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ORIGINAL ARTICLE

Promotion of acute-phase skin wound healing by *Pseudomonas aeruginosa* C_4-HSL

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Key words
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Abstract

A *Pseudomonas aeruginosa* quorum-sensing system, which produces *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C_{12}-HSL) and *N*-butanoyl-L-homoserine lactone (C_4-HSL), regulates the virulence factors. In our previous study, 3-oxo-C_{12}-HSL, encoded by *lasI* gene, was shown to promote wound healing. However, the effect of C_4-HSL, encoded by *rhlI* gene, remains to be elucidated. We addressed the effect of C_4-HSL on wounds in *P. aeruginosa* infection. Wounds were created on the backs of Sprague–Dawley SD rats, and *P. aeruginosa* PAO1 (PAO1) or its *rhlI* deletion mutant (Δ*rhlI*) or *lasI* deletion mutant (Δ*lasI*) was inoculated onto the wound. Rats were injected intraperitoneally with anti-C_4-HSL antiserum or treated with C_4-HSL at the wound surface. PAO1 inoculation led to significant acceleration of wound healing, which was associated with neutrophil infiltration and TNF-α synthesis. These responses were reversed, except for TNF-α production, when Δ*rhlI* was inoculated instead of PAO1 or when rats were co-treated with PAO1 and anti-C_4-HSL antiserum. In contrast, the healing process and neutrophil infiltration, but not TNF-α synthesis, were accelerated when C_4-HSL was administered in the absence of PAO1. This acceleration was not affected by anti-TNF-α antibody. These results suggest that C_4-HSL may be involved in the acceleration of acute wound healing in *P. aeruginosa* infection by modifying the neutrophilic inflammation.

Introduction

The wound healing process in skin consists of three stages, including the activation of inflammatory pathways leading to infiltration of leukocytes into the wound site, tissue degradation and tissue formation (1). Neutrophils quickly infiltrate the wound tissues, which are affected by interleukin (IL)-1β, tumour necrosis factor (TNF)-α, interferon (IFN)-γ and certain pathogen-associated molecular patterns (PAMPS), including bacterial components from *Pseudomonas*

Key Messages

- quorum sensing molecules, *N*-3-oxododecanoyl homoserine lactone (3-oxo-C_{12}-HSL) and *N*-butanoyl-L-homoserine lactone (C_4-HSL), secreted from *Pseudomonas aeruginosa* trigger the initiation of inflammatory responses
- it is not fully understood how C_4-HSL affects the wound healing and the host immune responses
- current study shows C_4-HSL may be involved in acceleration of the acute wound healing process in *P. aeruginosa* infection by modifying the neutrophilic inflammation

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aeruginosa (1,2). Although neutrophils are reported to inhibit some aspects of the repair process (3), they are intimately involved in natural wound healing, especially bacterial-contaminated wounds (4). Previously, we demonstrated that *P. aeruginosa* inoculation promoted skin wound healing by inducing neutrophil infiltration, in which TNF-α played a critical role (4). Macrophages were recruited after the infiltration of neutrophils and they supported the ongoing repair process, including the promotion and resolution of inflammation upon exposure to proinflammatory cytokines, lipopolysaccharides (LPS) or other microbial products (5).

P. aeruginosa is often isolated from chronic wounds as a primary causative agent of infection and the presence of this bacterium appears to be involved in the delay of wound repair (6). Acute infection with this bacterium leads to the production of proinflammatory cytokines and chemokines, such as TNF-α, IL-1α, IL-1β and IL-8, and a massive influx of neutrophils into the wound tissues (6). *P. aeruginosa* is rarely isolated from acute wounds, and thus, this bacterium appears to have different effects on the healing process between acute and chronic wounds (1,6). Recent studies from our and other laboratories have demonstrated that quorum-sensing (QS) molecules secreted from *P. aeruginosa* trigger the initiation of inflammatory responses (2,7–9). Two different QS molecules, *N*-3-oxododecanoyl homoserine lactone (3-oxo-C_{12}-HSL) and butanoyl-homoserine lactone (C_4-HSL), are encoded by the *las* and *rhl* genes, respectively. They are generated when the growth of this bacterium reaches a threshold level (10). A hierarchical relationship exists between the Las and Rhl systems, where the Las system controls the Rhl system, as shown by direct upregulation of *rhlR* transcription by the 3-oxo-C_{12}-HSL–LasR, receptor of its HSL, complex (11).

3-Oxo-C_{12}-HSL plays a critical role in regulating bacterial virulence (12) and modulating several mammalian cells including neutrophils (8,13) and macrophages (13). In addition, we demonstrated that 3-oxo-C_{12}-HSL was involved in the wound healing process by inducing TNF-α production (2). In contrast, although C_4-HSL is also involved in the regulation of bacterial virulence (14,15), it is not fully understood how this QS molecule affects the host immune responses. In our previous investigation, we determined that inoculation with a *P. aeruginosa* PAO1 strain promotes only the acute phase of wound healing by inducing a 'moderate' and 'evanescent' inflammatory response (4).

In the present study, we used a rat model with deep partial-thickness wounds to examine the involvement of C_4-HSL in acute skin wounds that healed following infection with *P. aeruginosa*. We also addressed the role of this QS molecule in leukocyte accumulation and TNF-α synthesis. We demonstrated that C_4-HSL helped to promote the wound healing process, as shown by increased wound re-epithelialisation, epidermal cell proliferation and neo-vascularisation and by accelerated inflammatory responses, such as the infiltration of neutrophils and macrophages, but not TNF-α synthesis.

Materials and methods

Animals

Male Sprague–Dawley (SD) rats (200–300 g, 7–10 weeks of age) were obtained from Clea Japan, Inc. (Tokyo, Japan) and maintained under a 12-hour light/12-hour dark cycle. Food and water were made available ad libitum. All handling of the animals was performed under anaesthesia that was induced by pentobarbital (40 mg/kg; NEMBTAL, Dainippon Sumitomo Pharma, Osaka, Japan). At the end of the experiments, the animals were euthanised by an overdose of pentobarbital. All experimental protocols described in the present study were approved by the Ethics Review Committee for Animal Experimentation of Tohoku University.

Bacteria

P. aeruginosa strain PAO1 and its *rhlI*-deletion mutant strain KG7002 (Δ*rhlI*) and *lasI*-deletion mutant strain KG7001 (Δ*lasI*) (16) were stored in a Microbank storage system (Pro-Lab Diagnostics Inc., Richmond Hill, ON, Canada) at −80°C. These bacteria were inoculated onto a sheep blood agar plate and incubated at 37°C overnight, and colonies were cultured in Brain Heart Infusion (BHI) broth (Eiken Chemical Co., Ltd, Tokyo, Japan) at 37°C for 24 hours and washed three times in normal saline. After the final suspension was mixed, bacterial counts were performed by measuring absorbance at 600 nm. In each experiment, a quantification culture was performed to confirm the inoculation dose.

Wound creation and tissue collection

Dorsal hair of the SD rats was shaved to fully expose the skin, which was then disinfected using 70% ethanol. Deep partial-thickness dermal wounds preserving the cutaneous muscle were created on the backs of the rats using a 6-mm diameter biopsy punch (Biopsy Punch, Kai Industries Co., Ltd, Gifu, Japan) and scissors under sterile conditions. Ten deep, partial-thickness wounds were made on the back of each rat. Wounds were separated from each other by at least 5 mm. A suspension (11 μl) of *P. aeruginosa* PAO1, Δ*rhlI* or Δ*lasI* was applied to the base of the wounds at 3·5 to $5{\cdot}5 \times 10^7$ cfu/wound in an individual rat. *P. aeruginosa* solution was prepared in every experiment to keep their viability, and therefore, it is not possible to prepare them at exactly the same inoculum dose. Uninoculated wounds in other rats were used as controls. Wounds were covered with a polyurethane foam dressing (Tegaderm Transparent Dressing, 3M Health Care, St. Paul, MN) and the whole trunk of the rats was covered with an elastic adhesive bandage (Hilate, Iwatsuki, Tokyo, Japan) to protect the wound from contamination and mechanical irritation. The day when the wounds were made was designated as day 0. Tissues were harvested from the wound sites at days one and three post-wounding by excising the area with an 8-mm diameter biopsy punch.

Reagents, antibodies and antiserum

C_4-HSL was purchased from Santa Cruz Biotechnology (Dallas, TX). 3-Oxo-C_{12}-HSL was synthetically produced by Wako Pure Chemical Industries (Osaka, Japan), dissolved in dimethyl sulfoxide (DMSO) at 3 mg/ml and then diluted with PBS to a final dose of 0·1% DMSO for in vivo use. Wounds were created using the above-mentioned methods, and just after

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wounding, a 10 µl suspension of 10 µM C_4-HSL, 3-oxo-C_{12}-HSL or control DMSO was applied to the base of the wounds. Anti-TNF-α monoclonal antibody (mAb) was purified from the culture supernatants of hybridoma cells (clone MP6-XT2.2-11) using a protein G column kit (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). To neutralise the TNF-α biological activity, rats were injected intraperitoneally with 1 mg of a mAb against this cytokine one day before wound creation. Rat IgG (ICN Pharmaceuticals, Inc., Aurora, OH) was used as the control Ab. To neutralise the biological activity of C_4-HSL or 3-oxo-C_{12}-HSL, rabbit antiserum against each compound, prepared as previously described (17), was injected intraperitoneally at a dose of 3 ml/kg, 18 hours before wound creation. Non-immune rabbit serum was used as a control.

Histology and immunohistochemistry

The wound tissues were collected using an 8-mm diameter biopsy punch (Biopsy Punch, Kai Industries Co., Ltd, Gifu, Japan) and dissected in the caudocranial direction at the centre of the wound. The tissues were fixed with a 4% paraformaldehyde-phosphate buffer solution and embedded in paraffin. Sections 3 µm thick were harvested from the central portion of each wound and stained with haematoxylin–eosin (HE), according to the standard method. Epithelial migration and the extent of re-epithelialisation were measured in these HE-stained sections by measuring the distance from the normal wound margin to the edge of the migrating epithelium at the centre of the wound. The re-epithelialisation index was determined based on the percentage of new epithelium present in the total wound ($n = 6$ per group). For immunohistochemistry, sections were treated with anti-PCNA antibody (1:400 dilution; Dako, Tokyo, Japan) or anti-CD31 antibody (1:100 dilution; Santa Cruz Biotechnology, Dallas, TX) after endogenous peroxidase and non-specific binding were blocked. They were then incubated with peroxidase-conjugated secondary antibodies (simple stain rat MAX-P®, Nichirei Co., Tokyo, Japan). To analyse keratinocyte proliferative activity in the neo-epidermis, the incidence of PCNA-positive basal nuclei in 500 cells between the wound margin and the extremity of the neo-epidermis was determined. The number of PCNA-positive basal cells is presented as a percentage of these 500 cells. The vascular density in the granulation tissue in five random fields (each 0·2 mm^2) was determined by counting the number of CD31-positive vessels. All analyses were performed under blinded conditions. Control sections were treated with isotype-matched irrelevant IgG in place of the first antibodies.

Preparation of leukocytes in the wound tissues

One group of rats was sacrificed 24 hours after wounding. Tissue samples from the wounds were collected from three rats in each group, as described below. The area was excised using a biopsy punch (8 mm in diameter) and teased apart using a stainless-steel mesh in RPMI1640 medium (Nipro, Osaka, Japan) supplemented with 10 mM HEPES, 10% foetal calf serum (FCS) (BioWest, Nuaillé, France), 1 mg/ml collagenase and 1 mg/ml hyaluronidase (Sigma-Aldrich, St. Louis, MO). The pellets were washed, then suspended in 4 ml of 40% Percoll solution (Pfizer, Inc., Täby, Sweden), added to 80% Percoll solution and centrifuged at 579 × g for 20 min at 20 °C. Pellets were washed three times with RPMI1640 supplemented with 1% FCS. Approximately 1×10^5 cells were applied onto a glass slide at 40 × g for 3 min using a Cytofuge-2 centrifuge (Statspin Inc., Norwood, MA) and stained using the Diff-Quik technique. The total number of neutrophils, lymphocytes and macrophages was estimated by multiplying the total leukocyte number by the proportion of each fraction in 500 cells.

RNA extraction and quantitative real-time RT-PCR

Total cellular RNA was extracted from the wound tissues using ISOGEN (Wako Pure Chemical, Osaka, Japan) Japan), and first-strand cDNA was synthesised using PrimeScript first-strand cDNA synthesis kit (TaKaRa Bio Inc., Otsu, Japan), according to the manufacturer's instructions. Quantitative real-time PCR was performed in a volume of 20 µl using gene-specific primers and FastStart essential DNA green master mix (Roche Applied Science, Branford, CT) in a LightCycler nanosystem (Roche Applied Science).

The primer sequences used for amplification are shown in Table 1. Reaction efficiency with each primer set was determined using standard amplifications. Target gene expression levels and that of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene as a reference were calculated for each sample using the reaction efficiency. We confirmed that expression of GAPDH was not affected by *P. aeruginosa* inoculation. The results were analysed using a relative quantification procedure and are presented as expressions relative to GAPDH expression.

Measurement of cytokine concentrations

Wound tissues were collected from three rats in each group and homogenised with RPMI1640 medium supplemented with 10 mM HEPES, 10% FCS, 1 mg/ml collagenase and 1 mg/ml hyaluronidase, and the concentration of TNF-α in the supernatants was measured using an enzyme-linked immunosorbent assay (ELISA) kit (Endogen, Cambridge, MA). The results are expressed as the average value present in one wound. The detection limit was 5·1 pg/ml.

Statistical analysis

Data analysis was performed using a statistical software package (SPSS 13.0 J for Windows, SPSS Japan, Tokyo, Japan). Data are expressed as the mean ± standard deviation (SD). Statistical analysis between groups was performed using the Mann–Whitney *U*-test, Student's *t*-test or one-way ANOVA. A P value $<0{\cdot}05$ was considered to indicate statistical significance.

Results

Effect of *P. aeruginosa* PAO1 or Δ*rhlI* inoculation on wound healing, accumulation of neutrophils and TNF-α expression

Previously, we reported that inoculation of *P. aeruginosa* PAO1 promoted the healing process at day 3, but not at other time

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Table 1 Primer sequences for real-time PCR

Gene product	Forward primer	Reverse primer
TNF-α	CCCAGACCCTCACACTCAGAT	TTGTCCCTTGAAGAGAACCTG
IL-1β	CACCTTCTTTTCCTTCATCTTTG	GTCGTTGCTTGTCTCTCCTTGTA
IL-6	AGCCCACCAGGAACGAAAGTCA	TGGCTGGAAGTCTCTTGCGGA
IL-17A	TTCCATCCATGTGCCTGATGCTG	TCGGCGTTTGGACACACTGAAC
CXCL-1	TGGAGAAAGAAGATAGATTGC	TTCTTCCCGCTCAACACCTTC
CXCL-2	CCTGGAAAGGAAGAACATGGG	ACCTCCCAACTACATAAGTAA
CXCL-3	TGGTCAAGAAGTTTGTCTCAACCC	CACAGGGAGGGGCTCTTCAGTA
GAPDH	TAGAGACAGCCGCATCTTCTTGTG	GCCAAATCCGTTCACACCGAC

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL-1β, interleukin (IL)-1β; TNF-α, tumour necrosis factor.

points, after wound creation (4). In the present study, we asked how deletion of either *rhlI* or *lasI* gene or both in *P. aeruginosa* influenced the wound healing effect caused by this bacterium on day 3. We created deep partial-thickness wounds, preserving the cutaneous muscle, in the backs of rats and histologically evaluated the process of re-epithelialisation. As shown in Figure 1A, re-epithelialisation was promoted on day 3 after wounding, as shown by the leading edges (arrows) of epidermis, which tended to extend farther in the PAO1-inoculated group than in the uninoculated group; these findings were not observed when wounds were inoculated with Δ*rhlI or* Δ*lasI* instead of PAO1. To quantitatively evaluate re-epithelialisation, the distance between the leading edges of the epidermis on either side of the wound was measured in the histological sections at day 3 after wounding, and the re-epithelialisation extent was calculated as the ratio of the distance between the leading edges to the distance between the wounded margins (arrowheads). As shown in Figure 1B, the re-epithelialisation extent was significantly greater in the PAO1 group than in the Δ*rhlI*, Δ*lasI* and uninoculated groups on day 3. As alternate indicators of wound healing, we evaluated the expression levels of proliferating cell nuclear antigen (PCNA) in the epidermis and CD31 in the dermal layer, which indicated epidermal cell proliferation and neo-vascularisation, respectively. As shown in Figure 1C and D, the expression levels of both indicators were significantly greater in the PAO1 group than in the Δ*rhlI*, Δ*lasI* and uninoculated groups. In addition, we confirmed that there were no differences in the amounts of bacteria in the wounds on day 3 among the groups and no other bacterial strain was detected in the wounded tissue after inoculation with *P. aeruginosa* PAO1, *rhlI* deletion or *lasI* deletion. Thus, these data demonstrate that *P. aeruginosa* PAO1 is involved in the promotion of the acute phase of wound healing.

Neutrophil accumulation at the wound sites after *P. aeruginosa* PAO1 or Δ*rhlI* inoculation

In our previous study, local neutrophil infiltration at the wound site reached a peak level at 24 hours after *P. aeruginosa* inoculation (4), and *lasI* gene contributed to neutrophil infiltration (2). In the next series of experiments, we addressed the question of whether the *P. aeruginosa rhlI* gene affected neutrophil infiltration. To quantitatively evaluate the cellular inflammatory responses, leukocytes were isolated from the wound tissue homogenates, and the total number of leukocytes and the proportions of neutrophils, macrophages and lymphocytes were morphologically analysed at 24 hours after wounding. As shown in Figure 2A, the number of total leukocytes and neutrophils was significantly greater in the PAO1 group than in the Δ*rhlI*, Δ*lasI* and uninoculated groups. There was no significant difference in the numbers of macrophages and lymphocytes between the four groups.

Inflammatory cytokine and chemokine expression after inoculation with *P. aeruginosa* PAO1, Δ*rhlI* or Δ*lasI*

Inflammatory cytokines and chemokines play a central role in leukocyte accumulation in the wounded tissues. Therefore, we next examined the expression of TNF-α, IL-1β, IL-6, IL-17A, and CXCL-1, CXCL-2 and CXCL-3. As shown in Figure 2B, cytokine and chemokine mRNA expression was strongly induced at 12 hours post-wounding in the PAO1 group compared with that in the uninoculated group. The increase in TNF-α, IL-6, IL-17A and CXCL-1 mRNA expression was significantly attenuated when the Δ*lasI* strain was inoculated instead of the PAO1 strain. However, attenuation was not observed when the Δ*rhlI* strain was used. Similar results were obtained when TNF-α production in the wounded tissues was measured using ELISA (Figure S1, Supporting information). However, IL-1β, CXCL-2 and CXCL-3 mRNA expression was comparable among the PAO1, Δ*rhlI* and Δ*lasI* groups.

Contribution of C_4-HSL to the wound healing promoted by *P. aeruginosa* PAO1 inoculation

To define the role of C_4-HSL in the wound healing promoted by *P. aeruginosa* PAO1, we first examined the effects of anti-C_4-HSL antiserum on the wound healing process. As shown in Figure 3A–D, treatment with anti-C_4-HSL antiserum significantly reduced the re-epithelialisation, epidermal cell proliferation and neo-vascularisation almost to the levels in the uninoculated group on day 3 after wounding, which was similar to the results observed using anti-3-oxo-C12-HSL antiserum. Non-immune rabbit serum did not show these effects. In addition, we confirmed that there was no difference among the groups in the bacterial counts in the wounds on day 3.

To evaluate the inflammatory responses, leukocytes were isolated from the wound tissues on day 1, and the number of leukocytes and neutrophils was counted. As shown in Figure 3E, treatment with anti-C_4-HSL or anti-3-oxo-C_{12}-HSL antiserum significantly reduced the increase in the number

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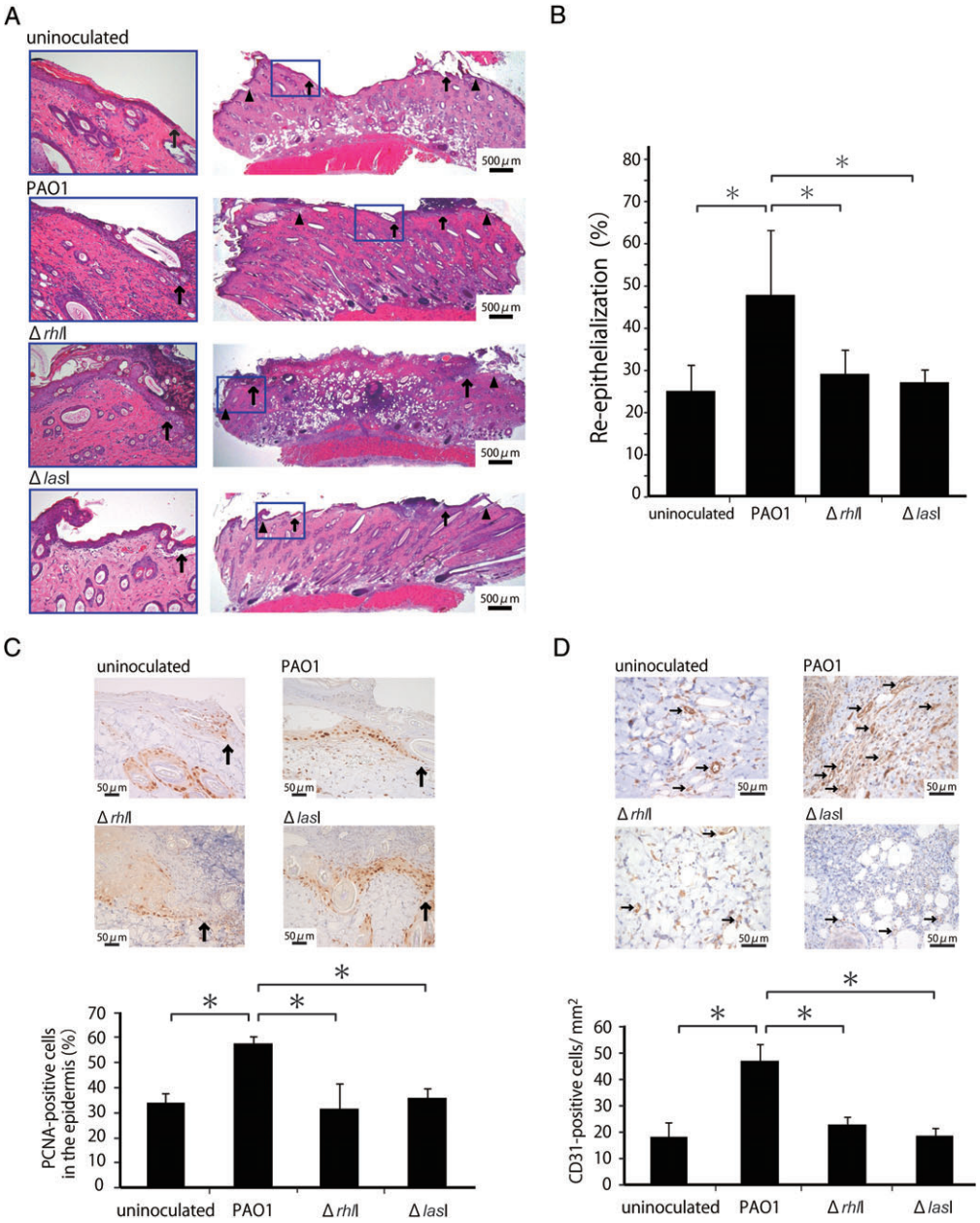

Figure 1 Effect of the *rhlI*-defect on skin wound healing promoted by *Pseudomonas aeruginosa*. Wounds were created and a suspension of PAO1 or Δ*rhl*I or Δ*las*I was inoculated into the wounds. Uninoculated wounds were created in other rats. (A) Representative histological views of skin wounds on day 3. Arrowheads and arrows indicate the original wound edges and re-epithelialised leading edges, respectively. (B) Re-epithelialisation extent was calculated on day 3 ($n=6$). (C) Cellular proliferation in the epidermis at the wound edges. Arrows indicate proliferating cell nuclear antigen (PCNA)-positive microvessels. The percentages of PCNA-positive cells among the 500 cells in the epidermis on day 3 ($n=3$). (D) Microvessel counts on day 3. Arrows indicate CD31-positive microvessels. The vascular density/mm^2 was determined by counting the positive vessels within six visual fields ($n=3$). Each column represents the mean ± standard deviation (SD). *$P<0{\cdot}05$.

of leukocytes and neutrophils induced by PAO1 inoculation, whereas a similar reduction was not observed by treatment with non-immune rabbit serum. In addition, as shown in Figure 3F, treatment with anti-3-oxo-C_{12}-HSL antiserum significantly reduced the increase in TNF-α synthesis in the wound skin homogenates caused by PAO1 inoculation, whereas such effects were not detected by treatment with anti-C_4-HSL antiserum or non-immune rabbit serum. These results suggested that C_4-HSL and 3-oxo-C_{12}-HSL may contribute to the wound healing processes through a different mechanism than that promoted by inoculation with *P. aeruginosa*.

C_4-HSL promotes wound healing

To further address the possibility that C_4-HSL may contribute to the wound healing processes promoted by

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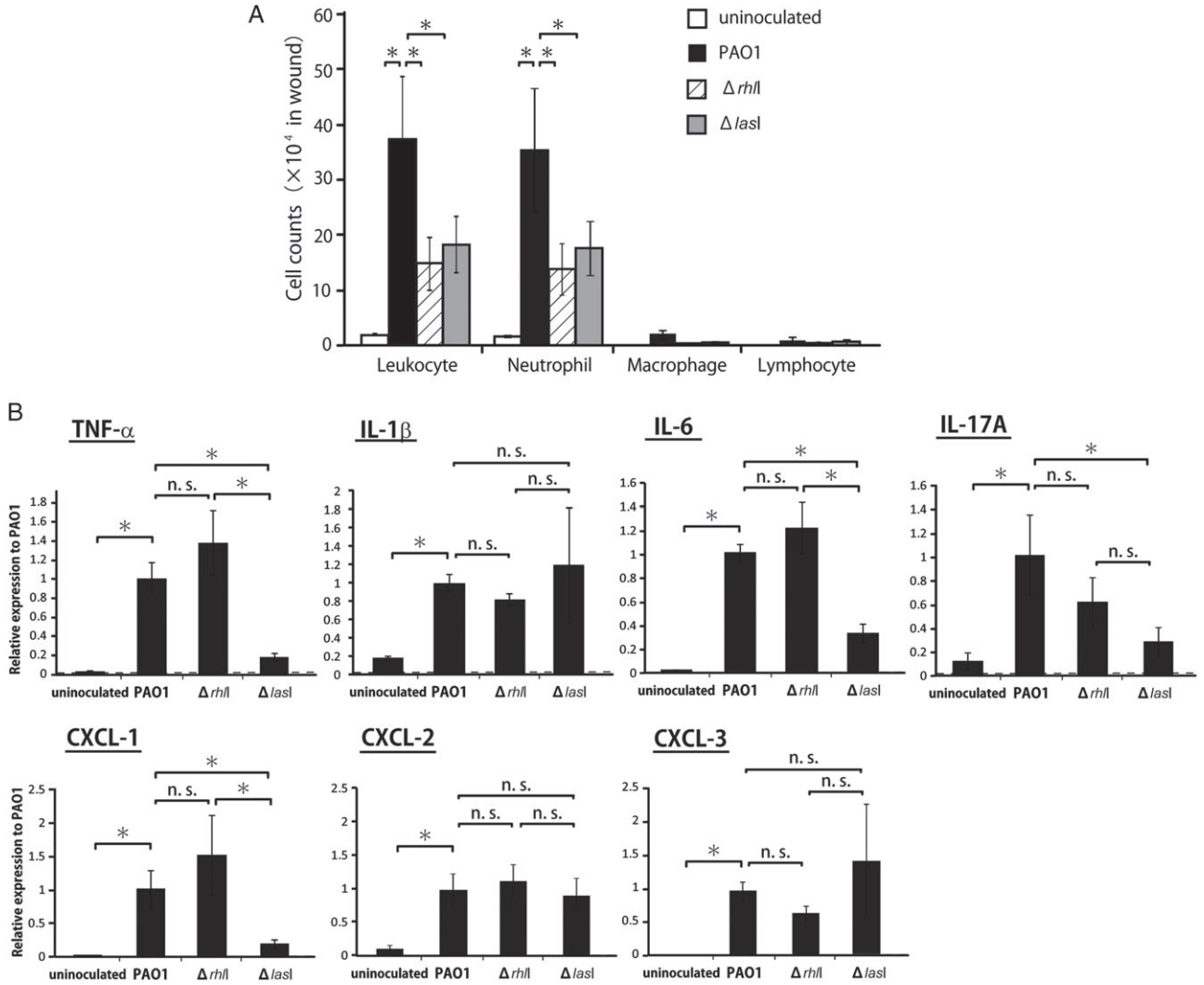

Figure 2 Cellular inflammatory response and expression of inflammatory cytokines after inoculation with *Pseudomonas aeruginosa* PAO1, Δ*rhlI* or Δ*lasI*. (A) Numbers of leukocytes, neutrophils, macrophages and lymphocytes in the wound tissues from rats inoculated with *P. aeruginosa* PAO1, Δ*rhl*I, Δ*lasI* or uninoculated rats were analysed at 24 hours following wound creation. (B) Tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-17A, CXCL-1, CXCL-2 and CXCL-3 expression in wounds was measured at 12 hours following wound creation. Each column represents the mean ± standard deviation (SD). *$P < 0{\cdot}05$; NS, not significant.

P. aeruginosa inoculation, we examined how treatment with 10 μM C_4-HSL affected the re-epithelialisation and inflammatory responses during wound healing. As shown in Figure 4A–D, the re-epithelialisation, epidermal cell proliferation and neo-vascularisation were significantly enhanced in the C_4-HSL-treated group compared to the control group, which was similar to the results obtained in the 3-oxo-C_{12}-HSL-treated group compared to the control group. We then examined the effects of C_4-HSL treatment on the inflammatory responses during the wound healing processes. As shown in Figure 4E, the number of leukocytes and neutrophils in the wound tissues on day 1 was significantly higher in the C_4-HSL-treated group than that in the control group. In contrast, local TNF-α synthesis in the wound skin homogenates was not affected by C_4-HSL administration (Figure 4F). To further address the role of TNF-α, we examined the effect of the neutralising anti-TNF-α mAb on the wound healing processes promoted by C_4-HSL. As shown in Figure 5A and B, treatment with anti-TNF-α mAb did not affect the re-epithelialisation promoted in rats receiving C_4-HSL, when compared with treatment with control IgG.

Discussion

In the present study, we demonstrated that C_4-HSL, a bacterial QS signalling molecule, was intimately involved in the acceleration of skin wound healing in *P. aeruginosa* infection, which was associated with the increased neutrophil infiltration. This is the first report that shows the involvement of C_4-HSL from *P. aeruginosa* in skin wound healing by modulating the inflammatory responses.

Previously, we demonstrated that *P. aeruginosa* infection accelerated wound repair in skin through secretion of 3-oxo-C_{12}-HSL that is encoded by the *lasI* gene, which

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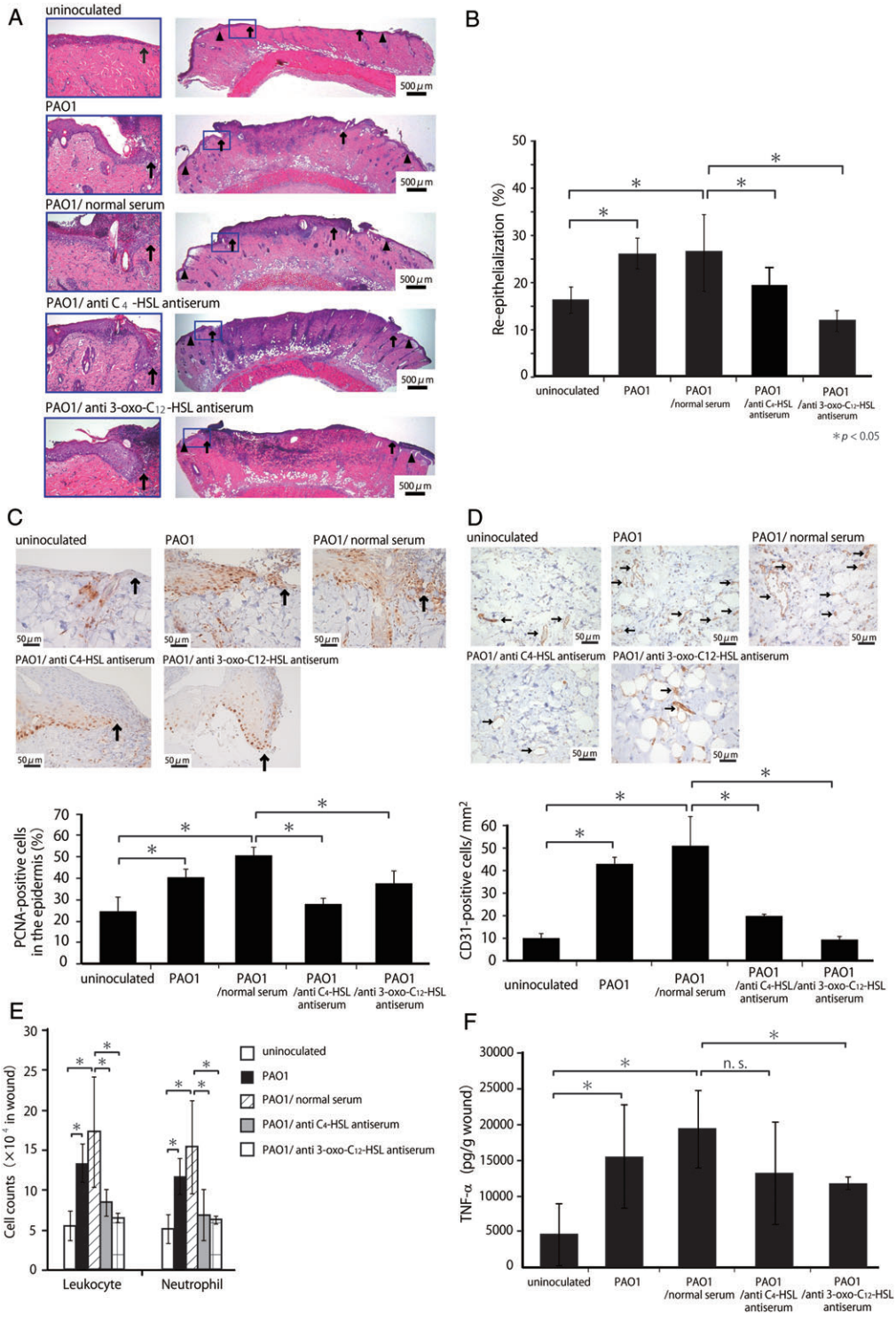

Figure 3 Effect of anti-C_4-HSL antiserum on wound healing promoted by *Pseudomonas aeruginosa* PAO1 inoculation. Rats were injected with anti-C_4-HSL antiserum, anti-3-oxo-C_{12}-HSL antiserum or preimmune serum. (A) Representative histological views on day 3. Arrowheads and arrows indicate the original wound edges and re-epithelialised leading edges, respectively. (B) Re-epithelialisation extent on day 3. (C) Cellular proliferation in the epidermis on day 3. Arrows indicate PCNA-positive microvessels. The percentages of PCNA-positive cells among the 500 cells in the epidermis were calculated ($n=3$). (D) Microvessel counts on day 3. Arrows indicate CD31-positive microvessels. The vascular density/mm^2 was determined by counting the numbers of positive vessels within six visual fields ($n=3$). (E) The number of leukocytes and neutrophils was analysed at 24 hours. (F) TNF-α concentrations were measured at 24 hours. Each column represents the mean ± standard deviation (SD). *$P < 0{\cdot}05$; NS, not significant.

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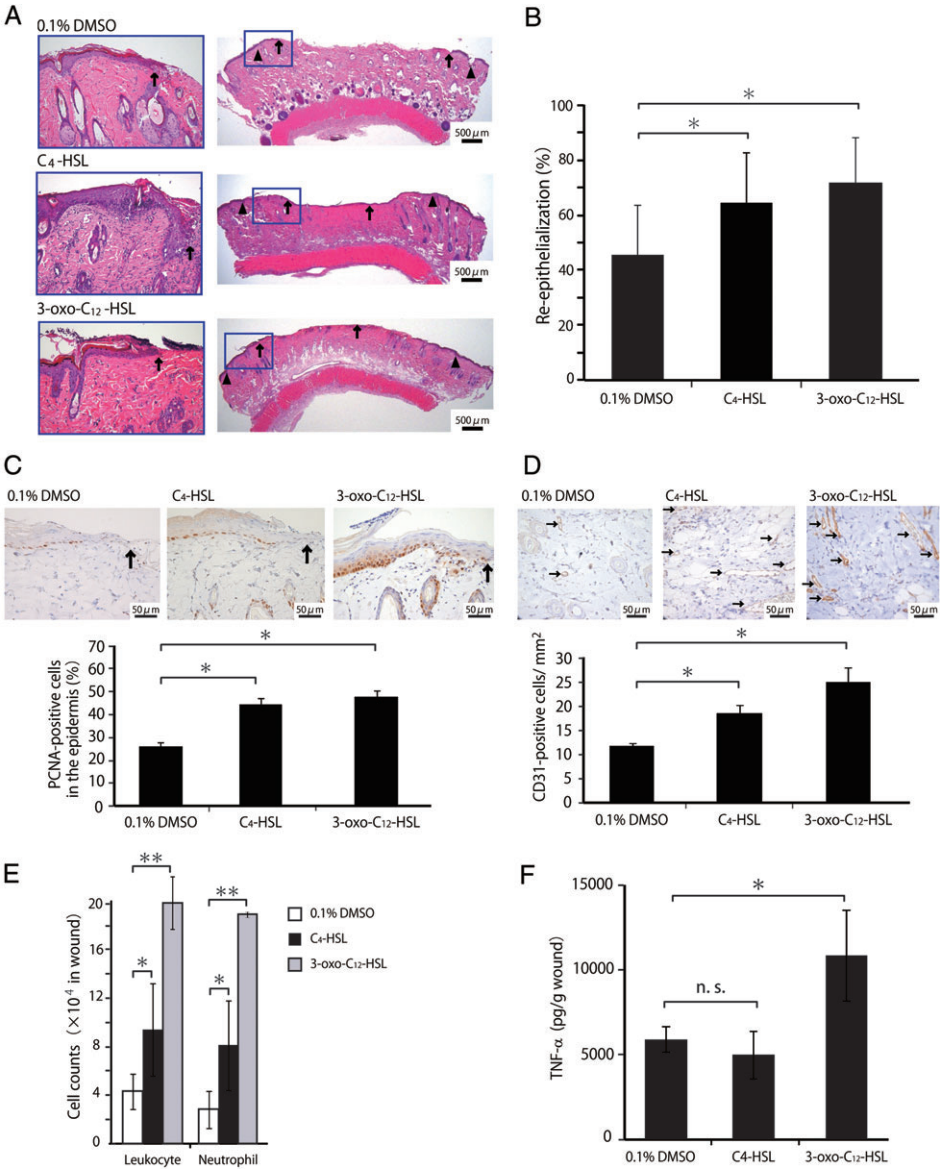

Figure 4 Effect of C_4-HSL administration on the wound healing. Wounded rats received C_4-HSL, 3-oxo-C_{12}-HSL or DMSO at the wound surface. (A) Representative histological views of wounds on day 3. Arrowheads and arrows indicate the original wound edges and re-epithelialised leading edges, respectively. (B) Re-epithelialisation extent on day 3. (C) Cellular proliferation in the epidermis on day 3. Arrows indicate PCNA-positive microvessels. The percentages of PCNA-positive cells among the 500 cells in the epidermis were calculated ($n = 3$). (D) Microvessel counts on day 3. Arrows indicate CD31-positive microvessels. The vascular density/mm^2 was determined by counting the numbers of positive vessels within six visual fields ($n = 3$). (E) The number of leukocytes, neutrophils was analysed at 24 hours (**$P < 0{\cdot}01$). (F) TNF-α concentrations in wounded tissue homogenates were measured at 24 hours. Each column represents the mean ± standard deviation (SD). *$P < 0{\cdot}05$; NS, not significant.

triggered neutrophil infiltration and TNF-α synthesis, and promoted wound healing processes such as re-epithelialisation and generation of new vessels (2). *P. aeruginosa* produces two distinct QS molecules, 3-oxo-C_{12}-HSL and C_4-HSL. In the current study, we focused on C_4-HSL, which is synthesised by an autoinducer synthase that is encoded by the *P. aeruginosa rhlI* gene. It has been reported that 3-oxo-C_{12}-HSL affects the host responses by stimulating a variety of cells (18), triggering the NF-κB translocation into the nucleus (9) and inducing apoptosis in these cells (13). However, few reports have shown the effect of C_4-HSL on host responses.

Many investigators have reported that the *rhlI* gene product is involved in the regulation of *P. aeruginosa* virulence factors, including rhamnolipid (19,20), elastase (15,20), hydrogen cyanide (15) and pyocyanin (19,20). Consistent with these findings, an *rhlI*-deficient mutant strain of *P. aeruginosa* was reported to show lower virulence, as indicated by the reduced mortality in a burn injury model (21), and the lower incidence of bacteremia and reduced mortality in a mouse model of pneumonia (22). In the current study, *P. aeruginosa* inoculation promoted neutrophil accumulation and accelerated repair of skin wounds. However, these effects were abolished when

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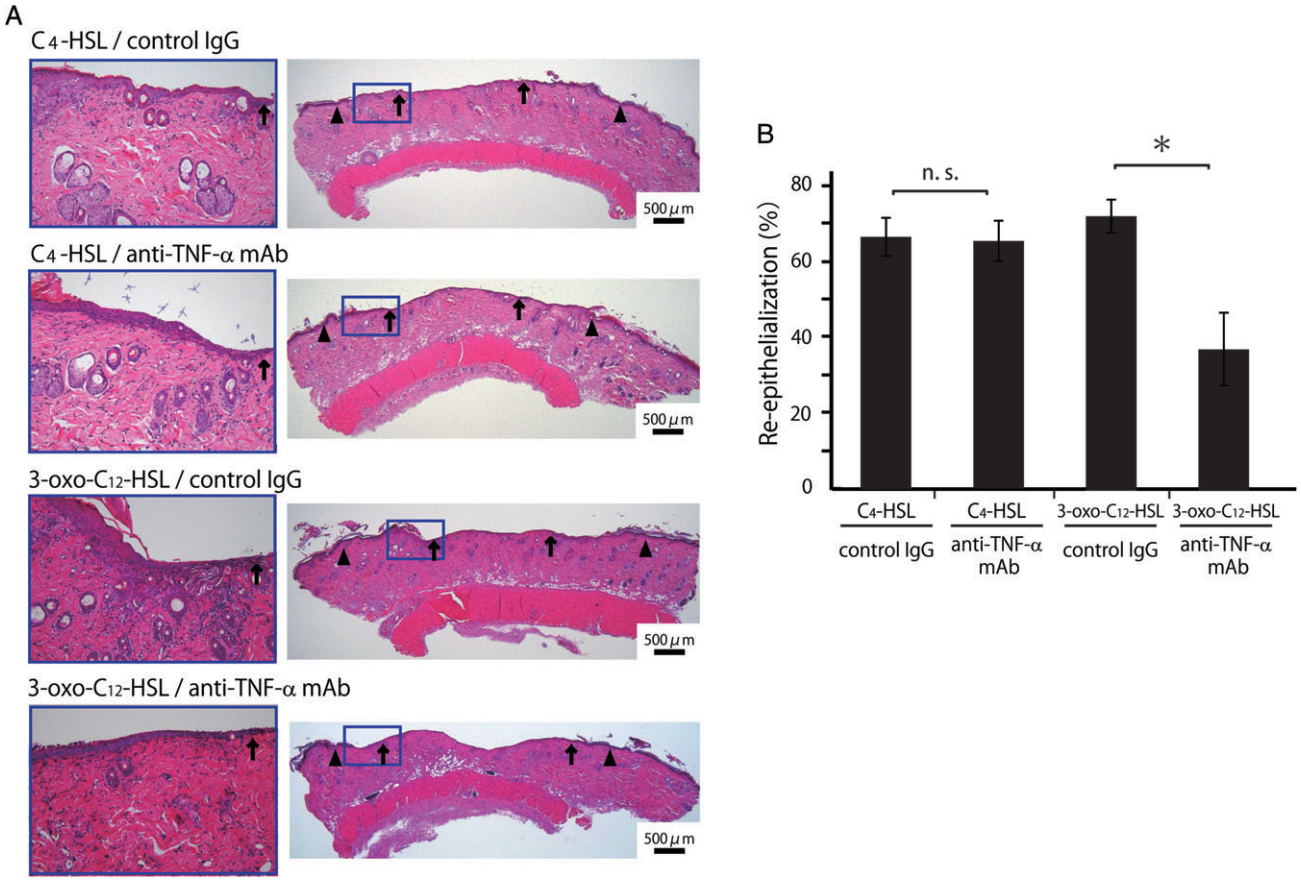

Figure 5 Effect of anti-TNF-α mAb on wound healing promoted by C_4-HSL. Rats were treated with anti-TNF-α mAb or control IgG on day 1 before wound creation. Wounded rats received C_4-HSL or 3-oxo-C_{12}-HSL at the wound surface. (A) Representative histological views of wounds on day 3. Arrowheads and arrows indicate the original wound edges and re-epithelialised leading edges, respectively. (B) Re-epithelialisation extent on day 3. NS, not significant.

mutant strains of *P. aeruginosa* were defective in either *lasI* or *rhlI*. Although Imamura *et al.* previously reported that the *rhlI* gene product was involved in neutrophil accumulation in pneumonia (23), the precise mechanism remains to be determined. A variety of chemoattractants are known to recruit neutrophils in *P. aeruginosa*-infected tissues, including *P. aeruginosa*-derived fMet-Leu-Phe (FMLP) and rhamnolipid and host-derived C5a, leukotriene B4 and IL-8 (1,24,25). In addition, neutrophil-derived TNF-α also contributes to neutrophil accumulation and the wound healing process in *P. aeruginosa* infection (4). Recently, we demonstrated that *lasI* is involved in TNF-α synthesis in *P. aeruginosa* infection during wound healing (2); this was in contrast with the current data, which indicates that the *rhlI* deficiency did not affect TNF-α production (Figure 2B, Figure S1). In addition, *lasI* deficiency led to a reduction in the expression of CXCL-1, IL-6 and IL-17A, which are chemokines and cytokines that are related to neutrophil attraction, in the wounded tissues during *P. aeruginosa* infection. This reduction was not observed in infection with the *rhlI*-deficient strain. These findings suggest that *P. aeruginosa*-derived factors that are regulated by *rhlI*, such as rhamnolipid, rather than by host factors, may contribute to the neutrophil accumulation. Trevani *et al.* indicated that DNA from other Gram-negative bacteria, *Escherichia coli* and *Acinetobacter baumannii*, but not mammalian DNA, induced human neutrophil activation (26), and these responses were induced through a TLR9-independent and MyD88-dependent pathway (27). These observations suggested that DNA from *P. aeruginosa* may also be involved in neutrophil attraction in the current study.

In addition to the experiments with anti-C_4-HSL antiserum, the current study demonstrated that C_4-HSL administration at the wound surface promoted wound healing, as evaluated by the increase in re-epithelialisation, epidermal cell proliferation and neo-vascularisation. The direct effects of C_4-HSL on wound healing have not yet been reported, and leukocyte accumulation after C_4-HSL administration was first identified in the current study. These were the same results as those for anti-3-oxo-C_{12}-HSL antiserum and 3-oxo-C_{12}-HSL administration, but the effect on TNF-α synthesis was different between C_4-HSL and 3-oxo-C_{12}-HSL. It is well known that TNF-α is directly activated in response to re-epithelialisation and angiogenesis in skin wound healing (4,28), but wound healing that is promoted by C_4-HSL is likely to be caused by other mediators, and further research is necessary.

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Both 3-oxo-C_{12}-HSL and C_4-HSL contribute to regulation in the production of virulence factors from *P. aeruginosa*, such as elastase, exotoxin A, alkaline protease and biofilm (10,29–31). In contrast, production of rhamnolipid and activation of the type III secretion system are regulated by C_4-HSL, but not by 3-oxo-C_{12}-HSL (14,15), suggesting that these HSLs act differently on the virulence factors from *P. aeruginosa*. In a clinical study by Bjarnsholt *et al.*, C_4-HSL and rhamnolipid synthesis was significantly increased in the mucoid type of *P. aeruginosa* compared with the non-mucoid type, which was isolated from the lungs of 238 cystic fibrosis patients (32). This suggests that C_4-HSL may regulate the production of alginate, a major component of mucus, in a manner independent of 3-oxo-C_{12}-HSL. Thus, two QS molecules from *P. aeruginosa*, C_4-HSL and 3-oxo-C_{12}-HSL, may possess distinct biological actions, as shown in the current study.

Many investigations have focused on the effect of 3-oxo-C_{12}-HSL, a *P. aeruginosa* QS molecule, on the host response. This QS molecule showed various effects on the inflammatory responses, including the production of TNF-α from neutrophils (2), IFN-γ from T cells (33) and IL-8 from human bronchiolar epithelial cells (7) and lung fibroblasts (18). It was also reported that 3-oxo-C_{12}-HSL impaired human airway epithelial cell activation (34) and neutrophil and macrophage apoptosis (13). By contrast, the current study shows, for the first time, the effect of C_4-HSL on the host response. Although 3-oxo-C_{12}-HSL receptors in the host cells remain to be defined, this QS molecule modulates inflammatory pathways involving NF-κB (35) and binds to peroxisome proliferator activated receptor (PPARγ), a family of nuclear membrane-associated transcriptional regulators that act as lipid sensors (36), and protease-activated receptor (PAR), which activates matrix metalloproteases (MMPs) (37). However, it is unclear how C_4-HSL acts on the host cells. To understand the significance of C_4-HSL during the wound healing process in *P. aeruginosa* infection, further research is necessary to define the C_4-HSL host cell receptor.

In the current study, we used the typically non-mucoid, wild-type *P. aeruginosa* PAO1 strain and its mutants, which is widely used in biofilm research (34,38). We also previously confirmed the formation of biofilm by *P. aeruginosa* PAO1 on the wounds, and our results suggested that the acute wound healing process in rats was unaffected by biofilm formation (39). In contrast, in chronic wounds, it has been suggested that microbial biofilms are implicated in both the infection of wounds and the failure of those wounds to heal (40). In the initial report by Davies *et al.*, *lasI* gene was found to be essential for the creation of mature, differentiated biofilms (38). The involvement of *lasI* and *rhlI* gene products with the biofilm formation within the chronic wounds has not yet been demonstrated. Perhaps clinical isolates of *P. aeruginosa* might be needed for an extended analysis of biofilm formation on the chronic wounds because *P. aeruginosa* PAO1 is not a particularly virulent wound isolate.

In conclusion, the current study demonstrated that acceleration of acute phase of wound healing in *P. aeruginosa* infection was mediated by the *rhlI* product, C_4-HSL, which triggered neutrophil infiltration and the *lasI* product, 3-oxo-C_{12}-HSL, as reported in our recent study (2). Although the mechanism by which C_4-HSL activates the neutrophil inflammatory response remains to be elucidated, the results presented here suggest the presence of a pathway that is distinct from pathways activated in response to 3-oxo-C_{12}-HSL-mediated effects. In addition, based on the data indicating that C_4-HSL alone promoted the repair of the wounded tissues, this compound could be a potentially promising therapeutic agent in the treatment of refractory skin wounds in the clinical setting. However, a high microbial burden results in redundant inflammatory responses, such as prolonged infiltration of neutrophils, which is believed to retard the healing of chronic wounds (41). Thus, the beneficial effect of *P. aeruginosa* infection and C_4-HSL treatment found in the current study may be applicable to acute wounds, but not to chronic wounds. This idea does not conflict with the dominant paradigm of current wound care, in which microbial infection contributes to the development of chronic wounds (6,41).

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Supporting Information

The following supporting information is available for this article:

Figure S1. Production of TNF-α at the wound sites after inoculation with *Pseudomonas aeruginosa* PAO1 or Δ*rhlI*. TNF-α concentration in the wound tissue homogenates from rats inoculated with *P. aeruginosa* PAO1, Δ*rhlI*, Δ*lasI* or from uninoculated rats were measured at 24 hours following wound creation. $^*P < 0{\cdot}05$; NS, not significant.

Additional Supporting Information may be found in the online version of this article.

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