Roles of Gr-1$^+$ Leukocytes in Postincisional Nociceptive Sensitization and Inflammation

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ABSTRACT

Background: Neutrophils are one of the predominant immune cells initially migrating to surgical wound edges. They produce mediators both associated with supporting (interleukin [IL]-1β, C5a) and reducing (opioid peptides) pain. Studies demonstrate neutrophil depletion/blockade reduces nociceptive sensitization after nerve injury and carrageenan administration, but enhance sensitization in complete Freund's adjuvant inflammation. This research identifies the contribution of infiltrating neutrophils to incisional pain and inflammation.

Methods: Antibody-mediated Gr1$^+$ neutrophil depletion preceded hind paw incisions. Sensitization to mechanical and thermal stimuli, effects on edema and local levels of IL-1β and C5a were measured. Local effects of C5a or IL-1 receptor antagonists PMX-53 and anakinra on sensitization after neutrophil depletion were examined. Groups of 4–8 mice were used.

Results: Anti-Gr1 antibody depleted more than 90% of circulating and infiltrating skin neutrophils after incision. Neutrophil depletion did not change magnitude or duration of mechanical hypersensitivity in incised mice. However, paw edema was significantly reduced and heat hypersensitivity was slightly increased in depleted animals. In depleted animals IL-1β levels were half of controls 24 h after incision, whereas C5a levels were increased in both. Prominent IL-1β immunohistochemical staining of epidermis was seen in both groups. PMX-53 and anakinra reduced incisional mechanical and heat nociceptive sensitization to the same extent, regardless of neutrophil depletion.

Conclusions: Neutrophil-derived IL-1β and C5a do not appear to contribute critically to peri-incisional nociceptive signaling. Other sources of mediators, such as epidermal cells, may need to be considered. Controlling inflammatory activation of resident cells in epidermis/deeper structures may show therapeutic efficacy in reducing pain from surgical incisions.

What We Already Know about This Topic

- Neutrophils responding to injury release mediators that enhance sensitization and pain after neuropathic injury in animals, but their role in incisional surgery is not known

What This Article Tells Us That Is New

- In mice, depletion of neutrophils reduced paw edema and tissue interleukin-1β concentrations after paw incision, but failed to significantly alter mechanical hypersensitivity
- Blockade of C5a and interleukin-1β signaling reduced hypersensitivity, suggesting that these factors are important to sensitization after incisional surgery, but are not dependent on local infiltration by neutrophils

PAIN after surgery remains problematic. Despite the heightened attention given to postoperative comfort, expanded use of patient-controlled analgesia devices, and increasing use of multimodal therapy, almost all patients experience some degree of postoperative pain, and 30–60% of patients undergoing surgery report moderate to severe pain levels.[1,2] On the other hand, progress has been made in understanding the mechanisms supporting this type of pain. Investigators have addressed a wide range of factors like wound dynamics, nociceptor sensitization, central nervous system changes, and patients' psychologic profiles to better understand postoperative pain. A good deal of attention has

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Received from the Anesthesiology Service, Veterans Affairs Palo Alto Healthcare System, Palo Alto, California, and Department of Anesthesia, Stanford University School of Medicine, Palo Alto, California. Submitted for publication September 3, 2011. Accepted for publication May 15, 2012. Supported by grant no. GM079126 from the National Institute of General Medical Sciences, Bethesda, Maryland.

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been focused on the liberation of local nociceptive mediators after incision, and the interaction of those mediators with primary afferent nerves.[3,4] The implicit hope of this research is that identification of key mediators and the sources of those mediators will further facilitate the development of specific therapeutic approaches.

One of the predominant immune cell types migrating to the injured tissue are neutrophils, which are present in wound edges within hours of incision, peak in abundance within 24 h, and then slowly decline in number. In addition to participating in fighting infection and regulating wound healing, these cells produce many known nociceptive mediators including cytokines, chemokines, proteinases, phospholipases, reactive oxygen species, and other molecules.[5] Cytokine-stimulated neutrophils can in turn activate additional incisional nociceptive mediators, such as complement system components.[6] Some of these mediators, namely interleukin (IL)-1β[7–9] and the complement fragment C5a,[10,11] have been shown to support nociception in rodent incisional pain models.

Additional evidence suggests neutrophils regulate nociceptive sensitization in other pain models. For example, depletion of circulating neutrophils reduces nociceptive sensitization early after peripheral nerve injury.[12] Also, blockade of neutrophil infiltration using the migration inhibitor fucoidin resulted in reduced mechanical hyperalgesia after carrageenan injection in the plantar tissue of rat hind paws, suggesting that in this pain model neutrophils might contribute to mediator production and sensitization.[13] On the other hand, neutrophils produce endogenous opioid peptides such as met-enkephalin and β-endorphin, potentially reducing pain.[14] In the complete Freund's adjuvant model of inflammatory pain, opioid peptides derived from neutrophils reduce nociceptive sensitivity, whereas depletion of neutrophils does not alter baseline sensitization in this pain model.[14–16] Thus, in some settings, neutrophils seem to provide a mechanism for endogenous peripheral analgesia.

We do not at this point understand whether the complex functions of neutrophils in incisional wounds lead to an overall enhancement, as would be suggested by mediator production, or reduction, as would be suggested by opioid peptide release, in nociceptive sensitization after incision. Furthermore, we do not understand for particular mediators already linked to sensitization in incisional wounds, such as IL-1β or C5a, whether local production by resident cells *versus* neutrophil infiltration and release is the mechanism responsible for the observed inflammation and sensitization. In these experiments we utilized an antibody-mediated neutrophil depletion protocol combined with assessments of the local consequences of incision to address these questions.

Materials and Methods

Animals

All experimental protocols were approved by Veterans Affairs Palo Alto Healthcare System Institutional Animal Care and Use Committee (Palo Alto, California) before beginning the work. Male mice 10–14 weeks old of the C57Bl/6J strain obtained from Jackson Laboratories (Bar Harbor, MA) were kept in our facility a minimum of 1 week before initiating experiments. All mice were kept under standard conditions with a 12 h light/dark cycle and an ambient temperature of 22 ± 1°C. Animals were allowed food and water *ad libitum*. All procedures followed the guidelines of International Association for the Study of Pain for care and use of laboratory animals. All experiments were done with 4–8 mice per group, guided by power analyses based on pilot and previous experimentation.

Hind Paw Incision

The hind paw incision model modified for mice was used in a similar way as previous studies investigating nociceptive effects and cytokine level changes following incision.[10,17,18] Briefly, mice were anesthetized using isoflurane (AErrane; Baxter Healthcare Corporation, Deerfield, IL), and after sterile preparation, a 5-mm longitudinal incision was made on the plantar surface of one hind paw. The underlying plantaris muscle was incised longitudinally; the wound was then closed with a single 6-0 nylon suture and antibiotic ointment was applied.

In Vivo Neutrophil Depletion

Treatment with functional grade purified antimouse Ly6G/Gr-1 (RB6-8C5 clone; eBioscience, San Diego, CA) or IgG2b control antibody (for controls) at 48 and 4 h before incision was carried out. Separate groups of mice received 4 mg/kg intraperitoneal injections of either antibody. Blood was collected by tail snip from anesthetized mice for neutrophil counts. For this purpose, blood samples were obtained before antibody treatment, before incision, and daily afterward for 5 days. Slides made using these samples were stained with Wright–Giemsa stain. Counting was completed manually.

Assessment of Mechanical Sensitivity

Mechanical sensitivity after incision was measured by using von Frey filaments following the "up-down" algorithm described by Chaplan *et al.*[10,18,19] After acclimating mice on wire mesh platforms inside clear cylindrical plastic enclosures, sequential application of filaments lateral to the central wound edge was carried out. Hind paw withdrawal because of fiber application was scored as a response. By using a data-fitting algorithm, the mechanical withdrawal threshold was calculated and subjected to parametric statistical analysis.[20] These experiments were done by a single experienced experimenter not blinded to treatment groups.

Assessment of Heat Sensitivity

Heat sensitivity after incision was measured using a modified method described by Hargreaves.[21,22] Mice were acclimated on a temperature-controlled glass platform (23.5 to 24.0°C) in a plastic enclosure and the beam of light was applied to the

area of hind paw incision. Withdrawal latency of the paw from the heat source was measured, and to prevent tissue damage, a 15-s cutoff was used. Three measurements were made per animal per test session, separated by several minutes. These experiments were done by a single experienced experimenter not blinded to treatment groups.

Assessment of Paw Edema

A laser sensor technique was used to determine the dorsal–ventral thickness of the hind paw, as previously described.[23] For laser measurements each mouse was briefly anesthetized with isoflurane and then held vertically so the hind paw rested on a table top below the laser. The paw was gently held flat on the table with a small metal rod applied to the top of the ankle joint. Using optical triangulation, a laser (4381 Precicura; Limab, Goteborg, Sweden) with a distance-measuring sensor (200-mm range, 0.01-mm resolution) was used to determine the distance to the table top and to the top of the hind paw, and the difference was used to calculate the dorsal–ventral paw thickness. Three measurements were made per paw, per animal.

Drug Administration

For some groups of mice, anakinra (Amgen, Thousand Oaks, CA), PMX-53 (Promix, Queensland, Australia), or saline vehicle was injected subcutaneously into the plantar skin of the hind paws of mice 2 h before incision and also daily 2 h ahead of behavioral testing. Preliminary experiments demonstrated this to be a point of maximal effect. For these injections mice were gently restrained. The injection volume was 15 μl administered through a 30-gauge needle, which raised a bleb similar to the length of the incisional wounds and approximately 1 mm of surrounding tissue.

Immunohistochemistry

The immunohistochemical analysis of mouse paw skin was done according to previously published methods.[9,10] Briefly, the primary and secondary antibodies used were IL-1β (H-153) rabbit polyclonal IgG, 1:50 (Santa Cruz Biotechnology, Santa Cruz, CA); fluorescein antirabbit IgG (H+L), 1:150 (Jackson ImmunoResearch Laboratories, West Grove, PA); rat antimouse neutrophil (allotypic marker) monoclonal antibody, 1:300 (AbD Serotec, Raleigh, NC); Texas red anti-rat IgG (H+L), 1:150 (Vector Lab Inc., Burlingame, CA); rat antimouse F4/80 antigen, 1:100 (AbD Serotec); fluorescein anti-rat IgG (H+L), 1:500 (Vector Lab Inc.); rabbit anti-β-endorphin, 1:100 (Peninsula Laboratories LLC, San Carlos, CA), and Texas red antirabbit IgG (H+L), 1:500 (Jackson ImmunoResearch Laboratories). Confocal laser-scanning microscopy was performed using Zeiss LSM 510 and LSM 510 META Laser Scanning Microscopes (Thornwood, NY). Control experiments included incubation of slices in primary and secondary antibody-free solutions, both of which led to low-intensity nonspecific staining patterns in preliminary experiments. After exposure with appropriate antibodies for neutrophils and macrophages, the number per high power field (x40) was counted by a blinded experimenter using SPOT Advanced software (SPOT, Sterling Heights, MI).

Cytokine (IL-1β) and C5a ELISA

An ovular patch of full-thickness skin providing 1 to 1.5 mm margins surrounding the hind paw incisions was collected rapidly after carbon dioxide asphyxiation of animals. These samples containing approximately 12 mg tissue per paw were placed immediately into ice-cold 0.9% NaCl containing a cocktail of protease inhibitors (Complete; Roche Applied Science, Indianapolis, IN). Samples were homogenized and centrifuged for 10 min at 12,000 x gravity at 4°C. An aliquot of the supernatant fractions was subjected to protein assay (DC Protein Assay; Bio-Rad Laboratories, Hercules, CA) and subsequently C5a and IL-1β protein levels were measured by a R&D Systems EIA kit (Minneapolis, MN), according to the manufacturer's protocol. The experimenter was blinded to the treatment groups.

Statistical Analysis

To compare time course and treatment effects for the behavior studies, a two-way ANOVA (time, treatment) with repeated measures for time was performed with Bonferroni correction for multiple comparisons. For data obtained from the peripheral blood and skin neutrophil determination experiments, a one-way ANOVA was performed with Bonferroni correction for multiple comparisons. All comparisons were run as two-tailed testing. All data are presented as means ± SEM, and differences were considered significant at $P < 0.05$ (Prism 4.0; GraphPad Software, San Diego, CA). No data were missing for any of the variables.

Results

Time Course of Neutrophil Depletion after Anti-Ly6G/Gr-1 Antibody Treatment

The results of preliminary experiments showed that two injections of anti-Gr-1 antibody were required to achieve substantial depletion of circulating neutrophils. In figure 1A, the time course of antibody depletion and recovery is presented. Depletion of more than 90% of circulating neutrophils was achieved by the time of incision, and persisted for at least the first 24 h. Circulating neutrophil counts remained depressed for at least 3 days.

Anti-Ly6G/Gr-1 Antibody Treatment Significantly Depletes Peri-Incisional Neutrophils

In order to assess the efficacy of the above two-dose depletion regimen in preventing neutrophil accumulation after incision, immunohistchemical analysis of skin samples from separate groups of mice for neutrophils was next carried out. This was done as the circulating neutrophil levels may not reflect the actual tissue content of these cells after local injury.

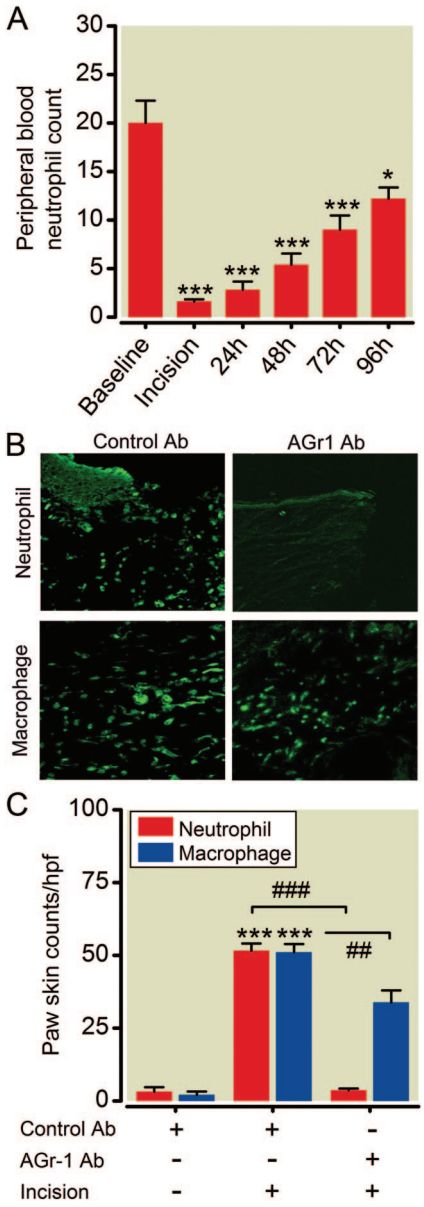

Fig. 1. Effects of Ly6G/Gr-1 antibody on circulating neutrophil and paw skin neutrophil and macrophage counts. (*A*) Timeline of peripheral blood neutrophil depletion following two doses of the antibody given 48 and 4 h before incision (n = 7). (*B*) Immunoflourescence staining for neutrophils (antimouse neutrophil monoclonal antibody) and macrophages (antimouse F4/80 antigen) with fluorescein anti-rat IgG (H+L) as secondary antibody, 24 h after incision on samples obtained from paw skin wounds. (*C*) Quantification of wound edge neutrophils and macrophages 24 h after surgical incision (n = 7). *$P < 0.05$, ***$P < 0.001$ difference from control antibody group, and ##$P < 0.01$, ###$P < 0.001$ difference from Ly6G/Gr-1 antibody group. Ab = antibody; AGr1 = anti-Gr-1.

Figures 1B and C show that 24 h after incision, there is a marked reduction of infiltrating skin neutrophils in the anti-Gr-1 antibody group compared with controls. The neutrophils were mostly seen as densely packed cells within the superficial part of the incision site. At the same time-point, the macrophage numbers were modestly decreased by the antibody treatment. The macrophages were mostly seen in the superficial dermal layers.

Effects of Anti-Ly6G/Gr-1 Antibody Treatment on Incision-induced Mechanical and Heat Hypersensitivity

As the migration of neutrophils into injured tissue has been implicated with both the production of pronociceptive mediators and the release of opioid peptides, we next determined if a reduction in these immune cells affected pain sensitivity in the incisional model. Neutrophil depletion did not change the magnitude or duration of mechanical hypersensitivity in incised mice compared with controls ($F_{1,50} = 0.43$, $P = 0.528$; fig. 2A). However, the neutrophil-depleted mice showed slightly increased heat hypersensitivity compared with controls 24 h after incision ($F_{1,72} = 5.84$, $P = 0.018$). No difference was observed in the preincisional heat sensitivity or 2 h after surgery, or at later time-points between the neutrophil-depleted and mice treated with control antibody (fig. 2B).

Anti-Ly6G/Gr-1 Antibody Treatment Significantly Reduces Paw Edema

In order to determine the contribution of neutrophils to a separate index of the inflammatory response in incised animals, we measured paw thickness at time-points up to 72 h in control and anti-Gr1-antibody-treated mice. There was a significant difference between the neutrophil-depleted animals and controls in the measure of paw edema at the 2 and 24 h time-points (fig. 2C).

Effects of Anti-Ly6G/Gr-1 Antibody Treatment on Skin C5a and IL-1β Levels

The effects of neutrophil depletion on proinflammatory mediator IL-1β and complement fragment C5a was next determined. Peri-incisional levels of IL-1β increased sharply in skin after incision, but anti-Gr-1 mice displayed only approximately 50% of the levels achieved in control mice at 24 h after incision. At 72 h, control and neutrophil-depleted mice had the same level of cytokine elevation. On the other hand, levels of the pronociceptive complement fragment C5a were increased to the same extent in both control and neutrophil-depleted animals 24 h after incision, but were lower in the depleted animals 72 h after incision (fig. 3A–B). Thus levels of the two mediators were altered differentially after incision in the setting of neutrophil depletion.

As C5 is produced by organs such as the liver and only activated to C5a locally, whereas IL-1β is locally produced after trauma, we pursued complementary immunohistochemical experiments for IL-1β to further localize its source in the control and neutrophil-depleted mice. Figure 4 demonstrates that neutrophils and dermal and epidermal cells produce IL-1β 24 h after incisions in control animals, and

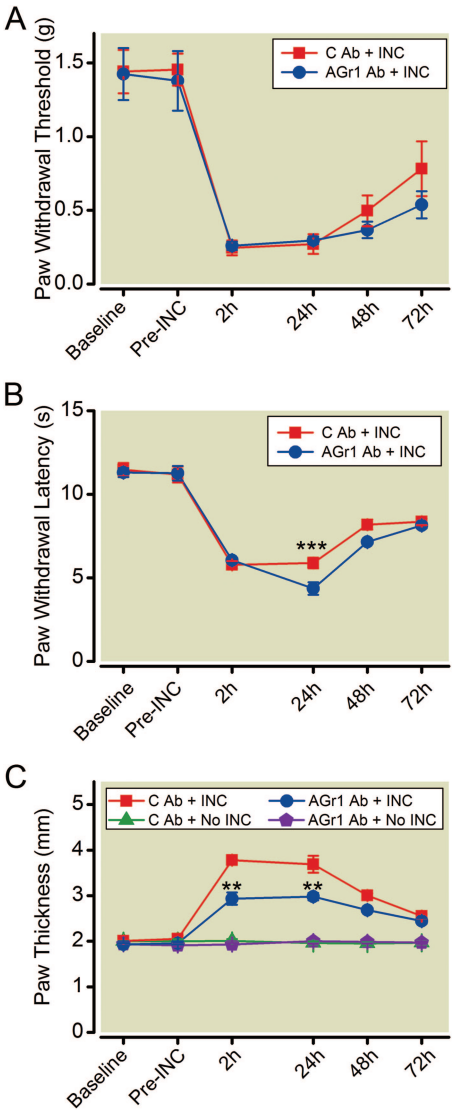

Fig. 2. Effects of Ly6G/Gr-1 antibody on pain and inflammation after hind paw incision. (*A*) Mechanical allodynia was measured in the neutrophil-depleted (AGr1 Ab) and control (C Ab) mice using calibrated von Frey filaments before and at different time-points after incision (n = 6 per group). (*B*) Paw withdrawal latencies to heat stimuli were measured in both antibody-treated groups using the Hargreaves method (n = 7 per group). (*C*) Measurement of paw edema using a laser assay in the antibody-treated mice (n = 7 per group). Mean ± SEM values of each group were analyzed by two-way ANOVA with *post hoc* Bonferroni correction comparing treatment groups at each time-point. $^{**}P < 0.01$, $^{***}P < 0.001$. Ab = antibody; AGr1 = anti-Gr-1; C = control; INC = incision.

that the dermal and epidermal sources remain prominent in neutrophil-depleted mice.

Effects of Anti-Ly6G/Gr-1 Antibody Treatment on Neutrophil-derived β-endorphin

Double immunostaining for β-endorphin and neutrophils in peri-incisional skin demonstrated many positive cells (fig. 5). In intact skin, β-endorphin positive cells were absent (figs. 5A, C). After anti-Gr-1-antibody treatment, there was an overall decrease in peri-incisional β-endorphin-positive neutrophils (figs. 4E, F) compared with control antibody group (figs. 5C, D).

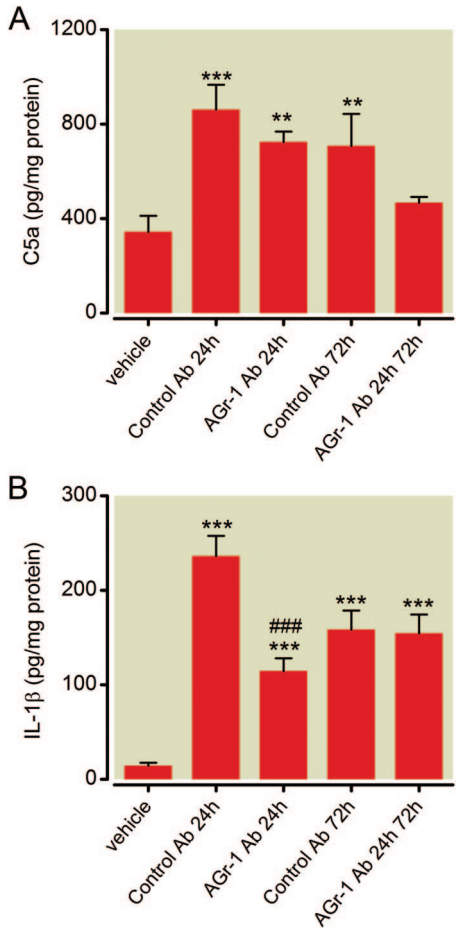

Fig. 3. Effects of hind paw incision on peripheral (*A*) C5a and (*B*) interleukin-1β levels. The levels of these mediators were measured in hind paw plantar skin at baseline and at the 2–72 h time-points after incision. The selected time-points were based on the behavior data presented in fig. 2. Different groups of mice were used for each time point (n = 6 per group). Data are presented as mean ± SEM and were analyzed by two-way ANOVA with *post hoc* Bonferroni correction. $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{###}P < 0.001$ difference from control antibody group. Ab = antibody; AGr1 = anti-Gr-1; IL = interleukin.

Effects of Treatment with IL-1R or C5a Antagonists on Nociceptive Hypersensitivity after Incision in AGr-1^{+}-depleted Mice

Lastly, we examined the contribution of local IL-1β and C5a signaling in skin after incision both under control and neutrophil-depleted conditions. The selective complement fragment C5a receptor antagonist PMX-53 (30 mcg/paw) reduced both mechanical and heat nociceptive sensitization to the same extent regardless of neutrophil depletion during the

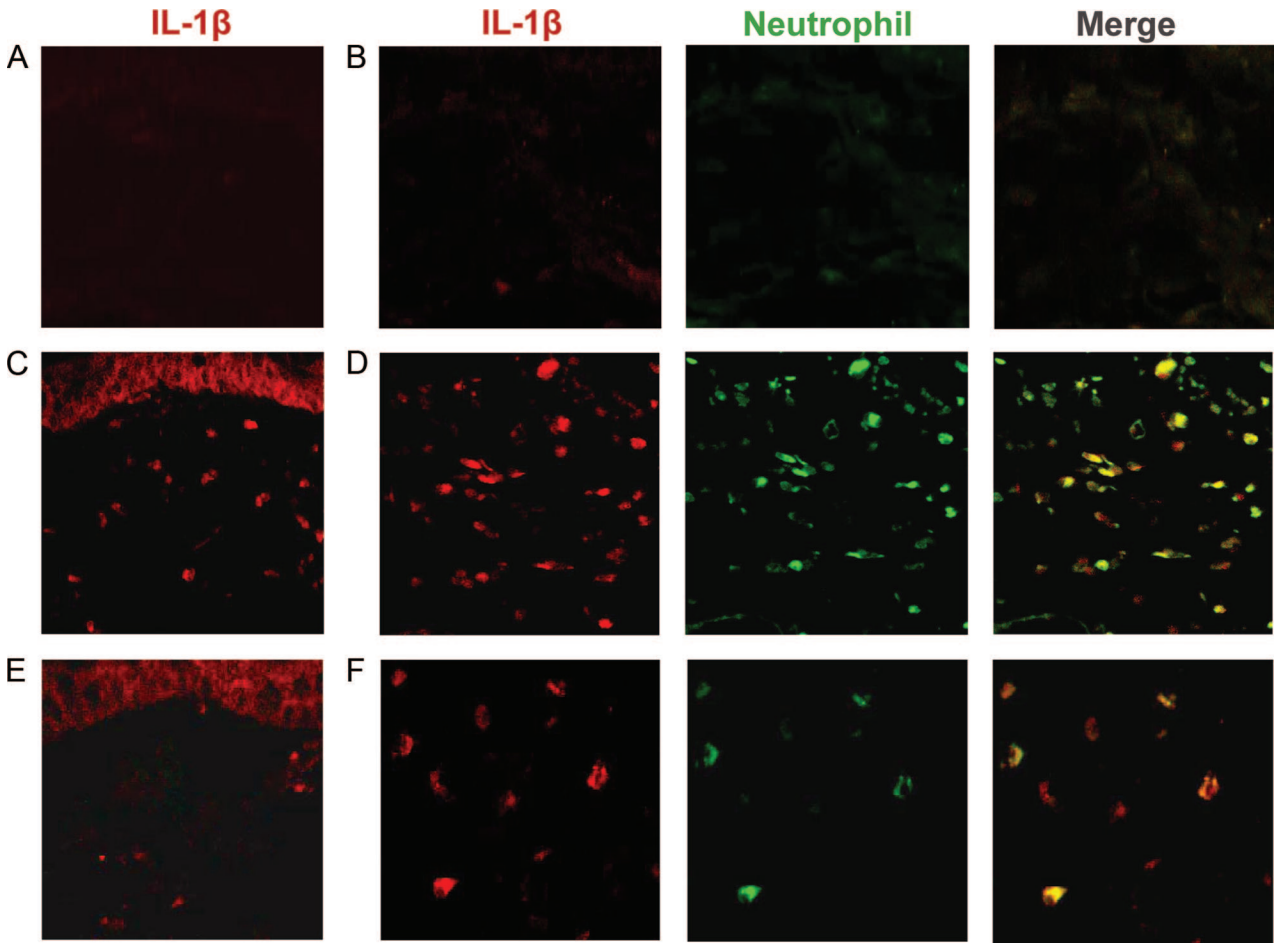

Fig. 4. Interleukin-1β (IL-1β) is produced by neutrophils and other cell types after hind paw incision. Immunostaining for IL-1β at 24 h after incision shows: (*A*) Control mice without incision have very low basal expression of the cytokine; (*B*) High-power image of the nonincised dermal layer; (*C*) Control antibody-treated mice after incision produce the cytokine in the epidermal and dermal layers; (*D*) High-power image of the incised dermal layer shows neutrophil abundance and colocalization with IL-1β; (*E*) Neutrophil-depleted mice have epidermal cytokine production; (*F*) High-power image of the incised dermal layer showing relatively rare IL-1β positive cell profiles. IL = interleukin.

72-h period following incision (figs. 6A, B). Similarly, there was no significant difference in the effect of treatment with the IL-1R antagonist anakinra (1.5 mg/paw) between the depleted and control groups with respect to its ability to reverse mechanical or heat nociceptive sensitization (figs. 6C, D). Thus, although IL-1β and C5a both seemed to support sensitization, neutrophil-derived IL-1β and C5a did not appear to contribute critically to peri-incisional nociceptive signaling.

Discussion

Investigations during the past several decades have identified many nociception-relevant components of the complex inflammatory soup generated after incision or tissue damage. Abundant evidence demonstrates that many of these mediators on their own or in combination can cause nociceptive sensitization. Some of these mediators, including IL-1β and C5a, two of the better examined nociceptive mediators in incisional pain models, can be generated by multiple cell types. Generally missing from these investigations have been experiments directed at understanding which sources of the mediators are most relevant to pain *versus* other wound processes, such as healing or fighting infection. Such information both aids in our understanding of incision-related nociceptive mechanisms and helps to define the cellular targets when designing analgesic strategies.

These studies used antibody-mediated neutrophil depletion to examine their function in the early period surrounding hind paw incision in mice. The antibody used here provided profound reductions in the levels of wound-area neutrophils in the early postincision period, causing small but significant changes in heat sensitization in the 24-h period after hind paw incision and no changes in mechanical nociceptive sensitization. Meanwhile, neutrophil depletion was effective in reducing postincisional edema. Looking more closely, the levels of IL-1β were reduced in the peri-incisional skin of neutrophil-depleted animals 24 h after incision compared with controls, whereas complement frag-

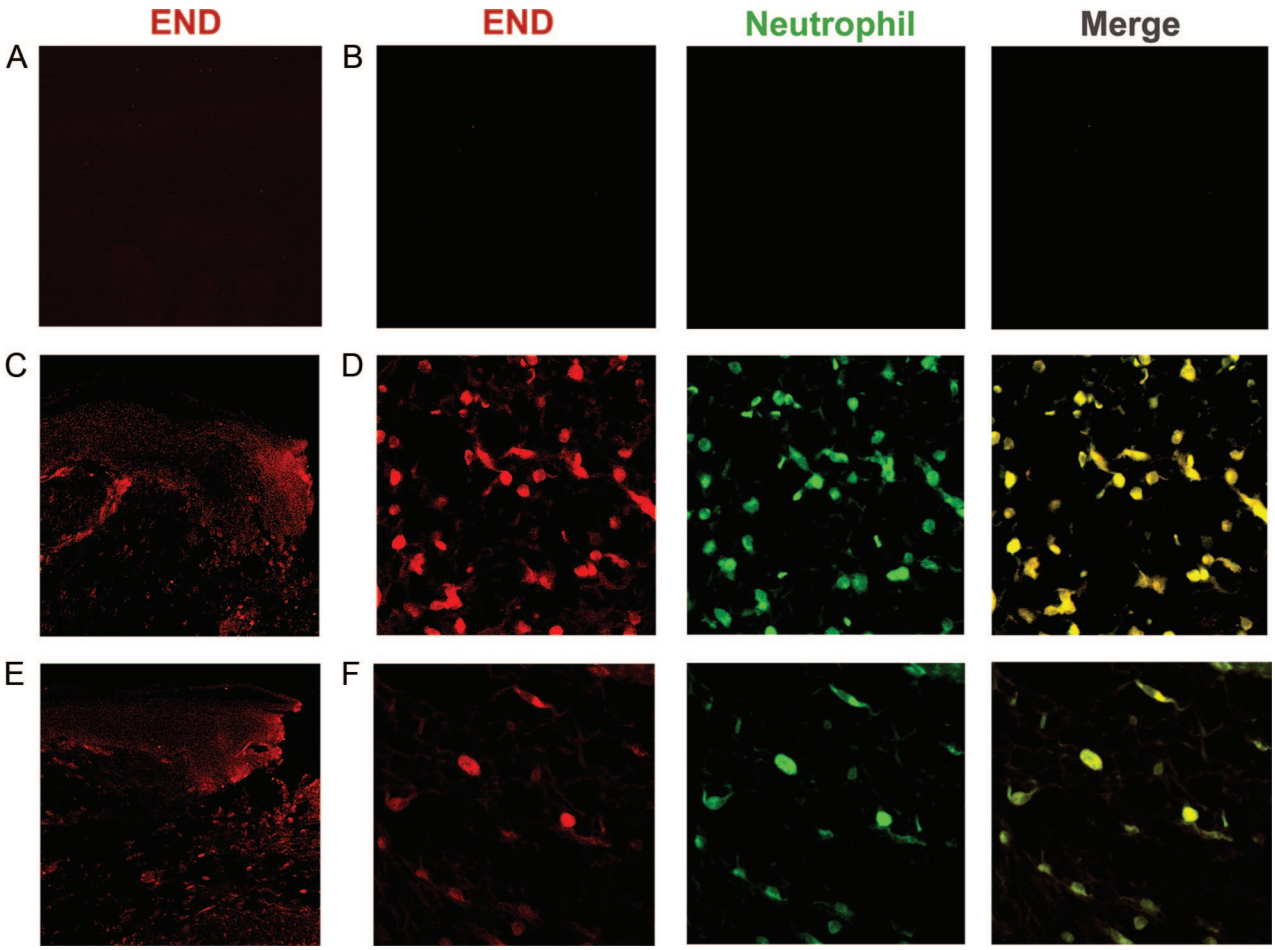

Fig. 5. β-endorphin (END) is produced by neutrophils and other cell types after hind paw incision. Immunostaining for END at 24 h after incision shows: (*A*) Control mice without incision have no expression of END; (*B*) High-power image of the nonincised dermal layer; (*C*) Control antibody-treated mice after incision produce END in the epidermal and dermal layers; (*D*) High-power image of the incised dermal layer shows neutrophil abundance and colocalization with END; (*E*) Neutrophil-depleted mice have epidermal END production; (*F*) High-power image of the incised dermal layer showing relatively fewer END/neutrophil positive cells. END = β-endorphin.

ment C5a was not reduced significantly in either group at this time-point. In both cases, they were still increased above baseline levels at a time-point when incisional sensitization was maximal. The local injection of the IL-1 receptor antagonist anakinra retained full effect in reducing mechanical allodynia even after neutrophil depletion, suggesting that the residual IL-1β production was sufficient to support nociceptive sensitization. It had been reported previously that the systemic administration of anakinra reduced nociceptive sensitization after hind paw incision.[8] Most IL-1β production in neutrophil-depleted mice appeared to be within the epidermal keratinocytes, shown previously to produce this cytokine after incision.[24]

Evidence suggests that in some systems the conversion of C5 to C5a is supported by a proteolytic reaction on the surface of neutrophils or by the action of neutrophil-derived myeloperoxidase products.[6,25,26] In fact, a positive feedback loop has been hypothesized in which C5a-mediated neutrophil recruitment leads to further augmentation of C5a production.[6] In our experiments, C5a levels were decreased moderately and the selective C5a receptor antagonist PMX-53 administered locally reduced nociceptive sensitization to the same extent in control and neutrophil-depleted mice. This agent has been shown to reduce nociceptive changes in mice after incision, perhaps by a mechanism involving a reduction in primary afferent nerve fiber activity.[11]

A strong circumstantial case can be made that infiltrating neutrophils support nociception in inflamed tissues. Activated neutrophils produce many mediators linked to nociception, including IL-1β and C5a, as well as reactive oxygen species, metalloprotinases, and other molecules.[5] The injection of exogenous IL-1β and C5a into rodent hind paw skin lowers nociceptive thresholds.[11,27] Moreover, neutrophils are recruited into paws after the injection of carrageenan model of inflammatory pain, and blocking the migration of neutrophils from the vasculature reduced mechanical hyperalgesia in these studies.[13] Conversely, complete Freund's adjuvant induced inflammation and hyperalgesia were unaf-

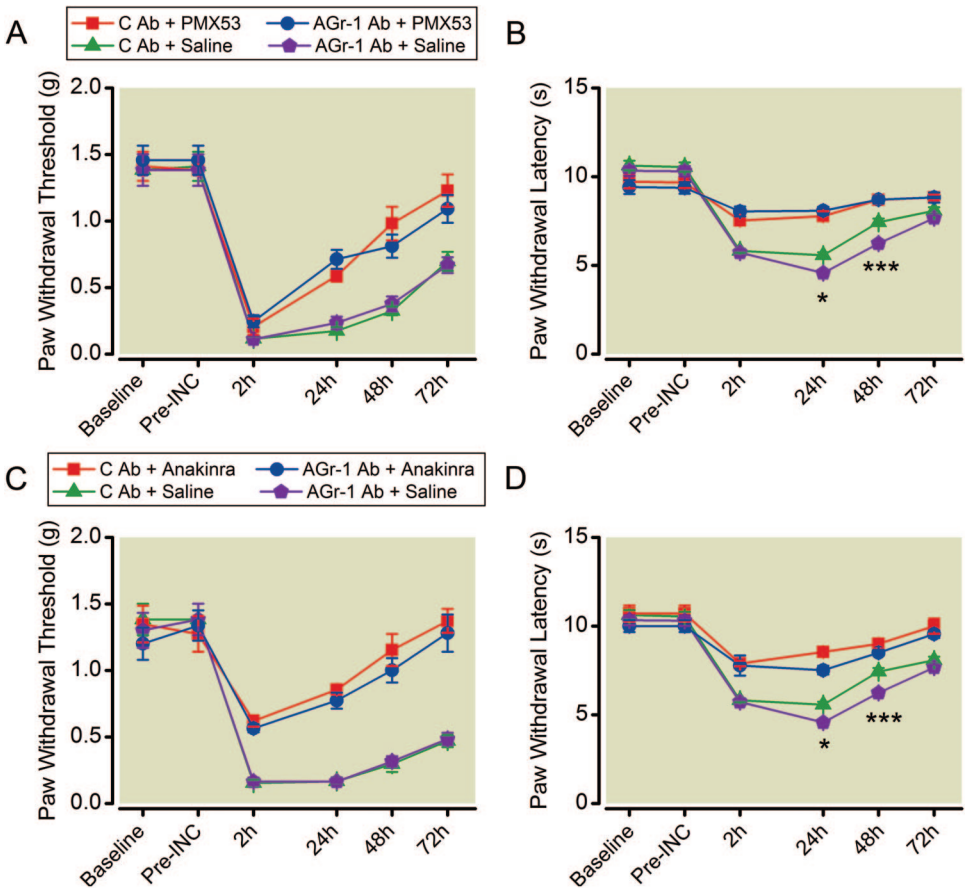

Fig. 6. Effect of inhibition of locally administered C5a and interleukin-1β receptor antagonists on incisional pain after neutrophil depletion. Mechanical allodynia and heat hyperalgesia were measured in the neutrophil-depleted (AGr1 Ab) and control (C Ab) hind paw-incised mice at different time points. Before behavioral measurements, mice received local PMX-53 (*A*, *B*), anakinra (*C*, *D*), or saline vehicle injection (n = 6 per group). Mean ± SEM values of each group were analyzed by two-way ANOVA with *post hoc* Bonferroni correction. $^{*}P < 0.05$, $^{***}P < 0.001$. Ab = antibody; AGr1 = anti-Gr-1; C = control; INC = incision.

fected by neutrophil depletion, despite reductions in IL-1β levels to a degree similar to that observed in our studies.[16] Importantly, the recruitment of activated neutrophils into hind paw tissues by virtue of local CXCL2/3 injection did not by itself cause hyperalgesia.[16] Moreover, neutrophils have been observed to infiltrate damaged nerves locally and at dorsal root ganglion level and have been linked to nociceptive sensitization in models of neuropathic pain.[12,28,29] Thus the apparent role of neutrophils in supporting nociception may depend both on the site and mechanism of inflammation and perhaps on the method of disruption of local neutrophil recruitment, *i.e.*, depletion *versus* inhibition of migration.

Our results indicate that depending on the site of production in wounds, mediators like IL-1β and C5a might have different effects; we were able to reduce inflammation as measured by a reduction in paw thickness, and we observed a similar degree of nociceptive sensitization in neutrophil-depleted animals. Previous studies show that IL-1β and C5a can increase endothelial leak and edema,[30,31] suggesting that a reduction in neutrophil-derived mediators could reduce edema. However, previous studies suggest that the richly innervated epidermal layer may be an important source of IL-1β after hind paw incision and in inflamed tissues.[24] Unclear at this time is the mechanism by which in the absence of infection keratinocytes are stimulated to produce mediators like IL-1β, although recent evidence suggests that neuropeptides and adenosine triphosphate, which are present at increased levels in injured tissue, may be able to stimulate the assembly and activation of IL-1β-producing inflammasomes.[32,33] Once produced and released, peripheral sensory nerves penetrating the epidermis may be exposed to high local concentrations of IL-1β and other mediators.[3] This model of mediator-neuron interaction accommodates both the observations that neutrophil depletion affects sensitization minimally, and the observation that locally administered anakinra retains its ability to reduce sensitization even after neutrophil-generated IL-1β is eliminated.

Conversely, a significant number of studies examining the properties of endogenous opioids produced by leukocytes have been provided.[34] Leukocytes, including neutrophils, produce proopiomelanocortin and proenkephalin under inflammatory conditions.[35–37] Also, the expression of opioid receptors on peripheral neurons is enhanced under inflam-

matory conditions.[38,39] Endogenous opioid peptides in sites of inflammation can be released spontaneously, though release is augmented by the presence of corticotropin-releasing factor or stress.[40–42] Importantly, in models of inflammation and metastatic bone cancer pain, the local injection of opioid receptor antagonists or antibodies enhance nociceptive sensitization by blocking local opioid signaling.[15,42,43] Also, the duration of inflammation is related to the strength of the role of endogenous opioids in limiting sensitization.[34] It is therefore possible that under the relatively acute conditions employed in our studies, analgesic mechanisms supported by inflammation and neutrophilic infiltration had not developed to the degree that their effects were more prominent. Indeed, our previous report shows that naloxone administration after incision causes a worsening of the heat hyperalgesia and mechanical allodynia, which are most prominent a few days after incision.[44] There is further support for the type of injury determining the duration and extent of neutrophil-mediated proalgesic *versus* analgesic mechanisms. In the setting of nerve injury, neutrophils steadily and continuously infiltrate endoneurium for 7 or 8 days, whereas they peak in 24 h after skin incision and decline rapidly to reach baseline levels in 3 days.[12,45] Neutrophil depletion attenuated nerve injury-induced hyperalgesia by potentially decreasing mediators responsible for induction of pain. However, local application of corticotropin-releasing factor to the site of nerve injury produced analgesia mediated by opioid-containing leukocytes.[40] Therefore, it seems that the neutrophils and opioid peptides produced by them are participating in an intensely localized inflammatory response. However, skin inflammatory response appears to be more diffused and involve many nonimmune cells. Keratinocytes and fibroblasts can produce and release pro-opiomelanocortin products, providing a potential nonimmunological origin for some portion of the peripheral opioids limiting sensitization after tissue injury.[46,47] Other immune sources of endogenous opioids, like memory T lymphocytes, which contain endorphin and migrate preferentially to inflamed tissues, could also contribute to limiting sensitization.[48]

It needs to be acknowledged that the present study has the following limitations. Although depleting neutrophils using antibodies to surface antigens has been the preferred technique for causing depletion with profound effects seen in these and other experiments, the technique is not entirely selective. The monoclonal antibody RB6-8C5 against surface antigen granulocyte receptor (Gr-1) has been widely used, but may result in depletion of select subpopulations of monocytes and macrophages that participate in the inflammatory process.[49,50] The alternative 1A8 antibody ostensibly has a better selectivity in depleting circulating neutrophils, but possesses a similar profile to RB6-8C5 in terms of depleting the wound macrophages.[51] Here we observed a modest decrease in wound area macrophage numbers, which could be attributed to the RB6-8C5 antibody as well as to the decrease in neutrophil-mediated chemotactic signals. In addition, the present study has the limitation of having the behavior assessments being done by the experimenter not blinded to treatment groups, as experimenter bias could affect the pain tests' results.[52]

Inflammatory mediators derive from multiple cell types in incisional wounds. Not all sources necessarily make the same contribution to nociceptive sensitization. Moreover, it should be kept in mind that any conclusions drawn concerning nociception may not apply to the source of mediators critical for wound healing or fighting of infection, which may rely more heavily on mediators derived from infiltrating immune cells. Likewise, our experiments were directed primarily at nociception deriving from skin and superficial tissues rather than deeper structures such as muscle and fascia, which may play a prominent role in pain-related behaviors observed in animals and pain scores reported in humans after incision.[53,54] Nevertheless, therapies directed at controlling the inflammatory activation of the epidermis may show efficacy in reducing pain from surgical incisions.

The authors thank Deyong Liang, Ph.D. (Anesthesiology Service, Veterans Administration Palo Alto Health Care System, Palo Alto, California, and Department of Anesthesia, Stanford University, Palo Alto, California), for valuable discussions throughout the preparation of the manuscript.

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