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Montelukast, an antagonist of cysteinyl leukotrienes signaling, impairs burn wound healing

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Abstract

Background: Burns are severe injuries often associated with impaired wound healing. Impaired healing is caused by multiple factors including dysregulated inflammatory responses at the wound site. Interestingly, montelukast, an antagonist for cysteinyl leukotrienes (cysLTs) and FDA-approved for treatment of asthma and allergy, was previously shown to enhance healing in excision wounds and to modulate local inflammation.

Methods: In this study, we examined the effect of montelukast in wound healing in a mouse model of scald burn injury. Burn wound tissues isolated from montelukast- and vehicle-treated mice at various times after burn injury were analyzed for wound areas (n=34–36), re-epithelialization (n=14), inflammation (n=8–9) and immune cell infiltration (n=3–6), and proliferation (n=7–8).

Results: In contrast to previously described beneficial effects in excision wounds, this study shows that montelukast delays burn wound healing by impairing the proliferation of keratinocytes and endothelial cells. This occurs largely independently of inflammatory responses at the wound site, suggesting that montelukast impairs specifically the proliferative phase of wound healing, in burns. Wound healing rates in mice in which leukotrienes are not produced were not affected by montelukast.

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Author contributions

AN and AMS designed experiments, analyzed data, and wrote the manuscript. AN, SEC, MW, MDB, GS and SY performed the experiments. HS and RRI provided scientific advice and analyzed data. All authors have read and approved the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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Ethics statement

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of the University of California, Davis.

Conclusions: Montelukast delays wound healing mainly by reducing the proliferation of local cells after burn injury.

2. INTRODUCTION

In the United States, over 30,000 patients are hospitalized annually due to burn injuries[1]. These injuries are often slow to heal, leading to lengthy hospitalization time, increased healthcare costs, and lower quality of life. Burn-associated impaired healing is linked to deregulated inflammation at the site of the injury driven by skin-resident and infiltrating peripheral immune cells[2–4]. Although certain inflammatory responses impair healing[5–8], others promote tissue repair and reconstruction[7–12]. Thus, defining pro-repair pathways associated with inflammation is a crucial step for the development of successful therapeutic strategies.

Leukotrienes (LTs) are short-lived lipid mediators derived from the conversion of arachidonic acid by 5-lipoxygenase (5-LO) and consist of LTB_4, and the cysteinyl leukotrienes (cysLTs): LTC_4, LTD_4, and LTE_4[13,14] (See Figure, Supplemental Digital Content 1, which shows the 5-LO pathway. A variety of stimuli can induce the release of arachidonic acid (AA) from membrane phospholipids by cytosolic phospholipase A2 (cPLA2; gene name *Pla2g4a*). 5-LO (gene name *Alox5*), together with 5-lipoxygenase activating protein (FLAP; gene name *Alox5ap*), converts free AA to form the unstable intermediate leukotriene A4 (LTA_4). LTA_4 can be either hydrolyzed by LTA4 hydrolase (LTA4H; gene name *Lta4h*) to form leukotriene B4 (LTB_4) or conjugated to reduced glutathione by LTC4 synthase (LTC4S; gene name *Ltc4s*) to form LTC_4, which can be further metabolized extracellularly to LTD_4 and LTE_4. The main receptors for LTB_4 are BLT1 and BLT2, while the main receptors for cysLTs are CysLT1, CysLT2 and the recently identified GPR99. Additionally, purinergic receptor and GPR17 have also been suggested as possible receptors for cysLTs, INSERT HYPER LINK). LTs participate in inflammatory responses and their overproduction is associated with asthma, atopic dermatitis, cardiovascular disease, and cancer[13–25], but also play roles in tissue homeostasis[26,27]. Members of the 5-LO pathway are expressed in various immune cells, such as neutrophils[28], macrophages[29], lymphocytes[30], dendritic cells[31], but also in non-immune cells such as neuronal cells and endothelial cells[32,33]. Skin-resident cells such as Langerhans cells[24,34] and keratinocytes[35,36] also express members of the 5-LO pathway. Both skin-resident and immune cells can produce leukotrienes[18,25,28,34,37,38] either independently or via transcellular synthesis[39–43].

In excision wounds, genetic deletion of Arachidonate 5-Lipoxygenase (*Alox5*, the gene encoding for 5-LO) or pharmacological antagonism of LT signaling accelerates healing[44,45]. Accordingly, montelukast, an FDA-approved cysLT antagonist currently used in the clinic for allergies and asthma[46], was shown to promote healing in wound models[45,47,48], suggesting that cysLTs impair wound healing. However, *in vitro* studies showed that cysLTs can also exert pro-healing functions by inducing proliferation of endothelial cells and intestinal epithelial cells[49–51], and by promoting collagen production by myofibroblasts[52].

In this study, we show that unlike its effects in excision wounds, montelukast inhibits wound healing after burn injury. This was associated with a slower proliferative phase in the absence of drastic changes in the inflammatory milieu at the burn wound site.

3. MATERIALS AND METHODS

Animals:

C57BL/6 and 5-lipoxygenase KO (*Alox5*$^{-/-}$) mice were purchased from the Jackson Laboratories and bred in-house. Experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee. Animals were age- and sex-matched for each experiment. Numbers of animals used in each experiment are indicated in figure legends.

Scald burn injury:

Dorsa of mice were shaved and depilated 24 hours prior to injury. Animals were scalded as we previously described[5].

Montelukast treatment:

Montelukast (Cayman Chemical) was prepared as powder dissolved in sterilized water to create an aqueous solution at a concentration of 10mg/mL. This was further diluted 1:3 in 0.9% USP-grade saline to create the injectable solution. Mice were treated daily with I.P. injections of 5mg/kg or 30mg/kg montelukast.

Excision wounding:

Dorsa of mice were shaved, and left-over hair was removed with a depilatory cream 24 hours prior to wounding. Animals were anesthetized via isoflurane inhalation and the dorsal skin was sterilized with a povidone-iodine solution. Mice were given analgesics via I.P. injection of 0.03 mg/mL buprenorphine. To create the wound, a 6mm diameter full-thickness area of skin was excised from the sterilized area, as previously described[53].

Analysis of wound areas and re-epithelialization:

Wounds were photographed and quantified as previously described[5,54]. Briefly, the wound borders were traced with the ImageJ software (NIH). Wound area was calculated as: (measured area/area of the wound on day 0) x 100. Re-epithelialization analysis for burns and excision wounds was performed on H&E-stained sections as previously described[5,53].

Immunohistochemical analyses of wound tissues:

For in-vivo assessment of cell proliferation, animals were injected intraperitoneally with 100μg/g body weight BrdU 24hrs, 4hrs, and 1hr before euthanasia. Immunohistochemical analysis was performed as we previously described[5]. Antibodies against Keratin 5 (Biolegend), CD31 (Abcam), CD11b (BD Biosciences), Ly6G (BD Biosciences), IBA1 (Fujifilm Wako), and BrdU (Santa Cruz Biotechnology) and appropriate isotype controls were used. Slides were imaged with Nikon A1 confocal microscope and analyzed through the ImageJ or NIS Elements (Nikon) software.

Hematoxylin & eosin (H&E) staining and picrosirius red staining:

H&E staining was performed as we previously described[5]. Picrosirius red staining was done with 1.3% picric acid and 0.1% Direct Red 80 (Sigma Millipore) for 1hr at room temperature. Sections were imaged under brightfield or polarized brightfield with the Olympus BX61 microscope. Collagen quantification analysis was performed using the MRI Fibrosis tool on ImageJ.

Quantitative PCR (qPCR):

qPCRs were performed as we have previously described[5]. *B2m* was used as the housekeeping gene. Primers used are listed in Table 1.

Flow cytometry:

Single cells suspensions were prepared from burn tissues and analyzed by flow cytometry as we have previously described[5]. Cells were analyzed using the Attune NxT flow cytometer (Life Technologies) and data were analyzed by FlowJo software.

Statistics:

For re-epithelialization, flow cytometric, qPCR, and proliferation analyses, significance was determined by Student's two-tailed t-tests to compare between treatments. For data that did not have equal variances, Welch's two-tailed t-test was used. For wound area quantitation, mixed effect analysis with Sidak's multiple comparisons was performed when there were uneven numbers of data points at various time points. Otherwise, two-way ANOVA with Sidak's multiple comparisons was used. Mann-Whitney test was used for analysis of normalized qPCR data. All analyses were performed on the Prism Graphpad software; p values <0.05 are considered significant.

4. RESULTS

Montelukast delays burn wound healing.

Transcripts of genes associated with the 5-LO pathway (See Figure, Supplemental Digital Content 1, **INSERT HYPER LINK**) were upregulated in murine burn-injured skin (Figure 1). Interestingly, *Ltc4s* was upregulated at later stages of wound healing compared to *Lta4h*. We speculated that the LTC4S arm of the 5-LO pathway may promote sustained inflammation, thus impairing burn wound healing. To test the effect of this pathway in burn wound healing, we employed the CysLT1 antagonist, montelukast.

We initially confirmed previous results in excision wounds showing that treatment of mice with 5 mg/kg/day of montelukast promotes wound closure.[45] (See Figure, Supplemental Digital Content 2, which shows the differential effect of montelukast treatment in excision and burn wounds. (A, B) Excision-injured mice were treated with 5mg/kg/day of montelukast. (A) Quantification of wound areas expressed as percent of initial wound area over time; n=6. (B) Representative images of H&E-stained sections on day 7 post-excision, followed by re-epithelialization rate analysis of vehicle- and montelukast-treated mice on day 7 post- excision injury; n=4. (C, D) Burn-injured mice were treated with 5mg/kg/day of montelukast. (C) Quantification of burn areas expressed as percent of initial burn wound

area over time; n=13–14. (D) Representative images of H&E-stained sections on day 17 post-burn, followed by re-epithelialization analysis of vehicle- and montelukast-treated mice on day 17 post-burn injury; n=10–12. Data are shown as means ± SD. Two-way ANOVA with Sidak's multiple comparisons or Student's two-tailed t-test were used to determine statistical significance; ***p<0.001, **p<0.01, *p<0.05, INSERT HYPER LINK.) In contrast to excision wounds, treating mice with 5 mg/kg/day of montelukast delayed healing in burn wounds. This effect was more pronounced when mice were treated with an increased dosage of 30 mg/kg/day of montelukast (Figure 2, **above**), a dose previously shown to be effective in other animal models[55–57]. For the remainder of the study, we employed the 30 mg/kg/day dose.

Compared to controls, burn wound areas of montelukast-treated mice were statistically significantly larger starting on day 3 post-injury and up to the end of the experiment (Figure 2, **above**). Accordingly, re-epithelialization rates on day 14 post-injury were statistically significantly lower in the montelukast-treated wounds when compared to control wounds (42%±13 vs 54%±15 respectively; p<0.01) (Figure 2, **below**).

To test whether the effects of montelukast on wound closure and re-epithelialization were due to off-target effects, we employed *Alox5*$^{-/-}$ mice, which are commercially available and devoid of all leukotrienes[58]. On day 14 post-burn injury, *Alox5*$^{-/-}$ mice treated with montelukast showed no differences in wound closure and re-epithelialization rates compared to their controls, suggesting the effects of montelukast in WT mice are mediated via activation of the 5-LO pathway and likely due to cysLTs. (See Figure, Supplemental Digital Content 3, which shows that montelukast does not affect healing in *Alox5*$^{-/-}$ mice. Burn-injured *Alox5*$^{-/-}$ mice were treated with either vehicle or 30mg/kg/day of montelukast. (A) Quantitation of burn areas expressed as percent of initial burn wound area of vehicle- and montelukast-treated *Alox5*$^{-/-}$mice; n=11–14. (B) Representative H&E-stained sections of burn wounds of vehicle- and montelukast-treated *Alox5*$^{-/-}$ mice. Re-epithelialization rates were analyzed on day 14 post-injury; n=11–15. Black arrows represent edge of the wound bed and grey arrows represent edge of neo-epithelium, INSERT HYPER LINK.)

A possible reason for the different outcome in healing between excision and burn wounds may be differential expression profiles of receptors known to be antagonized by montelukast at the wound site. In addition to CysLT1, GPR17, GPR99 ($CysLT_E$), and various purinergic receptors have also been shown to be antagonized by montelukast[59–67]. Previous reports have shown that these receptors may also respond to cysLTs[61,63]. Most of these receptors have been previously shown to be present in the skin[60,65]. We found that mRNA transcripts of *Gpr17*, *P2y1*, *P2y2*, *P2x5*, and *P2y12* were upregulated in burn tissues and remained upregulated during later stages of burn wound healing (day 14). (See Figure, Supplemental Digital Content 4, which shows wounded skin expression patterns of receptors previously shown to be antagonized by montelukast. Transcript levels of receptors in skin tissues at various time points post burn and day 7 post-excision; n=6–10. Transcript levels are expressed as fold change over values obtained from healthy tissues ($2^{-\Delta\Delta CT}$ ± SD). Student's two-tailed t-test was used for statistical analysis; ***:p<0.001, **:p<0.01, *:p<0.05. ND=not detected, INSERT HYPER LINK.) On day 7 excision wounds *Cysltr1*, *P2y1, P2y12* and *P2x5*, but not *Gpr17* or *P2y2*, were upregulated. These differing receptor

expression profiles may be partly responsible for the opposing healing outcomes between excision and burn wounds in response to montelukast.

Montelukast treatment does not drastically alter the inflammatory milieu at the burn wound site.

Burn wounded tissues were examined for inflammation levels on days 10 and 14 post-burn injury. There were no differences in the transcript levels of most of the cytokines/chemokines examined between the groups, except for *Ccl2*, which was upregulated in the montelukast-treated mice on day 14 post-burn. Interestingly, we also observed downregulation of *Nlrp3* in the montelukast group (Table 2).

Single cells were isolated from burn skin tissues on days 10 and 14 post-burn and analyzed by flow cytometry. (See Figure, Supplemental Digital Content 5, which shows that montelukast does not significantly modulate local immune responses at the burn site 10 and 14 days after injury. (A) Representative flow cytometric gating strategy for neutrophils (CD45+CD11b+Ly6G+), monocytes and macrophages (CD45+CD11b+Ly6Glo/−), and lymphocytes (CD45+CD11b-CD3+). (B) Percentages and absolute counts of neutrophils (CD45+CD11b+Ly6G+) and macrophages/monocytes (CD45+CD11b+Ly6Glo/−) on days 10 and 14 post-burn. Neutrophils were subdivided into N1 (CD45+CD11b+Ly6G+CD206−) and N2 (CD45+CD11b+Ly6G+CD206+) populations, and macrophages/monocytes were subdivided into inflammatory (CD45+CD11b+Ly6Glo/−Ly6C+CD206−) and reparative populations (CD45+CD11b+Ly6Glo/−Ly6C-CD206+). (C) Immunohistological analysis of burn tissues for neutrophils (Ly6G) and macrophages (IBA1) on day 14 post-burn. Dotted line represents the boundary between the eschar and dermis. Data are shown as means ± SD; day 10 analyses: n=3–6; day 14 analyses: n=8–9. Welch's two-tailed t-test was used for statistical analysis; **p<0.01; *p<0.05, INSERT HYPER LINK)

There were no statistically significant differences in the absolute counts of total CD45+ cells between montelukast- and vehicle- treated mice, in either day. Relative neutrophil (CD45+CD11b+Ly6G+) frequencies were statistically significantly upregulated only on day 14 in the wounds of montelukast-treated mice when compared with the vehicle-treated group (neutrophils: 44%±15% vs. 23%±14%, p<0.01). However, there were no differences in the absolute counts of total neutrophils between the groups in either time point. Neutrophil subsets CD45+CD11b+Ly6G+CD206- (N1; proinflammatory) and CD45+CD11b+Ly6G+CD206+ (N2; reparative) were also assessed. N2 neutrophils[68] were originally identified as tumor-associated neutrophils[69] and can exert pro-healing effects such as secreting laminin that is required for keratinocyte adhesion to the dermis[70,71]. There was a statistically significant decrease in the absolute numbers of N2 neutrophils in the montelukast group compared to the vehicle group (CD206+; $3.8\times10^4\pm1.3\times10^4$ vs. $9.1\times10^4\pm4.7\times10^4$, respectively; p<0.05), on day 10 only.

Relative frequencies of monocytes were decreased in the montelukast group when compared with the vehicle group only on day 14. Analysis of monocytic subsets showed that the relative frequencies of inflammatory monocytes/macrophages were statistically significantly increased in the montelukast group compared to the vehicle group (20%±7.1% vs. 11%

±7.4%, p<0.05, respectively). However, these differences did not translate into statistically significant differences in total absolute numbers of these cell subsets between the groups.

Immunohistological analysis showed that the distribution of neutrophils (Ly6G+) and macrophages (IBA1+) within the wound bed is similar between the vehicle- and the montelukast-treated mice. In both groups, neutrophils were more frequently localized toward the dorsal side of the wound near the eschar while macrophages were located deeper in the dermis. (See Figure, Supplemental Digital Content 5)

In combination, the above suggest that the migration and tissue infiltration by peripheral immune cells and the overall inflammatory environment were not significantly affected by montelukast at the time points examined.

Montelukast impairs the proliferative phase of burn wound healing.

To test the effects of montelukast on the proliferation of skin-resident cells, mice were injected with BrdU 24hrs, 4hrs, and 1hr before euthanasia on day 14 post-burn.

Montelukast-treated mice exhibited significant reductions in the numbers of proliferating keratinocytes (Krt5+BrdU+) in the neo-epidermis of burn wounds compared to vehicle controls (49±15 vs. 74±15 cells/field, respectively; p<0.01) (Figure 3, **above**). Additionally, wounds of montelukast-treated mice displayed fewer numbers of BrdU+ dermal endothelial cells in comparison to wounds of vehicle-treated mice (19±7.5 vs. 33±8.7 cells/field, respectively; p<0.01; CD31+BrdU+) (Figure 3, **center**). Moreover, montelukast-treated wounds had lower levels of type I collagen content when compared to wounds in the vehicle-treated group, on day 14 post-burn injury (Figure 3, **below**). No significant differences were observed in the numbers of proliferating keratinocytes in montelukast- and vehicle-treated burn wound *Alox5*$^{-/-}$ mice. (See Figure, Supplemental Digital Content 6, which shows that montelukast does not affect proliferation of keratinocytes in *Alox5*$^{-/-}$ mice. Representative images of sections immunostained for Krt5 and BrdU of vehicle- and montelukast-treated *Alox5*$^{-/-}$ mice on day 14 post-burn. Quantification of proliferating keratinocytes was examined between vehicle- and montelukast-treated *Alox5*$^{-/-}$ mice; n=3. Data are shown as means ± SD. Student's two-tailed t-test was used for statistical analysis, INSERT HYPER LINK.)

On day 10 post-burn, there were no differences in mRNA levels of growth factors shown to be implicated in the wound healing process between the montelukast- or vehicle-treated mice (Table 2). However, on day 14 post-burn, mRNA levels of *Arg1*, encoding for the enzyme arginase-1, were decreased in the montelukast-treated wounds. *Arg1* is associated with reparative macrophages and with collagen deposition[72,73]. In addition, mRNA levels of *Pdgfa* and *Tgfb*, both known to promote wound healing[7,74,75], were also downregulated in the montelukast group.

5. DISCUSSION

This study shows that montelukast impedes the proliferative phase of burn wound healing. In particular, montelukast diminished proliferation rates of keratinocytes and

dermal endothelial cells. Moreover, *Pdgfa* and *Tgfb*, crucial growth factors for wound healing as they induce cellular growth, repopulation, and differentiation[7,74,76,77], and *Arg1*, which promotes collagen deposition[72,73], were downregulated by montelukast treatment. Accordingly, montelukast treatment decreased type-I collagen content in the wound bed. These data agree with previous studies showing that cysLTs promote proliferation, neovascularization, collagen deposition, and cellular proliferation and migration[48,51,78–83].

Previous studies have shown that montelukast treatment dampens inflammatory responses in excision wounds[45], and that platelets, which are crucial for clotting and hemostasis[7,8], can be activated by cysLTs to produce inflammatory mediators[84,85]. Thus, we examined the effects of montelukast on the inflammatory status of the burn wound site. Reparative neutrophils were transiently and mildly downregulated (only on day 10) in the montelukast-treated group. There were no statistically significant changes in the monocyte/macrophage populations between the vehicle- and montelukast-treated mice, and spatial distribution of neutrophils and macrophages in the wounded dermis was similar between the groups at the time points examined. Interestingly, *Ccl2* transcripts were upregulated on day 14 in the montelukast group. CCL2 is a pro-inflammatory cytokine that recruits monocytes, lymphocytes, and dendritic cells to sites of inflammation[86,87]. However, *Ccl2* upregulation did not translate into changes in the absolute counts of immune cells between the two treatment groups. CCL2 has also been shown to promote the recruitment and generation of M2b monocytes, which have anti-inflammatory activity and have been shown to be upregulated in burn patients[88,89]. Furthermore, we found that *Nlrp3* was downregulated in the montelukast group. NLRP3 is a component of the inflammasome, a multimeric complex that induces the maturation of the cytokines IL-1β and IL-18[90]. Additionally, recent studies have demonstrated that burn-injured NLRP3−/− mice exhibited decreased *Tgfb* expression[91], and that LTD_4 stimulates the NLRP3 inflammasome[92]. We show that montelukast treatment reduced both *Tgfb* and *Nlrp3* expression, suggesting that cysLTs may induce cellular growth after burn injury via NLRP3 activity. In combination, these data suggest that montelukast does not drastically affect the inflammatory phase.

Previous studies suggest that montelukast enhances healing and protects against oxidative damage[45,47,48]. However, oxidative injury was previously assessed only 24 hrs after burn injury and not at later time points. Additionally, wound closure and re-epithelialization were not assessed[47]. A different study showed that burn-injured rats healed faster when treated with montelukast[48]. However, rats were treated with only up to day 10 post injury and analyzed four and ten days later. Interestingly, rats in the control group did not show signs of re-epithelialization by day 20 post injury. Nonetheless and in agreement with our data, the montelukast treated group exhibited impaired neoangiogenesis in that study[48].

Montelukast treatment results in an opposite outcome in excision wounds, where it promotes wound healing (ref. 45 and data presented in this study). The reasons for the differential effects of montelukast in wound healing between burn and excision wounds may be multiple and are currently unclear. Although montelukast is a marketed CysLT1 antagonist[46], it has been shown to also antagonize other receptors, such as GPR17 and various purinergic receptors[59–64,66,67]. *Gpr17*, *P2y1*, *P2y2*, *P2x5*, and *P2y12* were all upregulated in burned skin and remained upregulated up to the late stages of healing, compared to healthy skin.

The expression profile of these receptors was different in excision wounds in which neither *Gpr17* nor *P2y2* were upregulated when compared to healthy skin. The different expression profile of these receptors at the affected site may explain the opposite effect of montelukast in wound healing observed between excision and burn wounds. Interestingly, a recent study demonstrated that $P2Y_{12}$ activity promotes wound healing by inducing keratinocyte proliferation and collagen deposition[93], suggesting that $P2Y_{12}$ may play a major role in mediating burn wound healing.

It is possible that the route of montelukast administration can affect wound healing. Previous studies have administered montelukast via oral gavage[45,48,83] while we administered montelukast via intraperitoneal injection. Our data show that montelukast treatment resulted in decreased collagen maturation, which agrees with a previous study in which montelukast was given orally[83]. The pharmacokinetics and bioavailability of substances administered intraperitoneally were previously shown to be similar to those observed after oral administration and higher than those of subcutaneous administration[94,95]. Therefore, it is not likely that the route of administration affected the outcome of our study. However, other methods of administration, such as intravenous, intranasal, and subcutaneous routes, may have different effects on wound healing.

Montelukast treatment did not affect healing in *Alox5*$^{-/-}$ mice, suggesting that the effect is primarily actuated via a mediator produced by the 5-LO pathway, and likely cysteinyl leukotrienes. Interestingly, cysLTs have been previously shown to activate GPR17 and purinergic receptors[61,63,64], suggesting that cysLTs may act via one of these receptors in burn wound healing. However, other studies suggest cysLTs do not activate $P2Y_{12}$[96] or GPR17[97], suggesting that further studies are needed to clarify this issue in wound healing.

Several case studies show that prolonged use of montelukast resulted in the onset of cutaneous inflammatory disorders as the result of vasculitis onset associated with increased neutrophil and eosinophil recruitment[98,99]. Presentation of this type of adverse reactions is rare, and although the treatment period in our model was relatively short, we did not observe signs of vasculitis and increased granulocyte infiltration at the burn wound site in the montelukast-treated group. There is currently no clinical information on the effect of montelukast in wound healing in humans. A previous study demonstrated that montelukast reduced contractile forces exerted by human Tenon's capsule fibroblasts[100], suggesting that montelukast may confer similar effects in human wound healing.

Our study suggests that burn patients on montelukast may exhibit slower healing. Although further studies are needed to elucidate the effect of montelukast on human wound healing, our study suggests that burn patients who are on montelukast may need to be under additional observation. Furthermore, the differential effect of montelukast in excision vs burn wounds hints at wound healing mechanisms that are specifically activated in burn injury. Additional investigations in this field are necessary to elucidate these mechanisms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

LT	Leukotriene
5-LO	5-Lipoxygenase
cysLT	Cysteinyl leukotriene
SD	Standard Deviation

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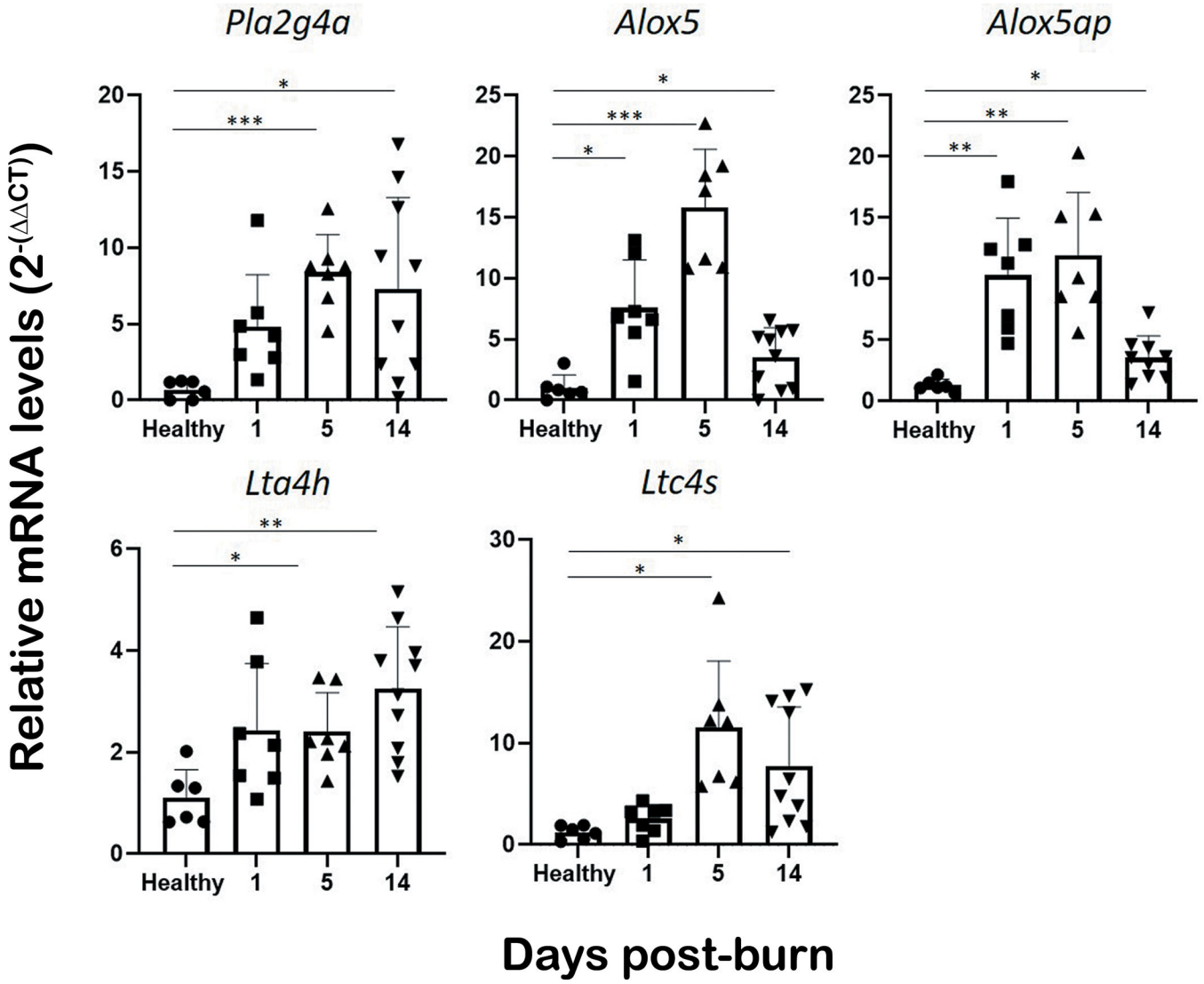

Figure 1. The 5-LO pathway is upregulated in burned skin.

Transcript levels of *Pla2g4a*, *Alox5*, *Alox5ap*, *Lta4h*, and *Ltc4s* in healthy and burned murine skin; n=6–10. Brown-Forsythe and Welch's ANOVA was used to determine statistical significance. ***p<0.001, **p<0.01, *p<0.05.

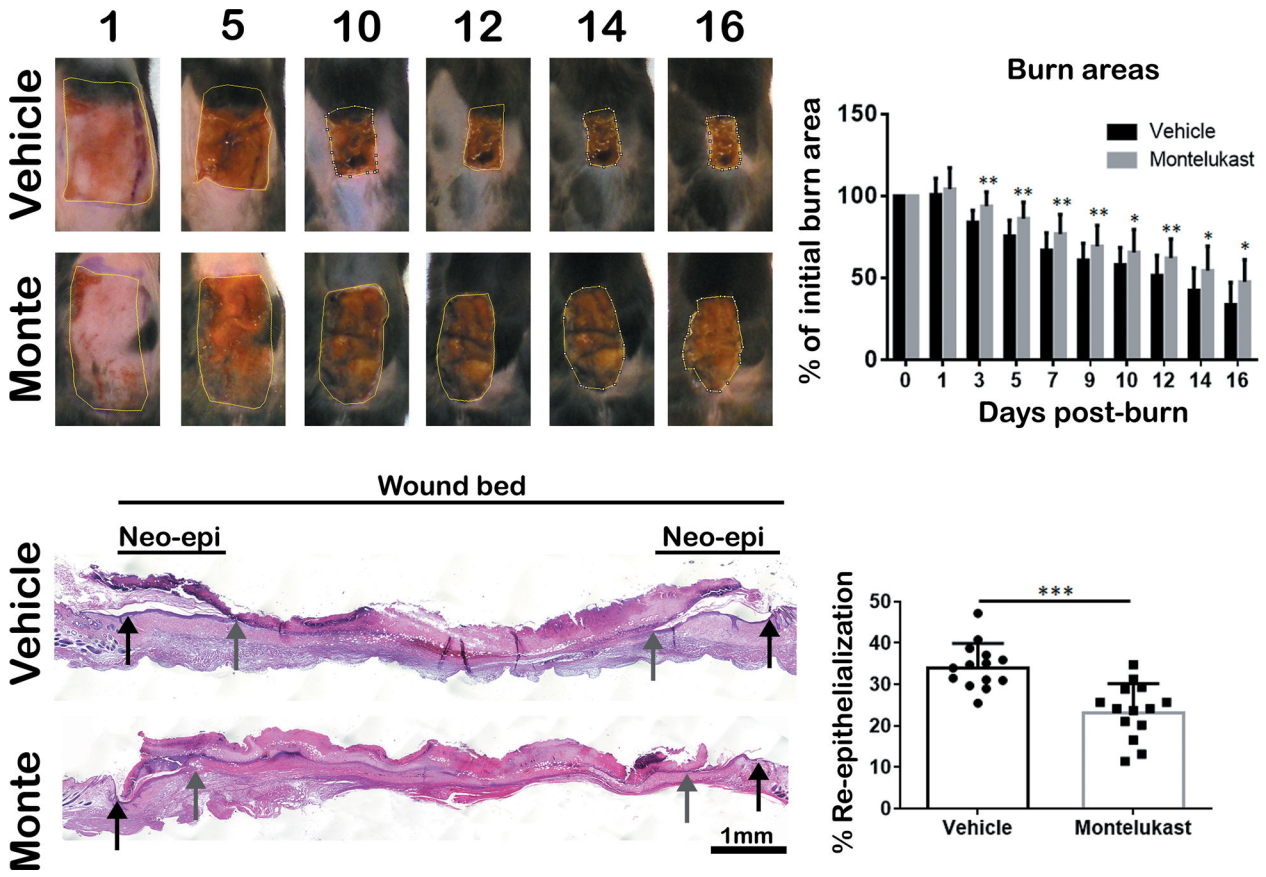

Figure 2. Montelukast impairs burn wound healing.

(**Above**) WT mice underwent burn injury, and immediately after received intraperitoneally montelukast or vehicle. Mice were randomly assigned to each treatment group. Mice were photographed daily; representative images are shown (left). Quantification of burn areas (right) was done with ImageJ and expressed as percent of the initial burn wound area; n=13–36 depending on time point. (**Below**) Representative images of H&E-stained sections of skin isolated on day 14 post-burn (left), and quantification of re-epithelialization rates (right). Black arrows represent edge of wound bed, and grey arrows represent the leading edge of neo-epithelium; n=14. Data are shown as means ± SD. Brown-Forsythe and Welch's ANOVA with Dunnett's multiple comparisons test were performed for statistical analysis for qPCR analyses. Mixed effects analyses with Sidak's multiple comparisons tests were performed for statistical analysis for wound area quantitation experiments. Student's two-tailed t-test was used for statistical analysis for re-epithelialization experiments; ***p<0.001, **p<0.01, *p<0.05.

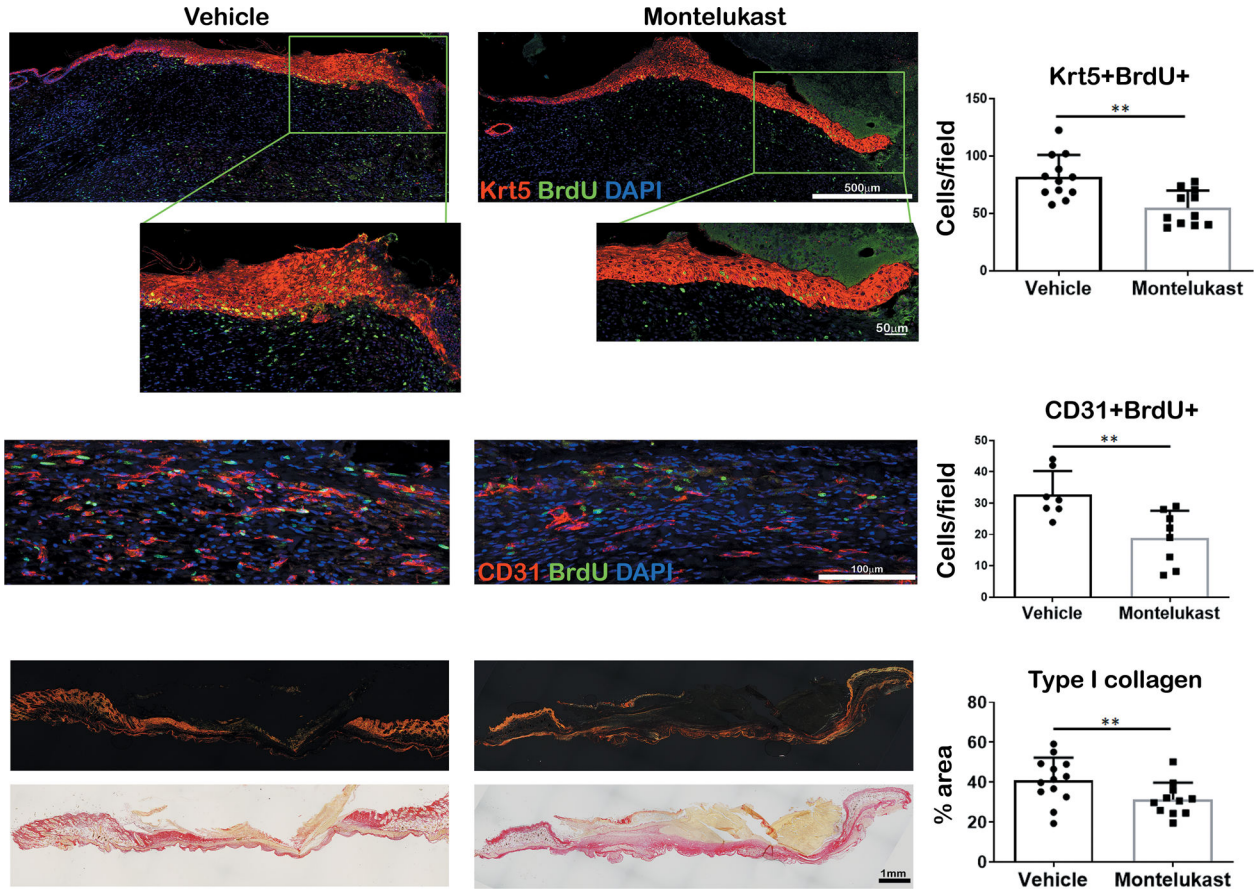

Figure 3. Montelukast treatment impairs the proliferative phase of burn wound healing.

Mice were injected with BrdU intraperitoneally 24hrs, 4 hrs, and 1hr before euthanasia. (**Above**) Proliferating keratinocytes were detected by immunohistological analysis of tissues for **Krt5+BrdU+** cells on day 14 post-burn (left). Lower images represent magnification of outlined areas. Right panel shows quantification of proliferating keratinocytes; n=12. (**Center**) Proliferating endothelial cells on day 14 post-burn were detected by immunohistological analysis of tissues for (**CD31+BrdU+**) (left). Right panel shows quantification of proliferating endothelial cells; n=7–8. (**Below**) Picrosirius red staining of day 14 post-burn tissues (left). Representative images of tissues scanned under brightfield (top) and polarized brightfield (bottom). Quantification of type I collagen was done using the MRI Fibrosis tool in ImageJ (right); n=11–13. Data are shown as means ± SD. **p<0.01, *p<0.05.

Table 1:

Primers used for qPCR analyses

Gene	Forward primer (5'–3')	Reverse Primer (5'–3')
B2m	TTCTGGTGCTTGTCTCACTGA	CAGTATGTTCGGCTTCCCATTC
Pla4g2a	CAGCACATTATAGTGGAACACCA	ATGGTCCAGCATATCGCCAAA
Alox5	Qiagen cat. No: QT01047557	
Alox5ap	Qiagen cat. No: QT01050574	
Lta4h	Qiagen cat. No: QT00160475	
Ltc4s	Qiagen cat. No: QT00153279	
Cysltr1	CCTCTCCGTGTGGTCTATTAT	ATGCAAACGAACCTGGCTTTT
Gpr17	CACCTGTCAAGTCCCTAAAG	GTGGGCTGACTAGCAGTGG
P2y1	GAGGTGCCTTGGTCGGTTG	CGGCAGTGAGTAGAACTGGAA
P2y2	CTGGAACCCTGGAATAGCACC	CACACCACGCCATAGGACA
P2x5	TCCCGGATGGCGAGTGTTCAG	GATGGGGCAGTAGAGATTGGTGGAG
P2y12	AGCAATGGGAAGAGAACCTGG	ATGGATATGCCTGGTGTCAACA
Arg1	CTCCAAGCCAAAGTCCTTAGAG	TAGCCATCAAACCTGTTGAGC
Retnla	CCAATCCAGCTAACTATCCCTCC	ACCCAGTAGCAGTCATCCA
Tgfb	Qiagen cat. No: QT00145250	
Igf1	CTGGACCAGAGACCCTTTGC	GGACGGGGACTTCTGAGTCTT
Vegf	Qiagen cat No. QT00160769	
Fgf7	Qiagen cat. No: QT00172004	
Pdgfa	GACGGTCATTTACGAGATACCTC	CTACGCCTTCCTGTCTCCTC
Nos2	CAGCTGGGCTGTACAAACCTT	CATTGGAAGTGAAGCGTTTCG
Nlrp3	ATTACCCGCCCGAGAAAGG	TCGCAGCAAAGATCCACACAG
Ccl8	TCTACGCAGTGCTTCTTTGCC	AAGGGGGATCTTCAGCTTTAGTA
Cxcl2	CCAACCACCAGGCTACAGG	GCGTCACACTCAAGCTCTG
Cxcl1	CTGGGATTCACCTCAAGAACATC	CAGGGTCAAGGCAAGCCTC
Ccl2	TTAAAAACCTGGATCGGAACCAA	GCATTAGCTTCAGATTTACGGGT
Il1b	Qiagen cat. No: QT01048355	
Tnfa	Qiagen cat No.: QT00104006	

Table 2:

mRNA levels of inflammatory and reparative genes in burn wounds shown as median of fold change against healthy skin (Interquartile range)

		Day 10		Day 14	
	Gene	**Vehicle**	**Montelukast**	**Vehicle**	**Montelukast**
Inflammatory	*Nos2*	447 (151–1405)	401 (188–792)	526 (11–724)	170 (18–357)
	Nlrp3	199 (4–342)	173 (125–261)	185 (74–410)	5 (0–97)
	Ccl8	29 (13–49)	21 (14–40)	23 (14–29)	19 (7–84)
	Cxcl2	4272 (2370–4941)	3902 (2344–6726)	2789 (1149–6020)	2099 (1214–5733)
	Cxcl1	1206 (878–2034)	2218 (772–4871)	609 (142–1734)	671 (261–1526)
	Ccl2	17 (10–34)	12 (6–21)	8 (4–26)	54 (13–82)
	Il1b	1760 (204–2807)	1586 (771–3217)	928 (459–1793)	669 (188–1122)
	Tnfa	156 (0–502)	283 (114–476)	133 (72–272)	120 (3–216)
Reparative	*Arg1*	154 (36–1254)	508 (174–816)	196 (105–638)	31 (19–136)
	Retnla	0.2 (0.1–0.7)	0.1 (0.1–0.2)	0.6 (0.4–1)	0.3 (0.2–0.8)
	Tgfb	174 (25–371)	122 (54–186)	87 (43–192)	22 (8–81)
	Igf1	13 (10–18)	9 (6–13)	10 (6–16)	8 (5–22)
	Vegf	16 (9–31)	26 (12–32)	22 (12–54)	14 (1–28)
	Fgf7	6 (4–16)	5 (3–9)	4 (3–18)	16 (6–22)
	Pdgfa	14 (8–28)	25 (12–28)	32 (4–47)	5 (0–13)

qPCR analyses of cytokines, chemokines and growth factors. Analyses were performed on days 10 day 14 post-burn tissues and expressed as fold change over values obtained from healthy skin. Data is shown as median of relative mRNA levels ($2^{-\Delta\Delta CT}$) and interquartile ranges. Shaded cells indicate statistically significant differences between vehicle and montelukast group. Mann-Whitney test was used for statistical analysis; day 10: n=10; day 14: n=12–13.