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ORIGINAL ARTICLE

Pirfenidone Ointment Modulates the Burn Wound Bed in C57BL/6 Mice by Suppressing Inflammatory Responses

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Abstract— An inflammatory response is the normal response to a burn-induced injury. The burn-associated inflammation can lead to further tissue damage as the tissue tries to repair the damage. Prolonged or excessive inflammation is associated with increased fibrosis of burn wounds and the development of hypertrophic scars. The high incidence of hypertrophic scar formation is one of the many challenges to treating deep partial-thickness burns. Prophylactic treatment to improve burn-induced hypertrophic scarring is lacking. For this reason, we evaluated prophylactic treatment of deep partial-thickness burns with pirfenidone in C57BL/6 mice. Pirfenidone is an FDA-approved anti-fibrotic drug for systemic use in the treatment of idiopathic lung fibrosis and other fibrotic disorders. Additionally, pirfenidone has anti-inflammatory activity. We tested treatment efficacy of pirfenidone using a mouse model of deep partial-thickness burns. Inflammatory cytokines including IL-1β, IL-2, IL-6, IL-13, G-CSF, and MIP-1α, along with neutrophil infiltration, were significantly reduced in wounds when mice were treated during the inflammatory phase of burn wound healing. Additionally, pirfenidone significantly reduced expression of αSMA 12 days after the induction of burns and modestly reduced hydroxyproline in 22-day-old burn wounds. Results show that pirfenidone treatment modulated the inflammatory response of the burn wound. The findings in this study indicate that further examination is required to validate the use of pirfenidone for prophylactic treatment to improve long-term outcomes of scarring and contracture in deep partial-thickness burn wounds.

KEY WORDS: pirfenidone; inflammation; partial-thickness burns; fibrosis; mice; hypertrophic scars.

INTRODUCTION

Burn wounds are some of the leading causes of morbidity and mortality. The immediate damage from the burn can be devastating and life threatening depending on the total body surface area (TBSA) involved. Superficial burns involve the upper dermis and require minimal intervention. Deep partial- and full-thickness burns require more medical care. Deep partial-thickness burns, in particular, can be a challenge to treat in part due to the difficulty in determining burn depth [9]. Moreover, burn wounds can continue to progress 2–4 days after thermal injury because of tissue

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damage exacerbated by the inflammatory response [5, 9]. When the inflammatory response continues unchecked past the initial inflammatory phase of wound healing, there is an increased potential of excessive fibrosis leading to hypertrophic scarring.

Hypertrophic scarring is a commonly occurring sequelae in patients that experience a deep partial-thickness burn. The hypertrophic scars can occur in as much as 50% of patients after a deep partial-thickness burn [3]. Deep partial-thickness burns retain some viable dermal tissue that contains keratinocyte stem cells and fibroblasts. Due in part to chronic inflammation, these cells, specifically fibroblasts, are dysregulated and prone to develop a profibrotic phenotype characterized by excessive production of collagen, as seen in hypertrophic scars [26, 32]. Hypertrophic scars have both functional impairment and detrimental psychological impacts on the patient [25]. Current treatment options for patients with hypertrophic scars are limited and minimally effective [1, 32]. Prophylactic treatment of burn-induced hypertrophic scarring is lacking [10]. We hypothesize that using prophylactic treatment options prior to the development of hypertrophic scars presents a viable treatment alternative. This approach may include treatment options to control the fibrotic or inflammatory response during the early phase of burn wound repair.

Pirfenidone (Pf) is an FDA-approved anti-fibrotic drug indicated for the treatment for idiopathic pulmonary fibrosis. It is a small molecule that is shown to have both anti-inflammatory and anti-fibrotic activity in several organs in both animal models and clinical trials [2, 11, 30, 34]. Our *in vitro* studies demonstrate that Pf is effective in dampening transforming growth factor (TGF)-β signaling that leads to myofibroblast differentiation and fibrotic responses [15]. Additionally, the drug targets p38 kinase signaling, known to be involved in inflammatory pathways [20, 31, 35]. We used our mouse model of deep partial-thickness [23] to evaluate the effectiveness of prophylactic treatment with Pf ointment formulations to ameliorate inflammatory responses that occur after experimental induction of burn wounds in C57BL/6 mice. We found that prophylactic treatment of deep partial-thickness burn wounds with topical Pf lessened inflammatory cytokines, α-smooth muscle actin, and hydroxyproline deposition in wound tissues.

MATERIALS AND METHODS

Mouse Scald Procedure. Research was conducted in compliance with the Animal Welfare Act, the implementing Animal Welfare Regulations, and the principles of the Guide for the Care and Use of Laboratory Animals, National Research Council. The facility Institutional Animal Care and Use Committee approved all research conducted in this study. The facility where this research was conducted is fully accredited by AAALAC International. Male C57BL/6 mice, 15 weeks old, weighing 24 to 35 g (Jackson Laboratory, Bar Harbor, ME), were individually housed for 1 week prior to initiation of burn procedures. After acclimation period, mice were scalded using a previously established model [23]. Briefly, all animals were anesthetized with 3–5% isoflurane, and their dorsal hair were shaved and depilated with a hair removal cream the day before the scald. To avoid any potential chemical burn, any residual hair removal cream was removed from the skin by repetitive cleansing using water-soaked gauze. After this procedure, they were randomly assigned to different treatment groups with six per group. Prior to burn, mice were injected subcutaneously with 1.2 mg/kg of buprenorphine SR Lab (Zoopharm, Windsor, CO) and allowed to rest for approximately 30 min for the drug to take effect. All animals were then anesthetized with a combination of ketamine (50–100 mg/kg) and xylazine (5–10 mg/kg), injected intramuscularly. Following anesthesia, mice were scalded at 54 °C for 20 s to induce a deep partial-thickness burn. One half to 1 mL of pre-warmed (36 °C) lactated Ringer's solution was injected intraperitoneally to aid in resuscitation and prevent dehydration. All mice were tattooed with tattoo paste (Ketchum Manufacturing Inc., Brockville, Canada) using lancets (Medipoint, Inc., Mineola, NY) to mark the corners and sides of the burn wounds. Mice were then allowed to recover in an incubator and returned to their cages once they were awake and ambulatory. Hydrogel food supplements (ClearH2O, Westbrook, ME) and other nutritional supplements were placed in the cage to reduce dehydration and weight loss. Throughout the experiment, the mice's general state was observed, although, the pain assessments were performed twice daily for 72 h postburn. Any additional fluids and pain medication were administered as needed based on assessments but generally were not required.

Treatment. Anesthetized mice were first treated immediately after the burn and again 48-h postburn. A treatment timeline is shown in (Fig. 1). The treatment groups included 1%, 3.5%, and 6.5% *w*/w Pf as well as vehicle ointment. Pf ointments were optimized to deliver Pf over 48 h. Development and characterization of the ointments were previously described in detail [7]. Mice were treated by adding 0.5 g of Pf-formulated ointment or vehicle on the burn area of the mouse. The wound and ointment were covered with Tegaderm (3 M, St. Paul, MN) film and sealed with Vetbond

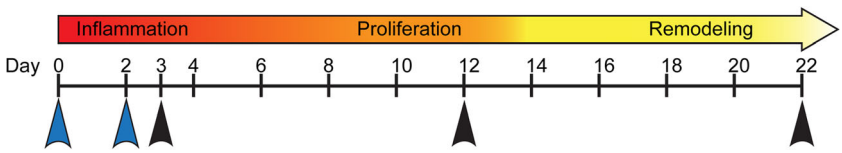

Fig. 1. Timeline depicting treatment and experimental procedures. Anesthetized mice were burned at day 0 and treated immediately after burn and 48 h later with vehicle, 1%, 3.5%, or 6.5% Pf. Mice were euthanized, and skin biopsies were collected at days 3, 12, or 22 after the induction of a deep partial-thickness burn. Blue arrow heads indicate treatment days, and black arrows indicate date of biopsy collections.

(3 M, St. Paul, MN) tissue adhesive. At 48 h after the initial treatment, the original Tegaderm was cut out on the inside of the Vetbond seal, the wound was wiped clean and replaced with the next dose of the corresponding ointment formulation. The wound was then covered with Tegaderm and resealed with Vetbond. Mice were anesthetized and then euthanized at the appropriate time points, and biological samples taken at post-operative days (POD) 3, 12, or 22.

Histology and Immunohistochemistry. Skin biopsies were fixed in 10% neutral buffered formalin for 48 h. Samples were processed using the Leica tissue processor ASP6025 (Leica Biosystems, Buffalo Grove, IL). Using Leica Biosystems reagents (Leica Biosystems, Buffalo Grove, IL), hematoxylin and eosin (H&E) staining were done according to the standard manufacturer provided protocol in a Leica Autostainer XL (Leica Biosystems, Buffalo Grove, IL). H&E-stained sections were scanned using the Aperio Versa 200 (Leica Biosystems, Buffalo Grove, IL) slide scanner. Immunohistochemical (IHC) staining was done on deparaffinized skin sections using a modified Abcam IHC protocol. Briefly, antigen retrieval was performed with sodium citrate buffer at 95–100 °C for 15 min and allowed to cool down for an additional 15 min. Skin sections were incubated with 1:500 anti-αSMA primary rabbit monoclonal antibody clone EPR5368 (Abcam, Cambridge, MA) overnight at 4 °C. Negative controls included IgG isotype and no primary antibody. Neither control showed nonspecific binding. Endogenous peroxidase activity was quenched with hydrogen peroxide blocking reagent (Abcam, Cambridge, MA), incubated with secondary goat anti-rabbit HRP-labeled antibody (Bio-Rad, Hercules, CA; 1:500), developed with a DAB peroxidase substrate kit (Vector Laboratories, Inc., Burlingame, CA) chromogen for 10 min, counterstained with hematoxylin QS (Vector Laboratories, Inc., Burlingame, CA) for 10 s, and scanned with the Aperio Versa 200.

Bioplex and αSMA ELISA Assays. Mouse skin samples were snap frozen upon collection. Tissues were stored at − 80 °C prior to use. Skin was pulverized and homogenized in tissue lysis buffer (10-mM HEPES pH 7.9, 100-mM KCl, 50-mM sucrose, 1% NP-40, 0.5% sodium deoxycholate) containing protease inhibitor mini tablets (Thermo Scientific, Waltham, MA). Samples were allowed to lyse for 4 h at 10 °C with gentle rocking. The homogenized samples were snap frozen. Lysates were then thawed on ice and centrifuged at 10,000 rcf for 5 min and transferred to new tubes in aliquots. Protein concentration was determined using a commercial BCA assay kit (Thermo Scientific, Waltham, MA). Samples were normalized to 900 μg/mL and cytokines assayed at a twofold dilution, using Bio-Rad Pro Mouse Cytokine 23-plex magnetic bead assay kit following included protocol. Assay was performed using the Bio-Rad Bio-Plex 200 system and Bio-Rad Bio-Plex Pro wash station (Bio-Rad, Hercules, CA). For αSMA ELISA, the same lysates were normalized to 1 mg/mL (Biomatik, Wilmington, DE) and assayed according to manufacturer's included protocol.

Myeloperoxidase Assay. A commercially available myeloperoxidase detection kit was purchased from Cell Technology (Cell Technology, Mountain View, CA). The assay was conducted according to manufacturer instructions. Briefly, frozen burn wound skin samples were pulverized. The samples were then homogenized with assay buffer according to protocol, centrifuged, and pellet solubilized with solubilization buffer. Samples were then sonicated using a Sonic Dismembrator Model 100 (Thermo Fisher, Waltham, MA) for 30 s in 5-s pulses. Finally, samples were put through two freeze thaw cycles before centrifuging and performing a BCA assay for protein concentrations. Samples were normalized to 150 μg/mL, and the myeloperoxidase assay was performed according to manufacturer's instructions.

Hydroxyproline Assay. A commercially available hydroxyproline colorimetric assay kit was purchased from BioVision (BioVision Incorporated, Milpitas, CA). Frozen skin samples were lyophilized for 24 h using a VirTis advantage Plus EL-85 lyophilizer (SP Scientific, Warminster, PA). The dried samples were weighed prior to preparing for assay. Samples were hydrolyzed and assayed according to manufacturer's instructions.

Statistics. Animal experiments were repeated twice, three mice each for a total of six mice ($n = 6$) per treatment group. All samples were evaluated as technical duplicates. Results were expressed as the mean ± standard deviation. Statistics were performed using GraphPad Prism v. 7.03 software. Student's two-tailed t test or two-way ANOVA was performed where noted. A P value of < 0.05 was considered significant.

RESULTS

Pirfenidone Reduces Inflammatory Cytokine Response in Deep Partial-Thickness Burn Wounds in C57BL/6 Mice

Pf is both an anti-fibrotic and anti-inflammatory drug. We evaluated the effects of Pf on the inflammatory response in a deep partial-thickness burn wounds in C57BL/6 mice. Skin biopsies were assayed for inflammatory cytokines. Figure 2a–f shows that Pf can significantly decrease the expression of inflammatory cytokines IL-1β, IL-2, IL-6, IL-13, G-CSF, and MIP-1α when comparing vehicle to Pf 6.5% ($P = 0.0042$, 0.005, 0.0297, 0.02, 0.0318, and 0.0072, respectively).

Pirfenidone Reduces Neutrophil Infiltration in C57BL/6 Mice Burn Wounds

We further evaluated the anti-inflammatory effects of Pf by evaluating the effects on inflammatory cell infiltration of burn wounds. Histology indicated a qualitative decrease in the number of cellular infiltrates in mice treated with Pf (Fig. 3a–d). Burn wounds were evaluated for myeloperoxidase activity to quantitate the presence of neutrophils. When comparing the vehicle control to Pf 6.5% treated mice, Pf significantly reduced the expression of neutrophil myeloperoxidase in burn wounds dose dependently ($P = 0.0369$) (Fig. 3e).

Pirfenidone Decreases αSMA Expression in Burn Wounds

Part of our long-term studies is to determine the effectiveness of Pf used as a prophylactic drug to prevent excessive scarring and contractures after a deep partial-thickness burn. To that end, we evaluated the anti-scar effect of Pf during treatment of the inflammatory phase of burn wound healing. Skin sections were evaluated by IHC for the presence of myofibroblasts using αSMA as a marker. Positive αSMA staining was seen in the dermis within the burn areas, indicative of myofibroblast trafficking to the site of injury (Fig. 4a–d). Additional staining was observed on the vasculature and muscles around the hair follicles of all treatment groups. Skin biopsies were assayed by ELISA to quantify the amount of αSMA expression by pirfenidone treatment. Treating the burn wounds in mice with Pf twice during the inflammatory phase of burn wound healing significantly reduced expression of αSMA ($P = 0.005$) (Fig. 4e). The treatment also reduced hydroxyproline deposition, an indicator of collagen content, during the remodeling phase of wound healing (Fig. 4f).

DISCUSSION

The initial phase of burn wound healing produces an inflammatory response that can exacerbate the tissue damage already present from the thermal injury. Part of the inflammatory response is due to the tissue destruction and the released damage-associated molecular patterns (DAMPs), such as HMGB1, S100A12, and degraded matrix constituents [5, 27, 33]. Additionally, pathogen-associated molecular patterns (PAMPs) such as LPS, flagellin, and peptidoglycan from normal flora and pathogenic bacteria, which can readily enter the breached skin barrier resulting from burns, can further exacerbate the inflammatory response [4, 5]. Exaggerated inflammation during the early phases of wound healing may contribute to excessive fibrosis and is associated with the development of hypertrophic scarring [5, 8, 26]. Our study found that Pf can significantly lessen the inflammatory response associated with a deep partial-thickness burn injury in C57BL/6 mice and dampen pro-fibrotic responses.

In our previous study, we demonstrated that the Pf ointment can significantly decrease IL-12p70 and TNFα in burn wounds [7]. The current study shows that Pf can also reduce expression of other inflammatory cytokines and chemokines which include IL-1β, IL-2, IL-6, IL-13, G-CSF, and MIP-1α. Overexpression of IL-1β is associated with hypertrophic scars [29]. Additionally, IL-2 and IL-6 mediate collagen deposition and fibroblast proliferation [6, 28]. Moreover, IL-13 is associated with skin fibrosis [24]. G-CSF is shown to support the proliferation, activation, and differentiation of neutrophils [16]. Finally, MIP-1α is a pro-inflammatory chemokine that can regulate a variety of immune cells by recruiting inflammatory cells to the sites of injury [18].

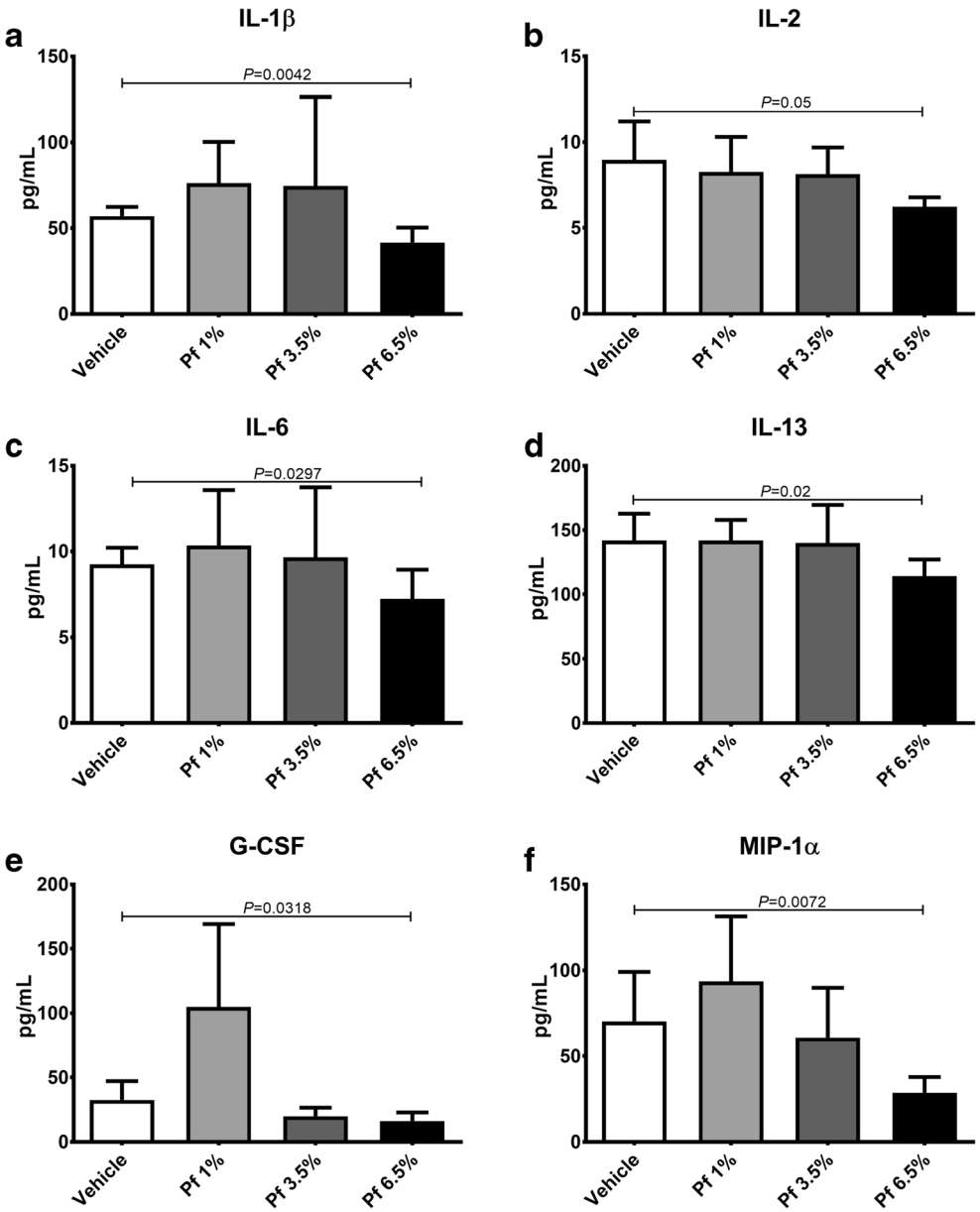

Fig. 2. Pirfenidone reduces pro-inflammatory cytokines during the inflammatory phase of wound healing. Skin samples were evaluated for inflammatory cytokines at the peak of inflammation, 3 days after the induction of a deep partial-thickness burn wound by Bioplex assay. Mice were treated with vehicle, 1%, 3.5%, or 6.5% Pf as described in Fig. 1. Student's *t* test analysis showed that cytokines IL-1β (**a**), IL-2 (**b**), IL-6 (**c**), IL-13 (**d**), G-CSF (**e**), and MIP-1α (**e**) concentrations in skin lysates were significantly decreased when comparing vehicle to Pf 6.5%.

These cytokines and chemokines have important roles during the early onset of the inflammatory response for wound healing. However, when dysregulated, these inflammatory mediators can also cause excessive inflammatory response harmful to healing. Our earlier *in vitro* data have shown that Pf inhibits p38 kinases in TGF-β1-stimulated human dermal fibroblasts [15]. Like other mitogen-activated protein (MAP) kinases, p38 kinases can activate the transcription factors driving the expression of inflammatory mediators [5]. It is conceivable that Pf could also target p38 kinases in inflammatory cells for lessening the early inflammatory phase of healing. A

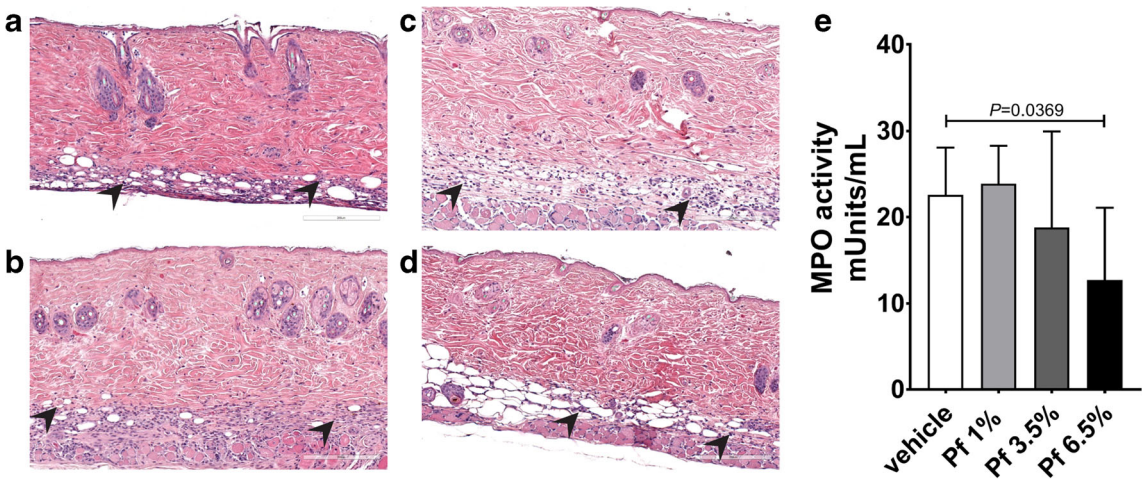

Fig. 3. Pirfenidone reduces expression of myeloperoxidase in burn wound bed. H&E stains are shown for deep partial-thickness wounds in mice treated with vehicle (**a**), Pf 1% (**b**), Pf 3.5% (**c**), and Pf 6.5% (**d**). Black arrow heads point to cellular infiltrates. There was a qualitative difference in the cell infiltrates for mice treated with the different ointment formulations. Wound beds were also evaluated for myeloperoxidase activity (**e**). Student's *t* test showed there was a significant difference in mice treated with Pf 6.5% compared to vehicle.

topical p38 kinase inhibitor has been used before to modulate the inflammatory response to reduce dermal cytokines in rat burn wounds, but long-term outcomes such as effects on αSMA and collagen deposition were not determined [20]. By contrast, excessive dampening of inflammatory response could negatively impact healing as shown in our other study in which clinical doses of indomethacin delay wound closure of rabbit ear wounds [26]. Therefore, it appears that treatments to dampen tissue-damaging inflammation for optimal healing need to be properly timed and titrated to reduce risks of stalled healing [5]. The use of Pf may offer a potential treatment that could bring the

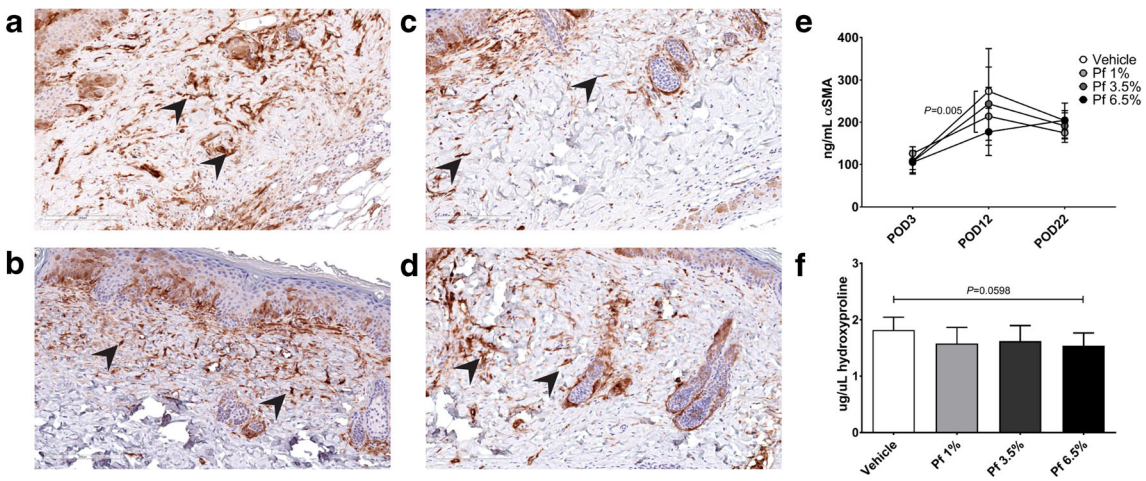

Fig. 4. Pirfenidone lowers expression of αSMA, and modestly lessens hydroxyproline deposition. IHC staining for αSMA for deep partial-thickness wounds in mice treated with vehicle (**a**), Pf 1% (**b**), Pf 3.5% (**c**), and Pf 6.5% (**d**). Black arrow heads point to αSMA-positive cells within the dermis. The effects of Pf on αSMA were evaluated over time in mice with a deep partial-thickness burn by ELISA (**e**). Two-way ANOVA analysis revealed a significant reduction in αSMA expression at 12 days after burn in mice treated with Pf 6.5% compared to vehicle. Hydroxyproline expression was evaluated with a colorimetric assay (**f**). Although not statistically significant, Pf treatment of burn wounds shows a reduction of hydroxyproline deposition.

inflammatory response into balance with the tissue's need for inflammation to repair the damage. In this context, we have reported previously that the use of Pf during the inflammatory phase of wound healing lessens inflammation but did not interfere with the rate of reepithelization of deep partial-thickness burn wounds [7].

The inflammatory responses associated with a burn wound include the influx of inflammatory cells to the site of injury. Compared to vehicle treatment, we saw a qualitative difference in the cell infiltrates in the burn wounds treated with Pf. The results were consistent with the reduction of dermal pro-inflammatory cytokines and chemokines seen in burn wounds treated similarly. Many of these cellular infiltrates were neutrophils. Neutrophils are some of the first cells that reach the site of injury [13]. Neutrophils are recruited to sites of injury by DAMPs and PAMPs, and are there to clear damaged tissue and to kill microbial pathogens that may have crossed the burn-induced damaged skin barrier [5, 19]. However, prolonged presence of neutrophils can cause damage to surrounding tissue and impair healing as shown in many chronic non-healing wounds. This again underscores the importance of balancing the inflammatory response in order to favor the wound healing dynamics toward healing. Interestingly, Martin et al. showed that mice without macrophages and functioning neutrophils are still able to heal and appear to have a reduced "scar" [22].

The burn wound inflammatory phase transitions into the proliferative phase where myofibroblasts proliferate and begin laying down extracellular matrix and keratinocytes proliferate and migrate over the wound bed as the body attempts to seal the broken skin barrier. This tightly controlled process can be dysregulated such as excessive deposition of extracellular matrix and delayed wound closure which could lead to increased fibrosis that carries into the remodeling phase [13]. Studies show that the level of inflammatory responses affect fibrosis during wound healing [26, 28, 29]; therefore, we have evaluated the effects of Pf treatment during the inflammatory phase on fibrosis markers into the proliferative and remodeling phases of wound healing. We detected a significant reduction in the expression of αSMA and a modest reduction in the hydroxyproline content of the wounds. Elevated levels of αSMA and collagen expression are found in hypertrophic scars due to activation of myofibroblasts [14]. Fibrosis, such as the type found in hypertrophic scars, is characterized by an increase in extracellular matrix and prolonged survival by myofibroblasts, which can be a result of prolonged or elevated inflammation [12, 14, 15]. αSMA together with mature focal adhesions is an essential component of the mechanoregulatory contractile machinery responsible for contractility seen in myofibroblasts when exposed to external mechanical and biochemical cues from extracellular matrix [21]. The decrease of αSMA as a result of Pf treatment could potentially dampen the contractility seen in myofibroblasts in these wounds. It may be possible that the use of Pf as a prophylactic treatment to inhibit p38 kinases during the early inflammatory phase of wound healing could improve long-term wound outcomes with reduced scarring and contracture as shown in this study and other studies [17].

A recent article from Finnerty et al. [10] argues that severe scarring is one of the greatest challenges that clinicians and patients face after a severe burn. The authors also express a need for prophylactic therapies to prevent scar formation [10]. Our findings indicate that Pf is a promising prophylactic treatment option for lessening dermal inflammation and fibrosis in mouse deep partial-thickness burn wounds. The study also indicates the need for testing this prophylactic treatment option for improving long-term outcomes of scarring and contracture as seen in humans resulting from deep partial-thickness burns using an animal model that can adequately recapitulate these adverse long-term outcomes.

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COMPLIANCE WITH ETHICAL STANDARDS

Research was conducted in compliance with the Animal Welfare Act, the implementing Animal Welfare Regulations, and the principles of the Guide for the Care and Use of Laboratory Animals, National Research Council. The facility Institutional Animal Care and Use Committee

approved all research conducted in this study. The facility where this research was conducted is fully accredited by AAALAC International.

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