wound expansion can be visualized by a merging of initially separated burn areas. We saw that wound surface area expanded similarly in both RvD2- and vehicle-treated groups for the first 48 hours postburn. Subsequently, wounds contracted (the predominant wound closure mechanism in rodents²²). Interestingly, contraction was slower in the RvD2 treatment group (Figure 1D), and, after closure, the surface area of the scar was consistently greater in RvD2-treated vs. control groups (Figure 1E).

RvD2 treatment prevents secondary thrombosis of the deep dermal microvasculature and reduces depth of necrosis

To understand the effects of RvD2 on the wound dynamics, we performed histology of partial thickness scald burn dorsal wounds covering 20% of the total body surface area. At 1 hour postburn, controls exhibited necrosis of the epidermis and hair follicles, while the deep dermal connective tissue above the hypodermal muscle layer was still viable as demonstrated by intact perfusion of the deep dermal vascular network (DDVN). At 4 hours postburn, thrombosis of the DDVN was visible in some areas (Figure 2A) and, at 48 hours, tissue necrosis extended down into the fascia, at which point the injury appeared to be full thickness (Figure 2B). In the RvD2-treated group, histology at 48 hours shows engorged vessels within the hypodermal muscle layer, unlike controls where these vessels were no longer patent (Figure 2B). Thus, RvD2 maintained perfusion of the DDVN, which likely explains the hemorrhagic appearance of RvD2-treated burn wounds (Figure 1C), unlike the controls. At later time points, controls showed dermal breakdown associated with scab formation and shedding. Conversely, the original dermal architecture and the DDVN remained intact in RvD2-treated groups (Figure 2C). No residual dermal components were seen at 14 days postburn in untreated groups and wound beds primarily showed granulation tissue and ongoing wound contraction. Conversely, deep dermal tissue components and the DDVN were still discernible in the RvD2-treated animals along with substantial thickening of the underlying deep body fascia (Figure 2D). The persistent retention of dermal components in the RvD2 treatment groups would likely inhibit wound bed contraction, thereby offering a plausible explanation for decreased contraction and increased scar area in the RvD2 treatment group (Figure 1E).

RvD2-treated animals preserve their vasculature throughout the burn wound

Taken together, the histological data suggest that RvD2 prevents thrombosis of the DDVN. To better characterize this phenomenon, we surgically exposed and visualized the DDVN through the hypodermal plane underneath the site of the burn injury (Figure 3A). Quantitative image analysis was performed to determine the percentage of surface covered by vasculature, expressed as relative vascular area (RVA). RvD2-treated animals preserved DDVN patency over the entire wound area, while vehicle-treated controls demonstrated progressive loss of functional vasculature starting from the center of the burn (Figure 3B). RVA was separately measured at the edge and the center of the wounds. There was a sharp decrease in the controls during the first 24 hours postburn. RvD2-treated animals demonstrated a roughly twofold and fivefold larger RVA at the edge and center areas of the wounds at that time, respectively, and RVAs of treated groups remained larger than controls

until at least 48 hours postburn (Figure 3C). These data are consistent with RvD2 preserving the DDVN and preventing subsequent necrosis of the deeper layers of the skin.

RvD2 effectively mitigates the hypoxia-driven response in burn wounds

The antithrombotic actions of RvD2 also correlated with a suppression of classical cellular responses associated with hypoxia and oxidative stress. Antioxidant enzymes such as Gpx-1, SOD1, and Nqo1 were significantly up-regulated in burn wounds at 24 hours, suggesting underlying reperfusion injury, while RvD2 abolished this response. Nfe2l-2 (or Nrf2), a prominent regulator of the antioxidant response in mammalian cells, was highly up-regulated in control wounds, but not in the RvD2-treated groups, also at 24 hours postburn. Hypoxia-inducible factor 3, alpha subunit (HIF3- α), but not HIF1- α , was up-regulated in burn wounds, while RvD2 treatment also suppressed this induction (Figure 3D). Taken together, these data suggest that RvD2 prevented the typical response to hypoxia and ischemia-reperfusion injury, consistent with maintenance of blood perfusion to the wounds.

RvD2 treatment alters adhesion molecule expression and decreases neutrophil-endothelial adhesion

RvD2 has been shown to stimulate endothelial generation of nitric oxide and prostacyclin, both of which are chemo-repellent against inflammatory cells, as well as regulated complement C5a-mediated responses.¹⁴ Here, we investigated the expression of cellular adhesion molecules, which are involved in the recruitment and activation of inflammatory cells within injured tissue.^{23,24} Burn injury elevated both PECAM-1 and vascular cell adhesion molecules (VCAM-1) mRNA levels at 24 hours compared to normal healthy skin. PECAM-1, predominately expressed on neutrophils and monocytes, was significantly suppressed by RvD2. Conversely, VCAM-1, primarily found on vascular endothelium, was unaffected by RvD2 (Figure 4A). Thus, the effect of RvD2 appeared mostly targeted to circulating inflammatory cells rather than the endothelium. In separate in vitro studies, RvD2 treatment of freshly isolated human neutrophils decreased adherence to cultured HSMCs stimulated by heat shock by an order of magnitude (Figure 4B).

RvD2 changes neutrophil distribution within the wound bed

RvD2 may therefore prevent neutrophil adhesion to the vascular endothelium in burn wounds. To investigate how RvD2 affects neutrophil sequestration, we measured neutrophil MPO activity in biopsies retrieved from the wound center and edges. Tissue MPO levels in normal healthy skin were very low, as expected. Untreated controls showed an initial peak in MPO in the wound centers which quickly returned to baseline within 24 hours, consistent with the notion that subsequent thrombosis of the DDVN prevented further neutrophil access. In the marginal unburned skin around the wounds, MPO activity transiently rose between 24 and 72 hours postburn. In addition, MPO activity measured in serum peaked at around 36 hours postburn, possibly due to a systemic effect of the burn injury. These findings are consistent with an early onrush of neutrophils into the wounds, followed by thrombosis of the DDVN, which then prevented further access of neutrophils to areas undergoing necrosis. RvD2 treatment significantly altered these dynamics. First, RvD2 prevented the rapid drop in MPO activity in the wound centers previously observed in the controls. Second, RvD2 abrogated the transient rise in MPO activity within the skin bordering the wounds (Figure 5A). Third, RvD2 led to consistently lower MPO levels in serum as to be expected due to the well-known inhibitory effects of resolvins on neutrophilic mobilization and chemotactic behavior (Figure 5B). However, treatment did not prevent neutrophils from homing into wounds in significant numbers. Therefore, although RvD2 may have exerted some chemo-repellent effects (directly or indirectly) on neutrophils, patency preservation of the DDVN and thus accessibility was likely the main factor regulating the impact of RvD2 on wound neutrophil infiltration. We also corroborated the

MPO activity measurements with histological evidence of neutrophils by counting nuclei per area above and below the hypodermal muscle layer of representative tissue samples. Clearly, the center areas of RvD2-treated wounds had greater cellular infiltration at any time point (Figure 5C). Thus, RvD2 prevented thrombosis but did not reduce the number of neutrophils in the wounds.

RvD2 reduces TNF- α levels in burn wounds

The fact that RvD2 prevented microvascular thrombosis but did not prevent neutrophil accumulation in the wound suggested possible other RvD2-related effects mitigating neutrophil-mediated damage. We therefore investigated TNF- α wound secretion levels, used as marker of inflammatory response activity, in tissue samples harvested at the wound center and edge. Controls showed a sharp early increase in TNF- α levels (Figure 5D). At the wound center, these levels dropped off very quickly, likely reflecting necrosis in this area. At the wound edge, the levels peaked (~ 10 -fold) at the 24-hour time point, followed by a significant decline at the 48-hour time point, although remaining at some elevated baseline afterwards. In the RvD2-treated group, we saw a completely different dynamics whereby there was a gradual increase of TNF- α levels, both at the wound center and edge, all the way to the end of the observation period of 72 hours. Therefore, in spite of the increased accumulation of neutrophils in RvD2-treated wounds, RvD2 also inhibited the early TNF- α secretion typically observed in the burn wounds, and thus correlates with the antithrombotic effects of RvD2 treatment.

Depletion of neutrophils prevents secondary thrombosis and injury

The fact that RvD2 does not inhibit neutrophil accumulation but inhibits TNF- α secretion suggests that RvD2 may work by inhibiting neutrophil activation in the wounds. Thus, we investigated whether or not neutrophils do mediate secondary thrombosis in this burn wound model. For this purpose, we investigated the phenomenon in neutrophil-depleted animals using rat anti-mouse Gr-1 (Ly-6) monoclonal antibody treatment 24 hours prior to burn. MPO activity in the burn wounds as well as in serum at 24 hours was significantly decreased compared to normal mice (Figure 6A). Similar to RvD2 treatment, in neutrophil-depleted animals, the RVA visualized on the underside of the burns was preserved (Figure 6B). Preservation of the DDVN in neutrophil-depleted animals was also visible by histology (Figure 6C).

Expression of pro-inflammatory markers is dependent on the presence of neutrophils in wounds

We therefore investigated the impact of RvD2 on several pro-inflammatory markers in the wound (Figure 6D), namely TNF- α and other markers related to the innate immune response, such as IL-1 β , interleukin 6 (IL-6), IP-10, and CCL-5. At 24 hours postburn, control wounds had significantly elevated mRNA levels of TNF- α , IL-1 β , IL-6, CCL-5, and IP-10. RvD2 and anti-Gr-1-mediated neutrophil depletion both decreased TNF- α and IL-1 β induction. The effect on TNF- α was roughly similar in both cases, but the IL-1 β decrease was more dramatic after anti-Gr-1 neutrophil depletion. These treatments did not prevent burn injury-induced IL-6, CCL-5, and IP-10 expression. In addition, both treatments appeared to mitigate the TNF- α /fatty acid synthase receptor (FAS-R)-driven extracellular proapoptotic pathway. At the level of transcription factors, the paninflammatory marker nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) was not up-regulated in burn wounds, but its inhibitory counterpart I κ B α was by over an order of magnitude. As NF- κ B activation induces I κ B α expression, it is likely that constitutively expressed NF- κ B was activated following burn injury.^{25,26} Both treatments demonstrated a strong suppression of this negative feedback response.

Discussion

In this study, we demonstrate that RvD2 effectively prevents thrombosis of the DDVN in early phases of burn injury, thus reducing secondary tissue damage and promoting long-term survival of deep dermal components after the initial thermal insult. We also show that secondary tissue damage is related to the recruitment of neutrophils into the wounds, as no such damage is observed in neutrophil-depleted animals. However, RvD2 did not prevent neutrophils from homing into wounds, and the data rather suggest that RvD2 may inhibit neutrophil activation and perhaps exert other anti-inflammatory actions in the wound.

Trafficking of innate immune cells in high numbers into tissues is an early event in a response to burn injury. RvD2 has been reported in several studies to limit neutrophil migration and to promote nonphlogistic phagocytotic properties of macrophages.^{27,28} This could effectively help “resolve” the inflammation and promote a transition from the inflammatory to the proliferative and remodeling phases of wound healing.²⁹ Therefore, the fact that RvD2 did not prevent neutrophil sequestration into the burn wounds seems paradoxical. Evidently, preservation of the DDVN, by maintaining vascular access to the area, played a key role in allowing neutrophils to reach the area. It is also possible that the burn wounds release endogenous chemoattractants that escape neutrophil counter-regulation via the RvD2 axis. Another possibility is that we should explore possible species differences in the effect of RvD2 on neutrophil chemotactic responses as most of the current understanding comes from murine studies.

As RvD2 did not prevent neutrophils from entering the DDVN, it is highly likely that RvD2 inhibited other key neutrophil functions which come to bear only after they reach the wound site. These anti-inflammatory actions were reflected by a decreased secretion and/or inhibited action of inflammatory mediators such as TNF- α and IL-1 β , and down-regulation of PECAM-1 expression, which, taken together, are more likely to explain the beneficial effects of RvD2 on burn wound healing. Not considered here are potential actions of RvD2 that are not directly related to neutrophils or other immune cells, such as pain regulatory effects via central and peripheral nerve actions.^{30,31} Further studies on potential effects of RvD2 on endothelial cells as well as systemic effects of resolvins are warranted as well.

Although we did not systematically investigate the impact of the administration schedule, the fact that microvascular thrombosis occurs within a few hours in controls suggests that the extent of the therapeutic window is likely to be limited to a few hours postburn. Nevertheless, the fact that the RvD2 administration was effective even as a single dose as low as 25 pg/g bw within an interval of up to 4 hours postburn, is very promising for future translation into a therapeutic approach. Indeed, within this time frame most burn patients would be admitted in a hospital or under the care of emergency medical personnel who could administer such a therapy. In a clinical context, tissue preservation by RvD2 may be significant by reducing the area of wound that needs surgical debridement and skin grafting.

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References

1. Mecott GA, Al-Mousawi AM, Gauglitz GG, Herndon DN, Jeschke MG. The role of hyperglycemia in burned patients: evidence-based studies. *Shock*. 2010; 33:5–13. [PubMed: 19503020]

2. Kempf M, Cuttle L, Liu PY, Wang XQ, Kimble RM. Important improvements to porcine skin burn models, in search of the perfect burn. *Burns*. 2009; 35:454–5. [PubMed: 18947931]
3. Loos MS, Freeman BG, Lorenzetti A. Zone of injury: a critical review of the literature. *Ann Plast Surg*. 2010; 65:573–7. [PubMed: 20948420]
4. de Macedo JL, Santos JB. Bacterial and fungal colonization of burn wounds. *Mem Inst Oswaldo Cruz*. 2005; 100:535–9. [PubMed: 16184232]
5. Singer AJ, McClain SA, Taira BR, Rooney J, Steinhauff N, Rosenberg L. Rapid and selective enzymatic debridement of porcine comb burns with bromelain-derived Debrase: acute phase preservation of noninjured tissue and zone of stasis. *J Burn Care Res*. 2010; 31:304–9. [PubMed: 20182376]
6. Nisanci M, Eski M, Sahin I, Ilgan S, Isik S. Saving the zone of stasis in burns with activated protein C: an experimental study in rats. *Burns*. 2010; 36:397–402. [PubMed: 19765907]
7. Parihar A, Parihar MS, Milner S, Bhat S. Oxidative stress and anti-oxidative mobilization in burn injury. *Burns*. 2008; 34:6–17. [PubMed: 17905515]
8. Wright K, Nwariaku F, Halaihel N, Schulman C, Yin HL, Turnage RH. Burn-activated neutrophils and tumor necrosis factor- α alter endothelial cell actin cytoskeleton and enhance monolayer permeability. *Surgery*. 2000; 128:259–65. [PubMed: 10923002]
9. Liu Q, Kishimoto TK, Mainolfi E, Deleon RP, Myers C, Moretz RC. Dynamic expression of L-selectin in cell-to-cell interactions between neutrophils and endothelial cells in vitro. *Exp Cell Res*. 1998; 243:87–93. [PubMed: 9716452]
10. Godin C, Caprani A, Dufaux J, Flaud P. Interactions between neutrophils and endothelial cells. *J Cell Sci*. 1993; 106(Pt 2):441–51. [PubMed: 8282752]
11. Arita M, Yoshida M, Hong S, Tjonahen E, Glickman JN, Petasis NA, Blumberg RS, Serhan CN. Resolvin E1, an endogenous lipid mediator derived from omega-3 eicosapentaenoic acid, protects against 2,4,6-trinitrobenzene sulfonic acid-induced colitis. *Proc Natl Acad Sci U S A*. 2005; 102:7671–6. [PubMed: 15890784]
12. Serhan CN, Gotlinger K, Hong S, Arita M. Resolvins, docosatrienes, and neuroprotectins, novel omega-3-derived mediators, and their aspirin-triggered endogenous epimers: an overview of their protective roles in catabasis. *Prostaglandins Other Lipid Mediat*. 2004; 73:155–72. [PubMed: 15290791]
13. Serhan CN, Hong S, Gronert K, Colgan SP, Devchand PR, Mirick G, Moussignac RL. Resolvins: a family of bioactive products of omega-3 fatty acid transformation circuits initiated by aspirin treatment that counter proinflammation signals. *J Exp Med*. 2002; 196:1025–37. [PubMed: 12391014]
14. Spite M, Norling LV, Summers L, Yang R, Cooper D, Petasis NA, Flower RJ, Perretti M, Serhan CN. Resolvin D2 is a potent regulator of leukocytes and controls microbial sepsis. *Nature*. 2009; 461:1287–91. [PubMed: 19865173]
15. Krishnamoorthy S, Recchiuti A, Chiang N, Yacoubian S, Lee CH, Yang R, Petasis NA, Serhan CN. Resolvin D1 binds human phagocytes with evidence for proresolving receptors. *Proc Natl Acad Sci U S A*. 2010; 107:1660–5. [PubMed: 20080636]
16. Schwab JM, Chiang N, Arita M, Serhan CN. Resolvin E1 and protectin D1 activate inflammation-resolution programmes. *Nature*. 2007; 447:869–74. [PubMed: 17568749]
17. Duffield JS, Hong S, Vaidya VS, Lu Y, Fredman G, Serhan CN, Bonventre JV. Resolvin D series and protectin D1 mitigate acute kidney injury. *J Immunol*. 2006; 177:5902–11. [PubMed: 17056514]
18. Filep JG. Lipid mediator interplay: resolvin D1 attenuates inflammation evoked by glutathione-conjugated lipid peroxidation products. *Br J Pharmacol*. 2009; 158:1059–61. [PubMed: 19845687]
19. Regas FC, Ehrlich HP. Elucidating the vascular response to burns with a new rat model. *J Trauma*. 1992; 32:557–63. [PubMed: 1588642]
20. Singer AJBL, Thode HC Jr, McClain SA. Standardized burn model using a multiparametric histologic analysis of burn depth. *Acad Emerg Med*. 2000; 7:1–6. [PubMed: 10894235]
21. Chen CJ, Kono H, Golenbock D, Reed G, Akira S, Rock KL. Identification of a key pathway required for the sterile inflammatory response triggered by dying cells. *Nat Med*. 2007; 13:851–6. [PubMed: 17572686]

22. Galiano RD, Michaels J, Dobryansky M, Levine JP, Gurtner GC. Quantitative and reproducible murine model of excisional wound healing. *Wound Repair Regen.* 2004; 12:485–92. [PubMed: 15260814]
23. Boyle JJ. Macrophage activation in atherosclerosis: pathogenesis and pharmacology of plaque rupture. *Curr Vasc Pharmacol.* 2005; 3:63–8. [PubMed: 15638783]
24. Bernot D, Peiretti F, Canault M, Juhan-Vague I, Nalbone G. Upregulation of TNF- α -induced ICAM-1 surface expression by adenylate cyclase-dependent pathway in human endothelial cells. *J Cell Physiol.* 2005; 202:434–41. [PubMed: 15389590]
25. Wang T, Zhang X, Li JJ. The role of NF- κ B in the regulation of cell stress responses. *Int Immunopharmacol.* 2002; 2:1509–20. [PubMed: 12433052]
26. Shen F, Cheng GF. Progresses in I κ B kinase and related signal transduction. *Sheng Li Ke Xue Jin Zhan.* 2001; 32:80–2. [PubMed: 12545787]
27. Schif-Zuck S, Gross N, Assi S, Rostoker R, Serhan CN, Ariel A. Saturated-efferocytosis generates pro-resolving CD11b low macrophages: modulation by resolvins and glucocorticoids. *Eur J Immunol.* 2011; 41:366–79. [PubMed: 21268007]
28. Fredman G, Oh SF, Ayilavarapu S, Hasturk H, Serhan CN, Van Dyke TE. Impaired phagocytosis in localized aggressive periodontitis: rescue by Resolvin E1. *PLoS ONE.* 2011; 6:e24422. [PubMed: 21935407]
29. Martin P, Leibovich SJ. Inflammatory cells during wound repair: the good, the bad and the ugly. *Trends Cell Biol.* 2005; 15:599–607. [PubMed: 16202600]
30. Xu ZZ, Zhang L, Liu T, Park JY, Berta T, Yang R, Serhan CN, Ji RR. Resolvins RvE1 and RvD1 attenuate inflammatory pain via central and peripheral actions. *Nat Med.* 2010; 16:592–7. 591p following 597. [PubMed: 20383154]
31. Park CK, Xu ZZ, Liu T, Lu N, Serhan CN, Ji RR. Resolvin D2 is a potent endogenous inhibitor for transient receptor potential subtype V1/A1, inflammatory pain, and spinal cord synaptic plasticity in mice: distinct roles of resolvin D1, D2, and E1. *J Neurosci.* 2011; 31:18433–8. [PubMed: 22171045]

Glossary

CCL-5	Chemokine C-C motif ligand 5
DDVN	Deep dermal vascular network
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Gpx-1	Glutathione peroxidase 1
HIF1-α	Hypoxia-inducible factor 1, alpha subunit
HIF3-α	Hypoxia-inducible factor 3, alpha subunit
HSMECs	Human skin microvascular endothelial cells
IκB-α	Inhibitor of NF- κ B alpha
IL-10	Interleukin-10
IL-1β	Interleukin-1 β
IP-10	Interferon gamma-induced protein 10 kDa
MPO	Myeloperoxidase
Nfe2l-2	Nuclear factor (erythroid-derived 2)-like 2
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
Nqo-1	NAD(P)H quinonic dehydrogenase 1

PECAM-1	Platelet–endothelial cell adhesion molecule-1
PMNs	Polymorphonuclear granulocytes (neutrophils)
RVA	Relative vascular area
RvD2	Resolvin D2
SOD1	Superoxide dismutase 1
TNF-α	Tumor necrosis factor- α
VCAM-1	Vascular cell adhesion molecule 1

Figure 1.

RvD2 treatment alters the appearance, onset of contraction, and resulting scar surface area of burn wounds. Model 1: A serrated brass block (A) was heated and applied to the depilated dorsal skin of mice for 10 seconds causing a pattern (B) of burn wounds separated by uninjured gaps. (C) Macroscopic appearance of wounds over time. At 48 hours postburn, control wounds appeared necrotic, while RvD2-treated wounds had a hemorrhagic appearance. (D) Wound area as a function of time ($n > 6$, mean $\pm$ SD). (E) Scar area observed 3 weeks postburn ($n > 8$, mean $\pm$ SD/SEM). * $p < 0.05$ vs. untreated control.

Figure 2.

RvD2 prevents progressive thrombosis of the deep dermal vascular network (DDVN) and necrosis of surrounding tissues. Model 2: (A) At 1 hour postburn, controls show patent vessels (red arrows). At 4 hours postburn, controls show signs of thrombosis (blue arrows). (B) Between 24 and 48 hours, complete thrombosis of the DDVN occurs in controls along with progression in the depth of dermal necrosis. Conversely, RvD2-treated animals still show patent and often largely engorged vessels at 48 hours. (C) At 6 days postburn, necrotic transformation in controls results in a scab forming on top of the underlying wound bed. Conversely, with RvD2 treatment, deep dermal structures were maintained. (D) At 14 days postburn, granulation tissue fills the defect in controls, whereas with RvD2 treatment, the wound bed shows residuals of the hypodermal muscle layer (black arrows) underlying the DDVN. Bars = 100 μ m.

Figure 3.

RvD2 prevents secondary thrombosis of the DDVN and expression of ischemic tissue markers. Model 2: (A) Representative images showing the DDVN 48 hours postburn in the RvD2 (500 pmol)-treated group compared to control. Bars = 5 mm. (B) Raw images from the center and edge of wounds were thresholded to quantify the degree of vascularization. (C) Changes of relative vascular area (RVA) over time in controls vs. treatment groups ($n = 4$, mean $\pm$ SD). Vehicle or RvD2 was given at 1, 12, 36, and 60 hours (see arrows). (D) Marker genes related to ischemia. Shown are mRNA fold changes normalized to healthy skin based on qPCR/ $\Delta\Delta C_t$ -method ($n = 3$, mean $\pm$ SD). * $p < 0.05$ vs. untreated control. Bars = 5 mm.

Figure 4.

RvD2 inhibits heat stress-induced neutrophil adhesion to endothelial cells in vivo and in vitro. (A) RvD2 suppression of PECAM-1 but not VCAM-1 in burns. Shown are mRNA fold changes normalized to healthy skin based on qPCR/ $\Delta\Delta$ Ct-method ($n = 3$, mean $\pm$ SD).

* $p < 0.05$ vs. untreated control. (B) RvD2 inhibited adherence of human neutrophils to human skin microvascular endothelial cells (HSMECs) in vitro. To assay for adherence, HSMECs on cover slips were heat shocked by immersion (IHS; 20 seconds, 50 °C). HSMECs were then co-cultured with neutrophils pretreated with RvD2 (100 pmol/mL). Shown are same field magnification images, blue = HSMECs, green = neutrophils. Cell adherence was quantified as number of adherent neutrophils normalized to density of HSMECs ($n > 3$, mean $\pm$ SD). * $p < 0.05$ vs. IHS untreated control. Bars = 150 μ m.

Figure 5.

RvD2 modulates myeloperoxidase (MPO) and TNF- α dynamics in distinct wound areas and serum. Model 2: (A) Time course comparison of MPO activity in center and edge areas of control burn wounds and RvD2-treated wounds ($n = 3$, mean $\pm$ SD). (B) Dynamics of MPO activity in serum in control vs. RvD2-treated wounds ($n = 3$, mean $\pm$ SD; * $p < 0.05$ vs. control). (C) Dynamics of neutrophil infiltration of burn wounds based on image analysis of representative histologies. (D) TNF- α levels as a function of time in center and edge areas of control and RvD2-treated burn wounds ($n = 3$, mean $\pm$ SD). In these experiments, RvD2 was given at 1 and 24 hours (see arrows).

Figure 6.

Neutrophils play a key role in the progressive thrombosis postburn. Model 2: Neutrophil depletion was induced by infusion of anti-Gr-1 antibody 24 hours prior to burn injury. (A) Comparison of MPO activity in serum and distinct areas of burn wounds at 24 hours postburn among untreated controls, anti-Gr-1-treated and RvD2-treated animals ($n = 3$, mean $\pm$ SD). * $p < 0.05$ vs. untreated controls. (B) Comparison of relative vascular area (RVA) among groups at 24 hours postburn. (C) Histological appearance: An accumulation of neutrophils is seen in and around thrombosed vessels (blue arrows) in controls opposed to anti-Gr-1-treated groups where vasculature remains patent (red arrows). Bars = 100 μ m. (D) Expression of pro-inflammatory marker genes in burns is mitigated by RvD2 treatment. Shown are mRNA fold changes normalized to healthy skin based on qPCR/ $\Delta\Delta$ Ct-method ($n = 3$, mean $\pm$ SD). * $p < 0.05$ vs. untreated control.