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SHORT REPORT

Unexpected Role of TNFα Signaling in the Resolution of Postoperative Pain in Mice

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Abstract: The mechanisms that govern the transition from acute to chronic pain remain poorly defined. Emerging evidence suggests that immune cells and acute inflammatory responses are not merely pathological but actively contribute to pain resolution and the prevention of chronic pain. Using a mouse model of postoperative pain induced by plantar incision, we demonstrate that inhibition of tumor necrosis factor (TNFα) signaling prolongs pain hypersensitivity. Intraplantar administration of either monoclonal or polyclonal neutralizing anti-TNFα antibodies or Etanercept, a TNF receptor decoy, significantly delayed the resolution of pain in both female and male mice. Unexpectedly, early blockade of TNFα signaling did not reduce pain hypersensitivity but instead extended its duration. These findings underscore a paradoxical yet critical role for TNFα and immune signaling in promoting the resolution of acute pain and preventing its persistence. Together it supports the concept that acute inflammation and immune cells are essential for initiating the resolution of pain.

Keywords: TNFα, postoperative pain, pain resolution, inflammation, mice

Introduction

More than 40 million Americans undergo surgical procedures each year, and acute pain is a common consequence. While most individuals recover without complication, an estimated 4 million develop chronic postsurgical pain, leading to persistent discomfort, emotional distress, and functional disability.[1,2] Chronic postsurgical pain also contributes significantly to the opioid crisis by prolonging opioid use and increasing the risk of dependence.[3,4] Effectively managing postoperative pain and preventing the transition to chronic pain remain major clinical challenges.

Although nonsteroidal anti-inflammatory drugs (NSAIDs) are effective in controlling acute postoperative pain, they frequently fail to prevent chronic pain. More concerning, recent data suggest that NSAID use may even promote the persistence of low back pain.[5] These findings highlight the complex and context-dependent role of the immune system in pain progression and resolution.

Both innate and adaptive immune cells are involved in the initiation, maintenance, and resolution of pain.[6–10] Myeloid cells, for instance, release both pro-nociceptive mediators such as interleukin (IL)-1β, which increase nociceptor excitability,[11,12] and anti-nociceptive cytokines such as IL-10,[13,14] which modulate it. IL-1β has been shown to reduce opioid analgesia, whereas IL-10 enhances opioid efficacy.[13,15] The shift from a pro-inflammatory to a pro-resolving immune profile may be mediated in part by specialized pro-resolving mediators (SPMs), which are produced by macrophages following the clearance of apoptotic neutrophils.[16,17] These observations underscore the dual and temporally dynamic roles of immune cells in shaping pain trajectories.

Among immune mediators, tumor necrosis factor alpha (TNFα) stands out due to its central role in inflammation. Single-cell (sc)RNA sequencing of skin immune cells after plantar incision revealed that *Tnf* is predominantly expressed by neutrophils and infiltrating monocytes,[18] two cell types recently implicated in facilitating pain resolution.[5,19] While TNFα sensitizes peripheral nociceptors via TNF receptor 1 (TNFR1),[20,21] activation of TNFR2 may exert neuroprotective and anti-nociceptive effects.[22,23] Indeed, TNFR2 agonism has been shown to accelerate recovery in models of

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neuropathic pain following nerve injury.[24] Additionally, TNF is also critical to activate neutrophils, which are important for the resolution of pain.[5,25]

Given the complex and paradoxical roles of TNFα signaling in pain modulation, we sought to determine how TNF contributes to the resolution or persistence of pain in a mouse model of postoperative pain. We antagonized TNFα signaling in the immediate aftermath of surgical incision and monitored pain sensitivity over time to assess its impact on pain resolution.

Method

Animal procedures were approved by Michigan State University (MSU) Institutional Animal Care and Use Committee (IACUC) and were in accordance with the National Institutes of Health (NIH) Guidelines. All the experiments were performed on 10–14 weeks old male and female C57Bl/6J mice (JAX# 000664). Mice were bred and housed in the Michigan State University Animal Care Facility prior to the start of behavioral testing. Animals had ad libitum access to food and water and were on a 12 h non-inverted light/dark cycle. Experimenters were blinded to treatment groups in behavioral experiments. Mice were randomized to treatment groups. Male and female mice were housed and tested jointly.

Postoperative pain model: Plantar incision was performed under vaporized isoflurane (2%) anesthesia. Incision surgery was performed by making a 5-mm longitudinal incision with a #11 scalpel blade in the skin of the left hind paw and the underlying muscle tissue 2 mm below the heel as previously described.[13,26,27] The wound was closed using a 5–0 silk suture followed by a 200 µL subcutaneous injection of the antibiotic gentamicin (Sigma-Aldrich, 5 mg/mL).

Mechanical pain hypersensitivity was assessed using calibrated von Frey filaments (Bioseb #577). Mice were placed in individual cubicles on an elevated mesh floor allowing stimulation of the plantar surface with the filaments. Mice were habituated at least 30 minutes before testing. Withdrawal threshold was calculated using the up-down method.[13,27,28]

Thermal pain sensitivity was assessed by the Hargreaves test using a Ugo Basile Thermal Plantar (Stoeltingco, 55370). Thermal stimulation was delivered to the plantar surface of mice using a light source following habituation. Paw withdrawal latency was recorded, with the infrared beam shutting off automatically upon withdrawal, which stopped the reaction time counter.

Intraplantar administration of neutralizing polyclonal anti-TNFα (RD System, #AF-410-NA, 5µg), isotype control IgG (Cell signaling Technology #7076S), monoclonal anti-TNFα clone XT3 (BioXCell, #BE0058, 5µg), isotype control InVivoPlus mouse IgG1 clone MOPC-21 (BioXCell, #BP0083), Etanercept (MedChemExpress #HY-108847) and PBS. Polyclonal antibody and IgG (Cell signaling technology) were reconstituted in phosphate buffered saline (PBS) and compared. Monoclonal antibody and IgG1 were reconstituted in InVivoPure dilution buffer (BioXCell, #IP0065) and compared. Etanercept was reconstituted in PBS and PBS was used as vehicle comparison. Injections were performed under anesthesia with Hamilton syringes in a volume of 5 µL at 2 and 24 h after incision.

Statistical differences between groups were assessed by two-way ANOVA followed by multiple comparison tests as indicated in figure legends.

Results

Incision induced marked mechanical pain hypersensitivity in both male and female mice. Mechanical sensitivity returned to baseline levels by day 10. Administration of neutralizing polyclonal anti-TNFα antibody in the early aftermath of surgery (2 and 24 hrs) significantly delayed the resolution of pain hypersensitivity in both sexes (Figure 1A, anti-TNF effect $F(1, 11) = 51.6$, $p<0.0001$). We repeated this experiment with a monoclonal neutralizing antibody (Clone XT3). In line with the previous data, neutralization of TNFα with monoclonal antibody drastically delayed the resolution of postoperative pain hypersensitivity (Figure 1B, anti- TNF effect $F(1,10) =46.5$, $p<0.0001$). To confirm the specificity of the TNF effect, TNF signaling was targeted by an alternative method. Intraplantar injection of the FDA approved TNF receptor decoy: Etanercept following surgery markedly impaired the resolution of both mechanical and thermal pain hypersensitivity in female and male mice (Figure 2 and 2A treatment effect $F(2, 14) = 34.5$, $p<0.0001$; Figure 2B treatment effect $F(1,9) = 38.6$, $p=0.0002$). Interestingly, inhibiting TNF signaling with etanercept had an early antinociceptive effect on mechanical sensitivity on day 6 in both sexes and with both doses but this antinociceptive effect did not persist.

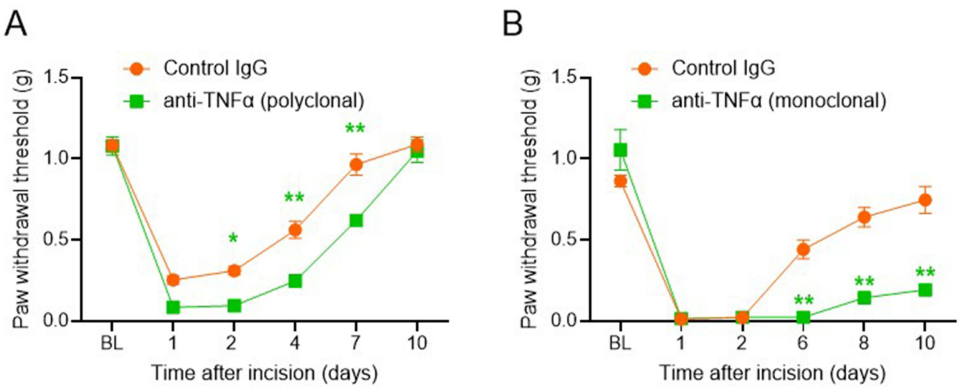

Figure 1 Neutralization of TNFα prolonged pain hypersensitivity induced by surgical incision. Mechanical sensitivity was assessed with von Frey filaments. (**A**) Neutralizing polyclonal anti-TNFα (5 µg) was injected at 2 and 24 h after incision (Control n= 4M and 3F, anti-TNFα n= 2M and 4F). Two-way ANOVA with Sidak's correction time x treatment interaction: F (5, 55) = 5.0, p=0.0008. (**B**) Neutralizing monoclonal anti-TNFα (5 µg) was injected at 2 and 24 h after incision (Control n= 3M and 3F, anti-TNFα n= 3M and 3F). Two-way ANOVA with Sidak's correction time x treatment interaction: F(2.6,25.9)= 18.6, p<0.0001). On the graph * = p<0.05, and ** = p<0.01. BL: indicates baseline measures performed before the surgery.

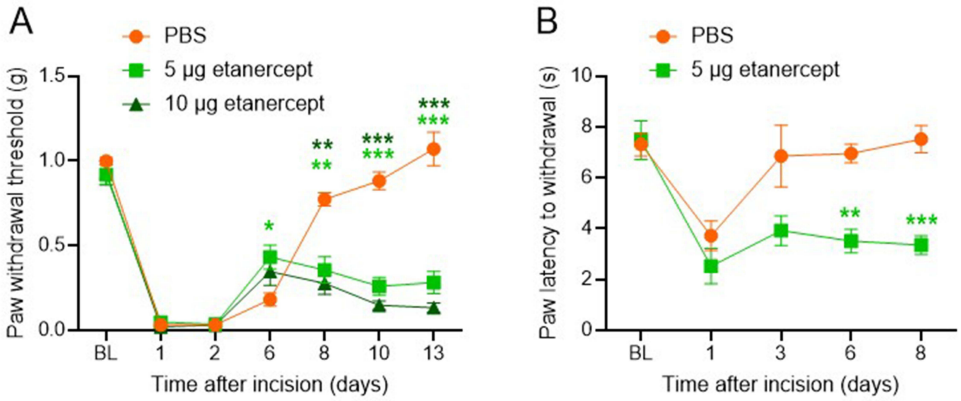

Figure 2 TNF receptor antagonism prolonged mechanical and thermal postoperative pain hypersensitivity. (**A**) Mechanical sensitivity, Etanercept was injected at 2 and 24 h after incision (n= 3M and 3F in each group). Two-way ANOVA with Tukey's correction time x treatment interaction: F(12, 84) = 25.5, p<0.0001. (**B**) Thermal sensitivity. Etanercept was injected at 2 and 24 h after incision (n= 3M and 3F in each group). Two-way ANOVA with Bonferroni correction time x treatment interaction: F(2, 19) = 3.31, p=0.05. On the graph * = p<0.05, ** = p<0.01, and *** = p<0.001. BL: indicates baseline measures performed before the surgery.

It is important to note that the impact of TNFα neutralization or the antagonism of TNF receptor were similar between male and female mice. No sex difference was observed (Figure 3). Moreover, blocking TNF signaling did not impair would healing (Figure 4) ruling out this potential confounding factor.

Discussion

Because inflammation is commonly associated with pain, immunosuppressive and anti-inflammatory strategies have been widely proposed for the treatment of persistent pain. However, anti-inflammatory therapies have shown limited efficacy in alleviating chronic pain conditions. In contrast, our findings reveal an unexpected role for TNF signaling in promoting the resolution of incision-induced hypersensitivity.

These results support the emerging paradigm that a robust acute inflammatory response is necessary to initiate endogenous resolution pathways (Figure 5). Recent studies have shown that the absence of proinflammatory immune cells, such as mast cells and neutrophils, key players in the acute inflammatory phase, impairs resolution and lead to persistent pain.[5,27] In parallel, pro-resolving immune populations, including IL-10–producing myeloid cells and regulatory T cells, are also required to prevent the transition from acute to chronic pain.[9,14,19,29] This concept is further reinforced by data from human cohorts with low back pain, where individuals with resolving pain displayed higher levels of inflammatory markers in the early post-injury phase.[5]

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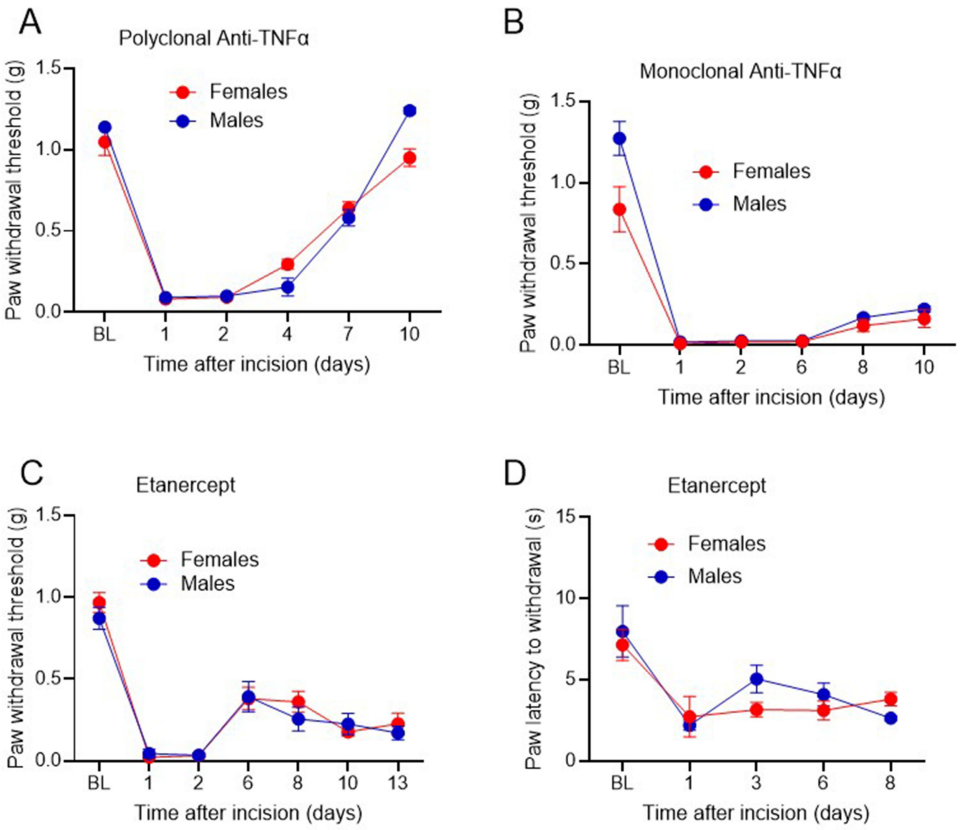

Figure 3 Absence of sex difference on the impact of TNFα signaling in acute postoperative pain resolution. (**A**) Effect of polyclonal antibody on mechanical pain corresponding to Figure 1A. (**B**) Effect of monoclonal antibody on mechanical pain corresponding to Figure 1B. (**C**) Effect of etanercept on mechanical pain corresponding to Figure 2A. (**D**) Effect of etanercept on thermal pain corresponding to Figure 2B. Effect of sex assessed by two-way ANOVA with multiple comparison test correction (**A**) p=0.14, (**B**) p=0.22, (**C**) p=0.59, and (**D**) p=0.53. BL: indicates baseline measures performed before the surgery.

Consistently, preclinical adoptive transfer studies have demonstrated that T cells must be primed by acute inflammation shortly after injury in order to promote pain resolution.[30]

The spatiotemporal dynamics of TNF signaling appear to be critical. In this study, TNFα inhibition was restricted to the early post-incision period and was localized to the site of injury. It is plausible that inhibition at later stages or at different anatomical sites would yield different outcomes on pain trajectories. Importantly, our findings do not contradict the established roles of TNFα in central sensitization or dorsal root ganglion signaling in models of neuropathic pain.[31,32] It is conceivable that TNFα may act sequentially; first modulating neutrophil activation and later contributing to nociceptor sensitization.[20,25]

Although anti-TNFα therapies have demonstrated some efficacy in alleviating pain in diseases such as rheumatoid arthritis (RA), it is worth noting that RA pain is chronic and often non-resolving. Moreover, not all RA-related pain is linked to ongoing peripheral inflammation, and successful suppression of inflammation, even with anti-TNFα agents, does not always correlate with pain relief.[33–40]

In conclusion, our study highlights that premature suppression of inflammatory mediators like TNFα may interfere with the natural processes required for pain resolution and may ultimately prolong postoperative pain. These findings underscore the need to re-evaluate the timing and context of anti-inflammatory interventions in the management of acute and chronic pain.

Conclusion

In summary, our findings reveal that TNF signaling plays an unexpected yet essential role in facilitating the resolution of postoperative pain. Contrary to the prevailing view of TNFα solely as a pro-nociceptive mediator, our study demonstrates

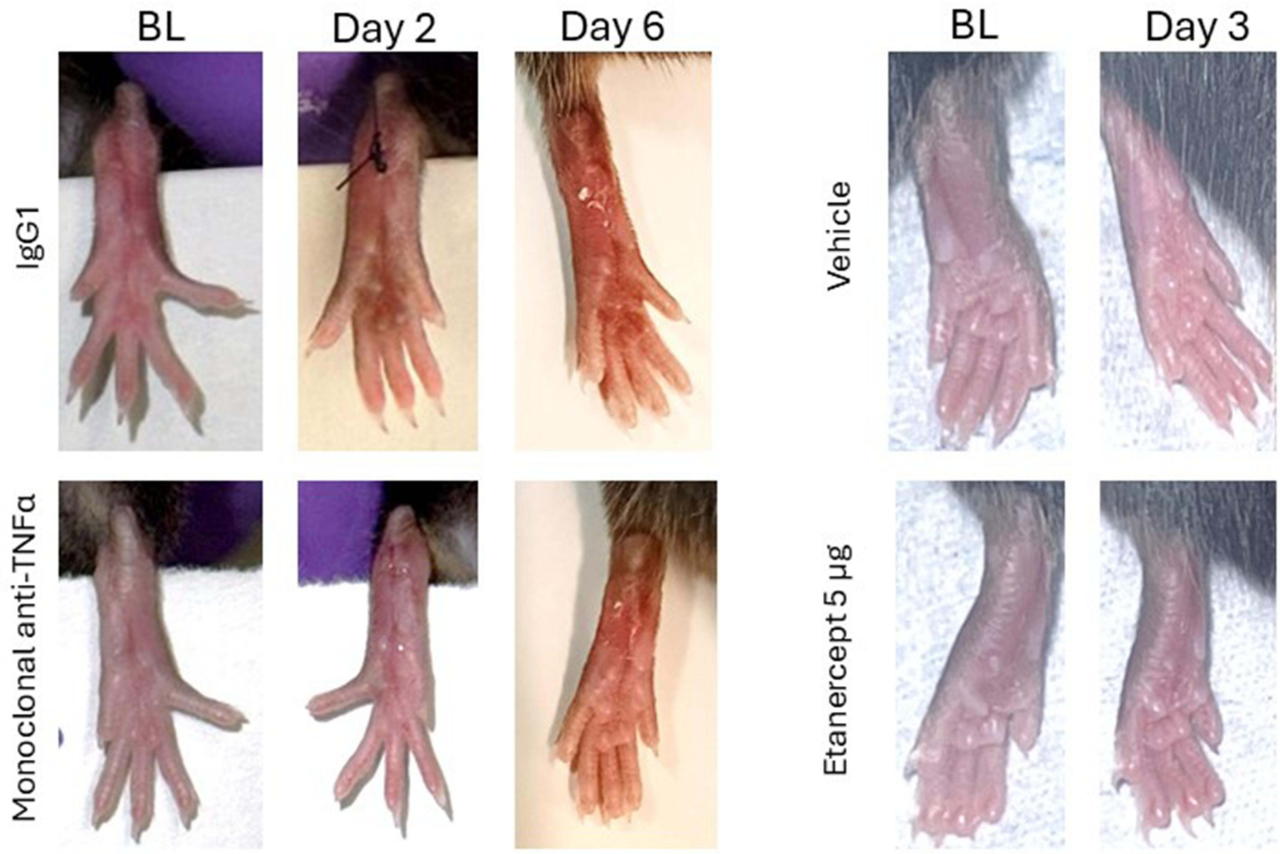

Figure 4 Blocking TNF signaling did not impact wound healing. Representative pictures of the incised plantar skin in vehicle-, etanercept-, and monoclonal anti-TNFα-treated mice. BL: indicates baseline measures performed before the surgery.

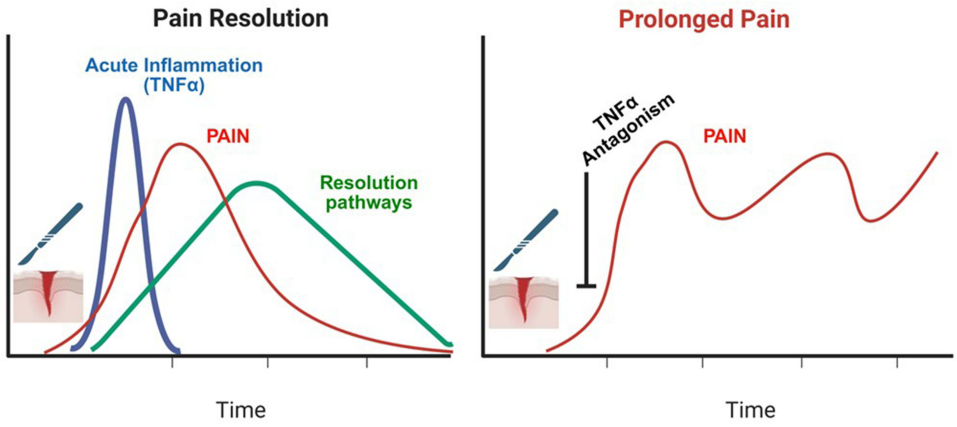

Figure 5 Working hypothesis: Robust TNFα-driven inflammation leads to resolution. A robust acute inflammatory response to tissue damage, such as surgical incision, is essential for activating endogenous resolution pathways that alleviate pain and prevent the development of prolonged pain. Inhibition of TNFα disrupts this acute inflammatory response, impairs the initiation of resolution mechanisms.

that early inhibition of TNFα delays recovery and prolongs pain hypersensitivity. These results underscore the importance of acute inflammatory signaling in activating endogenous resolution pathways and preventing the transition from acute to chronic pain. Clinically, they highlight the need for careful consideration of the timing and context of anti-inflammatory interventions, as premature suppression of key inflammatory mediators such as TNFα may hinder rather than promote recovery from postsurgical pain.

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Disclosure

None of the authors have any conflicts of interest to declare for this work.

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