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Transient Receptor Potential Ankyrin 1 Ion Channel Facilitates Acute Inflammation Induced by Surgical Incision in Mice

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Abstract

Background: Transient receptor potential ankyrin (TRPA) 1 is known as a peripheral initiator of acute inflammation and hyperalgesia. However, its role in the facilitation of innate immune responses followed by resolution of the inflammation triggered by a surgical incision has not been fully investigated. Therefore, we evaluated the mechanism by which TRPA1 regulates the inflammatory responses mainly facilitated by neutrophils and macrophages in the early course of wound repair after an incision. **Methods:** Plantar incision was performed in wild-type and TRPA1$^{-/-}$ mice. The infiltration of polymorphonuclear neutrophils, macrophage phenotype, and induction of inflammatory mediators were assessed for 7 days postoperatively. **Results:** TRPA1$^{-/-}$ mice exhibited decreased infiltration of polymorphonuclear neutrophilscompared with wild-type mice on day 1. Consistently, the influx of F4/80^{+} iNOS^{+} proinflammatory M1 macrophages to incised sites was markedly decreased on day 2. Similarly, F4/80^{+} CD206^{+}M2 macrophages, which regulate the resolution of inflammation and promote wound healing in the later phase of acute inflammation, were significantly decreased in TRPA1$^{-/-}$ compared with those in wild-type mice on day 7. In addition, the induction of heme oxygenase-1, which promotes wound healing by switching phenotype of macrophages, was decreased in the early phase of acute inflammation, whereas the expression of proinflammatory mediators such as tumor necrosis factor and cyclooxygenase-2, and 15-lipoxygenase, which are involved in the resolution of inflammation was increased in the late phase in TRPA1$^{-/-}$ mice. **Conclusions:** Early innate immune responses including neutrophil infiltration and macrophage polarization at incised sites were inhibited in TRPA1$^{-/-}$ mice, associated with increased pro-inflammatory mediators in later phase. Peripheral TRPA1 may

facilitate the acute inflammatory process, leading to the promotion of macrophage-mediated resolution of inflammation and wound repair after a surgical incision.

Keywords

Transient Receptor Potential Ankyrin 1, Surgical Incision, Macrophage, Inflammation

1. Introduction

Acute inflammation after tissue injury activates immune as well as neuronal cells that mediate pain and guarding behaviors to promote wound healing. Studies have shown that transient receptor potential (TRP) ion channels play significant roles in pain, immune response, and airway inflammation [1]. In particular, TRPA1, expressed on nociceptive TRPV1-expressing neurons and emerging as a key regulator of neurogenic inflammation, is of particular interest clinically due to its wide variety of ligands, such as microorganism, necrotic tissues, and reactive oxygen species, which are likely to be produced at surgical wound sites. Trevisan *et al.* recently reported that TRPA1 mediates trigeminal neuropathic pain in mice downstream of monocyte/macrophage-produced oxidative stress, indicating that the neuro-immune interaction through TRPA1 activation by local macrophages might play a role in the development of acute inflammation [2]. In addition, consistent with a previous report describing that anesthetic management affects inflammatory and repair processes at sites of surgical incision [3], a large group of toxic chemicals and commonly used drugs, such as nonsteroidal anti-inflammatory drugs, but also general anesthetics including propofol, desflurane, and isoflurane, activate TRPA1 [4]. These reports imply that anesthetics/analgesics used during the perioperative period as well as the surgical procedure can modulate the acute inflammatory process through TRPA1 activation. Although the role of TRPA1 in the incidence of surgical site infection and wound repair has not been thoroughly investigated, the loss of TRPA1 was previously found to suppress inflammation and fibrosis/scarring in the corneal stroma during wound healing following an alkali burn in TRPA1$^{-/-}$ mice [5]. Furthermore, Kun *et al.* reported that acute neurogenic inflammation evoked by bacterial endotoxins was primarily dependent on TRPA1 channel activation in nociceptive sensory neurons. Of proinflammatory mediators initially induced by surgical incision, interleukin (IL)-1 plays a critical host defense role against *S. aureus* by enhancing neutrophil recruitment [6]. Proinflammatory mediators such as IL-1 and tumor necrosis factor (TNF) were also shown to be upregulated in a colitis model of TRPA1$^{-/-}$ mice, independent of toll-like receptors [7]. In addition, oxygen partial pressure was found to regulate TRPA1 activation in sensory neurons [8], suggesting that neuronal TRPA1 regulates the changes in the local inflammatory responses dependent on the changes in tissue environ-

ment that are likely to occur after a surgical procedure.

Recently, the functional expression of TRPA1 has also been demonstrated on non-neuronal cells, including keratinocytes, macrophages, fibroblasts, and synoviocytes [9]. The exposure of these surgically important cells to inflammatory mediators during the wound repair process was found to induce the upregulation of TRPA1. The stimulation of TRPA1 in turn reduced the production of IL-6 and the neutrophil chemoattractant IL-8 in synoviocytes, suggesting that TRPA1-mediated resolution of inflammation in surgical wounds is mediated not only by nerve fibers, but also by local stromal cells involved in inflammation and the wound healing process. Consistent with this, TRPA1 was shown not only to be involved in the initiation of nociceptive sensation and neurogenic inflammation but also to prevent the transition of acute to chronic inflammation and pain development in a chronic pancreatitis model, by reducing the myeloperoxidase activity of neutrophils [10]. Therefore, TRPA1 may promote the resolution of inflammation in the later phase of wound inflammation in addition to the initiation of inflammation after surgical incision. We recently reported that the resolution of inflammation is promoted by the phenotype switching of macrophages from the proinflammatory M1 to the anti-inflammatory M2 phenotype, which is mediated by heme oxygenase (HO)-1 in macrophages [11]. In this study, we thus investigated the impact of TRPA1 on the local inflammatory changes during the postoperative period after incision.

2. Methods

2.1. Animals

Male TRPA1-deficient ($TRPA1^{-/-}$) mice aged 6 - 7 weeks were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and genotyped as previously described [8]. They were maintained as heterozygotes in our facility and crossed to obtain null mutants and WT littermates. The Animal Research Committee of Kagoshima University approved all experimental procedures, which were implemented in accordance with the guidelines of the National Institutes of Health and the International Association for the Study of Pain [12]. Mice were kept under a 12-h light/dark cycle and randomly assigned to the experimental conditions. The person performing the behavioral tests, immunohistochemistry and quantitative polymerase chain reaction (PCR) was blinded to which mice were used throughout the postoperative period.

2.2. Paw Incision Model

Plantar incision was performed as described previously [13]. Mice were anesthetized with 3% isoflurane (Abbott, Chicago, IL, USA) and oxygen via a nose cone. After antiseptic preparation of the left hind paw with 70% ethanol, a 5-mm longitudinal incision was made with a no. 11 blade 2 mm from the edge of the heel, through the skin and fascia there. The underlying muscle was elevated with curved forceps, leaving the muscle origin and insertion intact. The skin was closed with a single mattress suture of 8 - 0 nylon.

2.3. Immunohistochemistry

Mice were deeply anesthetized with sodium pentobarbital (50 mg/kg intraperitoneally) and perfused transcardially with saline. Tissues were fixed in 4% paraformaldehyde overnight at 4˚C and placed in 30% sucrose solution for 24 h at 4˚C. Sections (30 m thick) were incubated overnight with primary antibodies to the polymorphonuclear neutrophil (PMN) marker Gr-1 (1:100; Santa Cruz Biotechnology, Dallas, TX, USA), pan-macrophage marker F4/80 (1:100; Santa Cruz Biotechnology), iNOS (1:500; Abcam, Cambridge, UK), or CD206 (1:200; Santa Cruz Biotechnology) at 4˚C overnight. Sections were incubated for 1 h at room temperature with a secondary antibody labeled with Alexa Fluor 488 or 546 (1:500; Invitrogen, Life Technologies, Carlsbad, CA, USA) followed by nuclear staining with 4'6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA). Fluorescent images were obtained using an LSM700 imaging system (Carl Zeiss, Aalen, Germany). The average numbers of Gr-1$^+$, F4/80$^+$ iNOS$^+$, and F4/80$^+$CD206$^+$ cells with clearly visible cell bodies stained with DAPI in the subcutaneous tissue were counted using Image J 1.43u 2010 software (National Institutes of Health, Bethesda, MD, USA).

2.4. Quantitative PCR

Mice were deeply anesthetized with sodium pentobarbital (50 mg/kg intraperitoneally) and perfused transcardially with saline. Total hindpaw RNA was extracted using Sepazol reagent (Nacalai Tesque, Kyoto, Japan). The synthesis of first-strand cDNA was performed using the High-Capacity RNA-to-cDNA kit (Applied Biosystems, Life Technologies). TaqMan assays were performed for the quantification of IL-1 (*Il1b)* (assay ID:Mm00434228_m1), TNF- (*Tnf*) (assay ID: Mm00443260_g1), cyclooxygenase (COX)-2 (*Ptgs 2)* (assay ID: Mm00478374), heme oxygenase (HO)-1 (*Hmox1)*(assay ID: Mm00516005_m1), and 15-lipoxygenase (LO) (*Alox 15)*(assay ID:Mm00507789_m1) expression using TaqMan Fast Advanced Master Mix (Applied Biosystems) on an ABI Prism Step One Plus Real-time PCR System (Applied Biosystems). Target gene expression was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) (assay ID: Mm99999915_g1).

2.5. Statistical Analysis

Differences between groups were analyzed by two-way analysis of variance (ANOVA) followed by Bonferroni's *post hoc* test for multiple comparisons. Values are presented as the mean ± standard error of the mean (SEM). Statistical analyses were conducted using GraphPad Prism version 6.0 (GraphPad Software, La Jolla, CA, USA). A P value of < 0.05 was considered statistically significant.

3. Results

3.1. Migration of Gr-1$^+$PMNs and Expression of IL-1β were Decreased in TRPA1$^{-/-}$ Mice

To investigate the involvement of TRPA1 in the initiation of inflammation after

surgical incision, we first evaluated the migration of PMNs, which are the first front-line immune cells to be engaged in the elimination of necrotic tissue and pathogens at wound sites (Figure 1(a)). The influx of Gr-1$^+$DAPI$^+$ PMNs was significantly lower in TRPA1$^{-/-}$ than in WT wounds on day 1 (WT *vs.* TRPA1$^{-/-}$: 2452 ± 287 *vs.* 1226 ± 232 cells/mm^2, $P < 0.01$) (Figure 1(b)). Consistent with the decrease in the influx of Gr-1$^+$DAPI$^+$ PMNs in the TRPA1$^{-/-}$ wounds, expression of the *Il1b*genewas significantly decreased at the incision site of TRPA1$^{-/-}$ mice compared with that in WT mice on day 1 (WT *vs.* TRPA1$^{-/-}$: 378.0 ± 102.9 *vs.* 152.4 ± 74.6, P < 0.01) after incision (Figure 1(c)).

3.2. Phenotype Switching of Macrophages to both M1 and M2 Phenotype is Delayed in TRPA1$^{-/-}$ Mice

To evaluate whether TRPA1 is involved in the phenotype switch of local macrophages in incision wounds, we counted local macrophages after immunos-

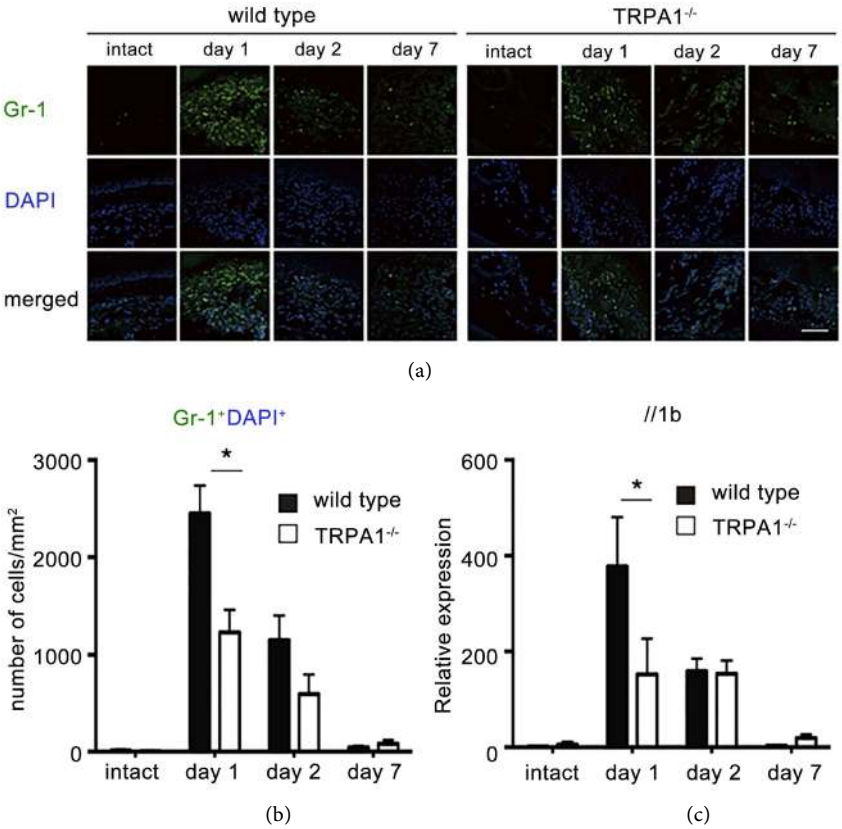
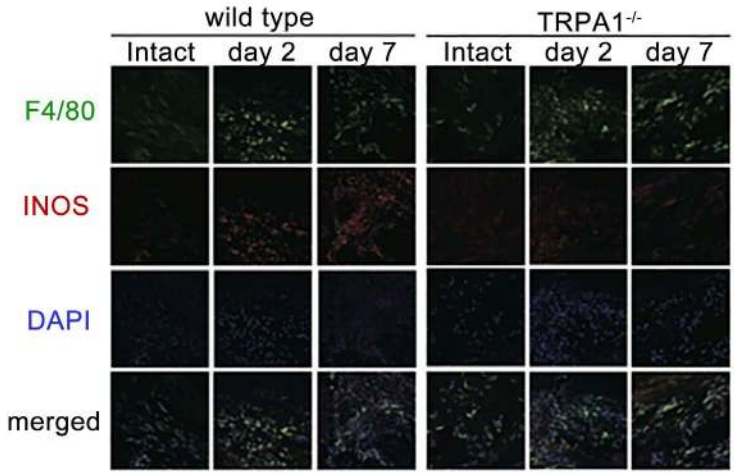
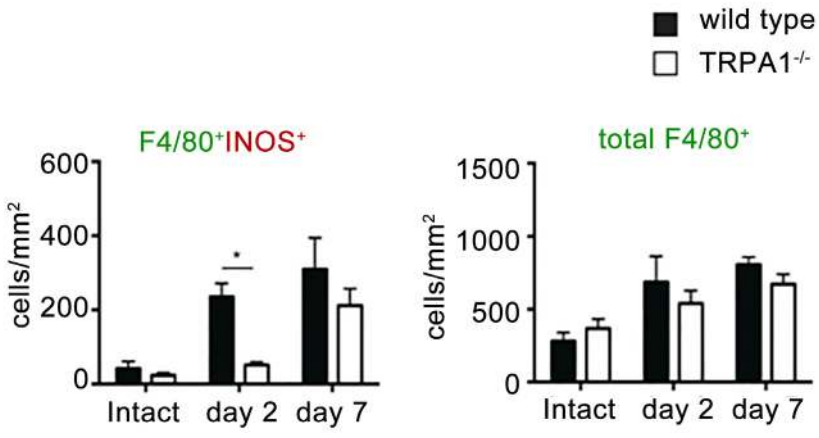


Figure 1. Local infiltration of Gr-1$^+$ PMNs and the expression of IL-1 (Il1b) were decreased in TRPA1$^{-/-}$ mice. (a) Gr-1$^+$ PMNs on days 1, 2, and 7 were evaluated using nuclear staining with 4', 6-diamidino-2-phenylindole (DAPI). (b) The total number of Gr-1$^+$DAPI$^+$ PMNs in subcutaneous tissue was counted. Two-way ANOVA (intera- ction: F3, 39 = 5.47, P < 0.01) with Bonferroni's post hoc test for comparing the mean number of Gr-1$^+$ PMNs at each time point. Green, Gr-1; blue, DAPI. Scale bar, 50 m. (c) Il1b expression was quantified by real-time PCR. Two-way ANOVA (interaction: F3,40 = 3.02, P < 0.05) with Bonferroni's post hoc test for comparison among groups was performed at each time point. n = 6 – 7 for each. Each column represents the mean ± SEM. *P < 0.05.

taining for the pan-macrophage marker F4/80, with an M1-specific marker, iNOS (Figure 2), or an M2-specific marker, CD206 (Figure 3). The number of F4/80$^+$iNOS$^+$ M1 macrophages recruited in the early phase was significantly decreased in the incision sites of TRPA1$^{-/-}$ mice on day 2 (WT *vs.* TRPA1$^{-/-}$: 235 ± 36 *vs.* 51 ± 8 cells/mm^2, $P < 0.05$), without altering the total number of F4/80$^+$ pan-macrophages (Figure 2(a), Figure 2(b)) implying that the infiltration of monocytes from the circulation to the wounds was not impaired in the early phase but that macrophage polarization to the M1 phenotype might be impaired in TRPA1$^{-/-}$ mice. Consistently, the number of F4/80$^+$CD206$^+$ M2 macrophages was markedly decreased in TRPA1$^{-/-}$ mice on day 7 (WT *vs.* TRPA1$^{-/-}$: 428 ± 69 *vs.* 153 ± 46 cells/ mm^2, $P < 0.01$) (Figure 3(a), Figure 3(b)). These results suggest that macrophage polarization to both M1 and M2 is inhibited by the ablation of TRPA1.



(a)



(b)

Figure 2. Phenotype shift of macrophages to M1 types is impaired in TRPA1$^{-/-}$ mice. (a) Infiltration of F4/80$^+$iNOS$^+$ M1 macrophages was evaluated on days 2 and 7. (b) The numbers of F4/80$^+$iNOS$^+$ and total F4/80 macrophages per area. Two-way ANOVA (F4/80$^+$iNOS$^+$: interaction: F2, 42 = 1.88, P = 0.1650; total F4/80+: interaction: F2, 42 = 0.96, P = 0.3906). n = 8 for each. The results are presented as mean ± SEM. *P < 0.05. Green, F4/80; red, iNOS; blue, DAPI. Scale bar, 100 m.

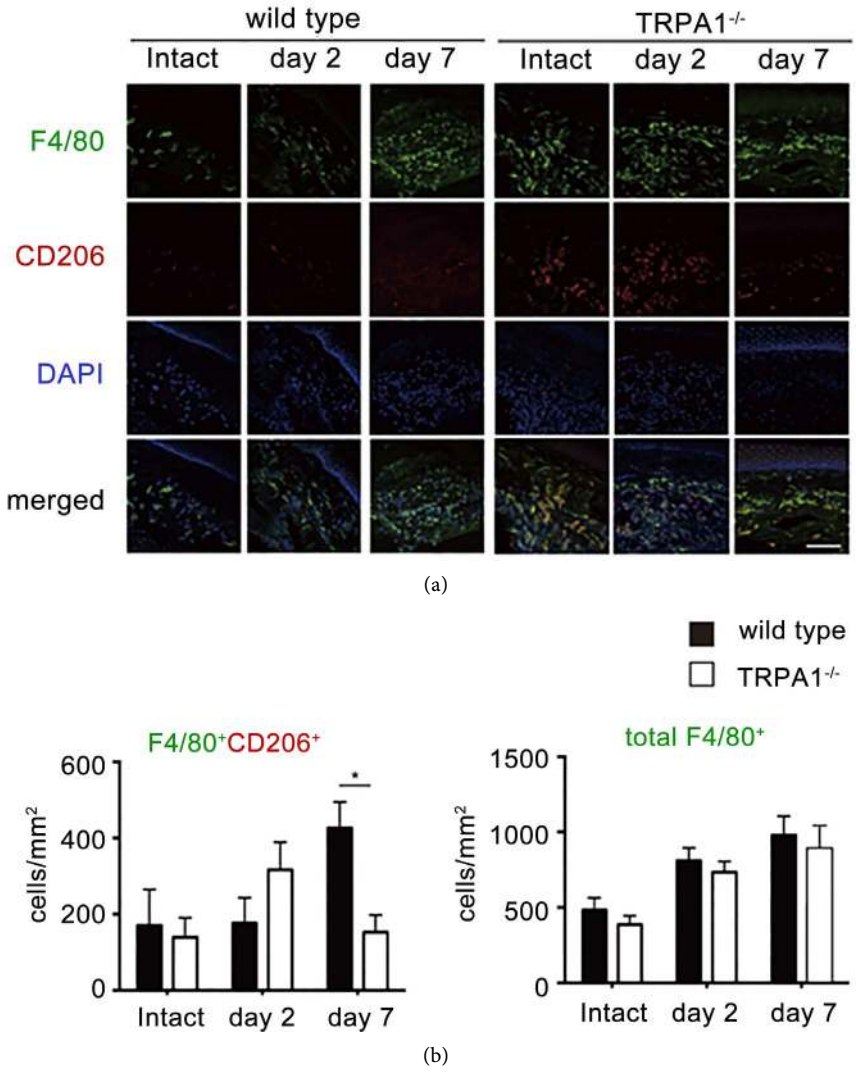


Figure 3. Phenotype shift of macrophages to M2 types is impaired in TRPA1$^{-/-}$ mice. (a) Infiltration of F4/80^{+}CD206^{+} M2 macrophages was evaluated on days 2 and 7. (b) The numbers of F4/80^{+}CD206^{+} and total F4/80 macrophages per area. Two-way ANOVA (F4/80+CD206+: interaction factor: F2,42 = 4.60, P < 0.05; total F4/80^{+}: interaction factor: F2,42 = 2.87, P = 0.0681). n = 8 for each. The results are presented as mean ± SEM. *P < 0.05. Green, F4/80; red, CD206; blue, DAPI. Scale bar, 100 m.

3.3. Impact of TRPA1 on the Expression of Inflammatory Mediators and the Resolution of Inflammation

We next investigated the change in the expression of proinflammatory mediators, TNF-αand COX-2 after plantar incision. The expression levels of TNF-*α* (*Tnf*) and COX-2 (*Ptgs 2*) genes were similar between WT and TRPA1$^{-/-}$ mice on day 2; however, the increases in their expression levels were observed on day 7 in TRPA1$^{-/-}$ mice (*Tnf*: WT *vs.* TRPA1$^{-/-}$: 1.5 ± 0.4 *vs.* 3.8 ± 0.2, P < 0.05; *Ptgs 2*:WT *vs.* TRPA1$^{-/-}$: 0.9 ± 0.4 *vs.* 6.2 ± 1.0, P < 0.05) (Figure 4). Consistent with the increase in COX-2 expression, the expression level of the 15-LO gene (*Alox 15*), involved in the cascades of arachidonic acid (AA) as well as COX-2, was increased on day 7 in TRPA1$^{-/-}$ mice (WT *vs.* TRPA1$^{-/-}$: 1.7 ± 0.4 *vs.* 4.6 ±

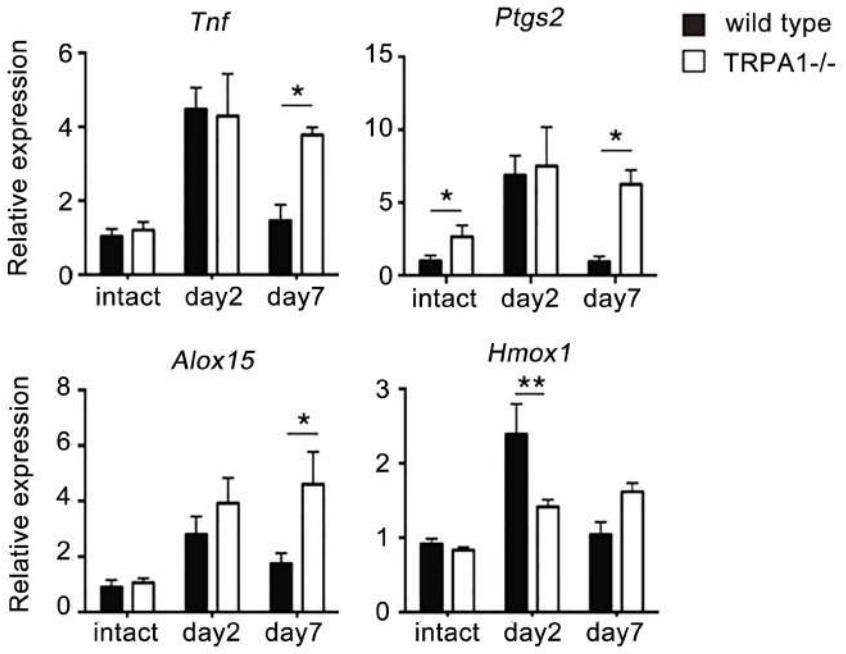


Figure 4. Gene expression of TNF- (Tnf), COX-2 (Ptgs 2), 15-LO (Alox15) and HO-1 (Hmox1) was evaluated after a surgical incision. It was quantified by real-time PCR. Two-way ANOVA (interaction:Tnf: $F_{2,30} = 3.857$, $P = 0.0747$; Ptgs 2: $F_{2,36} = 1.60$, $P = 0.2166$; Alox 15: $F_{2,30} = 1.98$, $P = 0.1553$, Hmox1: $F_{2,33} = 9.10$, $P < 0.001$) with Bonferroni's post hoc test for comparison at each time point. n = 6 – 7 for each. The results are presented as mean ± SEM. *P < 0.05, **P < 0.01.

1.2, P < 0.05). In addition, gene expression of HO-1 (*Hmox1*), which is induced by 15d-PGJ_2, the metabolites of AA, in macrophages and promotes macrophage polarization from the M1 to the M2 phenotype, was significantly decreased in the wounds of $TRPA1^{-/-}$ mice on day 2 (WT *vs.* $TRPA1^{-/-}$: 2.4 ± 0.4 *vs.* 1.04 ± 0.2, P < 0.01). These findings suggest that the impairment of phenotype shift to M1 macrophages on day 2 (**Figure 2(b)**) may have led to the decrease in HO-1 expression as well as the sustained increases in COX-2, TNF-*α*, and 15-LO, resulting in the decrease in M2 macrophages on day 7 in $TRPA1^{-/-}$ mice.

4. Discussion

We demonstrated that initial defense responses including the influx of PMNs and the polarization of macrophages to the M1 and M2 phenotype were all impaired in $TRPA1^{-/-}$ surgical wounds. IL-1 secreted by local macrophages promotes the migration of $Gr\text{-}1^+$ PMNs [14]. In this study, the migration of $Gr\text{-}1^+$ PMNs was markedly decreased in $TRPA1^{-/-}$ mice (**Figure 1(a)**, **Figure 1(b)**) associated with the decrease in IL-1 expression (**Figure 1(c)**). Sahbaie *et al.* previously reported that the depletion of $Gr\text{-}1^+$ PMNs in incised paw, associated with IL-1 levels half those of the controls 24 h after incision, did not alter the magnitude or duration of mechanical hypersensitivity [15], indicating that the increase in $Gr\text{-}1^+$ PMNs and the expression of IL-1 in peripheral tissue might contribute less to the development of mechanical hypersensitivity.

In contrast, TNF- and PGs play pivotal roles in neutrophil recruitment in initial inflammatory responses [16] [17]. In an inflammatory state, neutrophils undergo apoptosis after exposure to TNF- and post-phagocytosis [18], while PGE2, which is synthesized by COX-2, has a role in the resolution and healing phase as well as in the early stages of the inflammatory response [19]. In our study, the peripheral expression levels of COX-2 and TNF- were similar between WT and TRPA1$^{-/-}$ mice on day 2, but both increased in TRPA1$^{-/-}$ mice on day 7 (**Figure 4**). These data suggests that the resolution of inflammation is impaired in TRPA1$^{-/-}$ wounds.

We recently reported that the resolution of inflammation through the clearance of PMNs is accelerated by the alteration of macrophage polarity from the M1 to the M2 phenotype during the course of postoperative pain development [11] [20]. In addition, HO-1, contributing to the phenotype switch of macrophages, enhances the phagocytosis of PMNs [21]. The inhibition or genetic lack of HO-1, which was induced on days 2 and 3 after wounding, led to delayed wound closure, the suppression of re-epithelialization, and the formation of extensive skin lesions, accompanied by impaired neovascularization [22]. The decrease in the expression levels of HO-1 in the wounds of TRPA1$^{-/-}$ mice (**Figure 4**) might induce insufficient phagocytosis of PMNs at the wounds (**Figure 1(a)**, **Figure 1(b)**), leading to impaired macrophage polarization (**Figure 2**, **Figure 3**) and sustained increase in TNF- and COX-2 (**Figure 4**). Similar to HO-1, 15-LO regulates the resolution of inflammation and orchestrates the clearance of apoptotic cells through phagocytosis by monocytes [23]. Consistent with the prolonged expression of COX-*2*, the expression of 15-LO was also increased in the wounds of TRPA1$^{-/-}$ mice on day 7 (**Figure 4**). Although both HO-1 and 15-LO promote macrophage polarization to M2 phenotype, the expression of HO-1 was decreased while that of 15-LO was increased on day 7. Although HO-1 induction was found in a previous study to be independent of COX-2 products in a wound healing model [24], further study is required to clarify the mechanism by which COX-2 and 15-LO, involved in AA cascades, and HO-1 orchestrate the resolution of inflammation through the phenotype switch of macrophages.

Bacterial component such as lipopolysaccharide (LPS) facilitates switching to the M1 phenotype [25]. It has been indicated that TRPA1 is involved in LPS-induced initiation of local immune responses. Although the link between TRPA1 and macrophage polarization to the M1 phenotype is unclear, the lack of TRPA1 may be critical for the impairment of macrophage polarization in TRPA$^{-/-}$ mice.

Recently, it has been reported that TRPA1 as well as TRPM7 is functionally expressed on macrophages [7]. In addition, the specific inhibitors of TRPM7 prevent the polarization of macrophages towards the anti-inflammatory M2 phenotype [26]. The current density of TRPM7 is significantly greater in the M2 phenotype than in untreated cells, or in the M1 phenotype. Therefore, further investigation is required to clarify the involvement of TRPA1 expressed on macrophages in the inflammatory process after a surgical incision.

5. Conclusion

We demonstrated that TRPA1$^{-/-}$ mice exhibit decreased neutrophil infiltration, resulting in decreases in the influx of M1 and M2 macrophages in the early and late phases of acute inflammation, respectively. In addition, we showed that there is a difference in the induction of inflammatory mediators involved in the phenotype switching of macrophages. Further investigation is required to clarify the mechanisms by which TRPA1 regulates the local environment at surgical sites.

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Conflict of Interests

The authors have no conflicts of interest to declare, financial or otherwise.

Ethics Approval

The Animal Research Committee of Kagoshima University approved all experimental procedures, which were implemented in accordance with the guidelines of the National Institutes of Health and the International Association for the Study of Pain.

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Abbreviations

TRP, transient receptor potential; IL, interleukin; TNF, tumor necrosis factor; HO, heme oxygenase; PG, prostaglandin; COX, cyclooxygenase; LO, lipoxygenase; PMN, polymorphonuclear neutrophil; AA, arachidonic acid; LPS, lipopolysaccharide.