Tissue injury elicits an inflammatory response that facilitates host defense. Resolution of inflammation promotes the transition to tissue repair and is governed, in part, by specialized pro-resolving mediators (SPM). The complete structures of a novel series of cysteinyl-SPM (cys-SPM) were recently elucidated, and proved to stimulate tissue regeneration in planaria and resolve acute inflammation in mice. Their functions in mammalian tissue repair are of interest. Here, nine structurally distinct cys-SPM were screened and PCTR1 uniquely enhanced human keratinocyte migration with efficacy similar to epidermal growth factor. In skin wounds of mice, PCTR1 accelerated closure. Wound infection increased PCTR1 that coincided with decreased bacterial burden. Addition of PCTR1 reduced wound bacteria levels and decreased inflammatory monocytes/macrophages, which was coupled with increased expression of genes involved in host defense and tissue repair. These results suggest that PCTR1 is a novel regulator of host defense and tissue repair, which could inform new approaches for therapeutic management of delayed tissue repair and infection.

skin, intestine, skeletal muscle, eye, gut, and periodontium. Importantly, SPM facilitate host defense by stimulating macrophages and neutrophils to phagocytose and kill bacterial pathogens. A novel series of cysteinyl-SPM (cys-SPM) comprising peptide conjugates within the resolvin, protectin, and maresin families were discovered and were coined conjugates in tissue regeneration (CTRs) based on their roles in promoting tissue regeneration in planaria. The cys-SPM used in this study were synthesized via total organic synthesis and their stereochemistry conformation of the nucleoside analog 5-ethynyl-2',3'-deoxyuridine (EdU) during DNA synthesis using the incorporation of the nucleoside analog 5-ethynyl-2',3'-deoxyuridine (EdU) during DNA synthesis using the cys-SPM in multiple injury contexts. Recent studies confirm and extend the potent inflammation-resolving actions of cys-SPM in mammalian tissue repair programs, including re-establishment of epithelial barriers, have yet to be addressed. Here, evidence is presented that PCTR1 directly stimulates migration of human keratinocytes in vitro which translates to accelerated closure of full-thickness skin wounds in vivo. Importantly, PCTR1 was produced in wounds infected with the common skin pathogen, Staphylococcus aureus, and PCTR1 accelerated bacterial clearance, suggesting novel roles of this SPM in both facilitating host defense, as well as engaging in tissue repair programs.

Materials and Methods

Reagents and Animals

The cys-SPM used in this study were synthesized via total organic synthesis and their stereochemistry confirmed by nuclear magnetic resonance. Isotopic cys-SPM, including PCTR1-(15)C2,15N: 16R-glutathionyl (glycine-13C2,15N)-17S-hydroxy-4Z,7Z,10Z,12E,14E,19Z-docosahexaenoic acid, PCTR2-(13C4,15N): 16R-cysteinylglycinyl (glycine-13C2,15N)-17S-hydroxy-4Z,7Z,10Z,12E,14E,19Z-docosahexaenoic acid, and PCTR3-(13C3,15N): 16R-cysteinyl (13C3,15N)-17S-hydroxy-4Z,7Z,10Z,12E,14E,19Z-docosahexaenoic acid, were also synthesized to facilitate identification of cys-SPM in tissues. Their complete synthesis will be reported separately. The structural integrity of cys-SPM was validated using liquid chromatography-tandem mass spectrometry (LC-MS/MS) prior to experimental procedures (Supplemental Figure S1). Primary human epidermal keratinocytes (HPEKp) were obtained from CELLnTEC (Bern, Switzerland). Male C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME) and were maintained on a normal laboratory chow diet. Mice were housed in a temperature-controlled environment with a 12-hour light/dark cycle and were used for experiments when they were 8 to 12 weeks of age. All murine procedures were performed under protocols approved by the Brigham and Women’s Hospital Institutional Animal Care and Use Committee (#2016N000131) and institutional biosafety committee (#2017B000050). Animals were randomly assigned to experimental groups, but investigators were not blinded to the treatments or group assignments in downstream data analysis.

Scratch Wound Human Keratinocyte Migration Assay

An in vitro scratch assay was performed to assess the effects of cys-SPM on cell migration. HPEKp were seeded at a density of 0.2 × 10^6 cells per well in 24-well culture plates and allowed to reach confluency in CnT-Prime complete medium (CELLnTEC) overnight. The cells were then serum starved in CnT-PR-BM.1 basal medium (CELLnTEC) for 16 to 18 hours. A scratch wound was generated midway across each cell monolayer with a sterile 200-µL pipette tip, and the wound was washed with phosphate-buffered saline (PBS) twice to remove displaced cells. The cells were incubated in the presence or absence of cys-SPM (1 or 10 nmol/L) in basal medium for 24 hours. Epidermal growth factor (EGF; 100 ng/mL; Thermo Fisher Scientific, Waltham, MA) was used as the positive control. Scratch wounds were photographed before and after treatment using a Moticam 2 digital microscope camera (Motic, Richmond, BC, Canada). To ensure that the same region was photographed after 24 hours, the left edge of each scratch wound was marked with a pen on the underside of the plate. Wound closure was determined using ImageJ software version 1.52s (NIH, Bethesda, MD; http://imagej.nih.gov/ij) by comparing the area not occupied by cells at 24 hours with the original wound area before treatment (ie, 0 hours). In addition, the time course of cell migration following PCTR1 treatment (10 nmol/L) was assessed by taking images of the scratch wounds at 0, 3, 6, 12, and 24 hours after wounding.

In some experiments, the cells were treated with PCTR1 (10 nmol/L) in the presence or absence of protein kinase A inhibitor H89 (5 µmol/L, Tocris; Bio-Techne, Minneapolis, MN).

Human Keratinocyte Proliferation Assay

Cell proliferation was tested on PCTR1-treated HPEKp by the incorporation of the nucleoside analog 5-ethynyl-2'-deoxyuridine (EdU) during DNA synthesis using the...
Click-iT EdU Microplate Assay (Molecular Probes, Eugene, OR). For this, HPEKp were seeded at a density of $1.0 \times 10^4$ cells per well in a 96-well plate and cultured overnight. Following serum starvation in CnT-PR-BM.1 basal medium for 18 hours, the cells were treated with vehicle (0.1% ethanol) or PCTR1 (0.1, 1, and 10 nmol/L) in basal medium. EdU (10 μmol/L) was added into each well, and the cells were incubated under serum-free conditions for 24 hours. CnT-Prime complete medium was used as the positive control. The incorporated EdU was coupled with Oregon Green 488 dye based on a copper-catalyzed covalent reaction between an azide and an alkyne, and subsequently incubated with a horseradish peroxidase–Oregon Green 488 antibody, which was allowed to react with Amplex UltraRed substrate generating a fluorescent product, as described in the manufacturer’s protocol. The fluorescence signal was measured using the excitation wavelength of 530 nm and the emission wavelength of 590 nm using a SpectraMax M5 Microplate Reader (Molecular Devices, San Jose, CA).

**cAMP Enzyme-Linked Immunosorbent Assay**

cAMP was measured in PCTR1-treated HPEKp using the cAMP Select Enzyme-Linked Immunosorbent Assay Kit (Cayman Chemical, Ann Arbor, MI). For this, HPEKp were seeded at a density of $0.5 \times 10^6$ cells per well in 6-well culture plates and allowed to reach 80% confluency. Following serum starvation in CnT-PR-BM.1 basal medium for 18 hours, the cells were treated with PCTR1 (10 nmol/L) for 15 minutes. Untreated cells were included as the negative control. After freeze–thaw, cell lysates were collected in enzyme-linked immunosorbent assay buffer and centrifuged at 14,000 × g for 15 minutes at 4°C. The supernatants were transferred to new microcentrifuge tubes for cAMP measurement as described in the manufacturer’s protocol. Optical density was measured in duplicate at 410 nm on a SpectraMax Plus 384 Microplate Reader (Molecular Devices). A standard curve was generated with a four-parameter logistic curve fit using GraphPad Prism software version 8.0.0 (GraphPad Software, La Jolla, CA).

**Excisional Cutaneous Wound Healing Model**

Full-thickness excisional cutaneous wounds were created as described previously. Briefly, 1 day prior to wounding, the dorsal skin surface was exposed using depilatory cream to remove hair. Mice were anesthetized using 1.5% to 2% isoflurane (2 L/minute oxygen) on a temperature-controlled water blanket and given a local subcutaneous pre-operative administration of buprenorphine (0.5 mg/kg) approximately 12 to 15 mm from the area to be wounded. The mouse was then gently rotated to its side, and using smooth forceps, the dorsal skin was grasped and pulled away from the underlying subcutaneous tissue. With the tissue still pinched in the forceps, two full thickness wounds were created using a 5-mm biopsy punch. The excised skin was then carefully removed from the wounds. The newly created wounds were then topically treated with sterile saline or PCTR1 (100 ng; 10 μL); silicone ring splints were attached to the skin using n-butyl-ester cyanoacrylate and covered with a semi-permeable polyurethane dressing. Finally, a self-adherent wrap was applied around the animal, covering the injury site. An additional local subcutaneous dose of buprenorphine was administered within 24 hours. After wounding, the wrap and dressings were removed and replaced daily to allow for direct topical administration of saline or PCTR1. Serial images were acquired to assess wound closure by tracing the wound edge using ImageJ software version 1.52s.

At the indicated time points following wounding, animals were euthanized by inhalation of carbon dioxide followed by cervical dislocation. The dorsal skin was removed, and the entire wound was collected using a 12-mm biopsy punch and was either flash frozen in liquid nitrogen, placed in ice cold methanol, or fixed for histology (see below).

**Histology**

Skin wound biopsies were collected from mice at day 7 after wounding after daily topical treatment with PCTR1 (100 ng per wound per day) or saline. Wounds were bisected and fixed in 10% buffered formalin (Thermo Fisher Scientific) and paraffin embedded using standard protocols by the Beth Israel Deaconess Medical Center Histology Core (Boston, MA). Cross sections (4 μm per section) were obtained from two distinct regions, 240 μm apart, starting from the center of each wound using a Leica RM2135 microtome (Leica Biosystems, Buffalo Grove, IL). These sections were subjected to hematoxylin and eosin staining in accordance with the instructions provided by ScyTek Laboratories Hematoxylin and Eosin Stain Kit (Logan, UT). VectaMount Permanent Mounting Medium (Vector Laboratories, Burlingame, CA) was used to adhere a coverslip to each section. Images were obtained using a Nikon Eclipse E400 microscope equipped with Spot acquisition software version 3.5 (Nikon, Melville, NY). Granulation tissue area was traced and measured by ImageJ software version 1.52s. Data are expressed as averages of the areas resulting from the two regions. For collagen area quantification, sections from the first region (closer to the center of the wound) were subjected to Picosirisir Red staining. For this, sections were deparaffinized in SafeClear II xylene substitutes (Thermo Fisher Scientific) and rehydrated in graded alcohol. Weigert’s iron hematoxylin solution (Electron Microscopy Sciences, Hatfield, PA) was used for nuclei staining. Picosirisir Red solution was prepared by dissolving 0.5 g of Direct Red 80 (Sigma-Aldrich, St. Louis, MO) in 500 mL of saturated picric acid (1.2% w/v; Genesee Scientific, San Diego, CA). Sections were incubated in Picosirisir Red solution for 1 hour, washed in acidified water, dehydrated in alcohol, and finally cleared in SafeClear II solution.
Figure 1  Structure-activity analysis of cys-SPM in promoting human keratinocyte migration. **A:** Cys-SPM biosynthetic pathways and structures, depicting key epoxide intermediates involved in the biosynthesis of protectins (PD1 and PCTRs), D-series resolvins (RvD1, RvD2, and RCTRs), and maresins (MaR1, MaR2, and MCTRs). **Right panel,** assessment of closure in scratch-wounded monolayers of human primary keratinocytes stimulated with EGF (100 ng/mL) or structurally distinct synthetic cys-SPM (1 and 10 nmol/L; 24 hours). **B:** Representative images of control and PCTR1-treated keratinocytes at baseline and 24 hours after wounding, with the **black dotted line** indicating the cell border. **C:** Assessment of proliferation by 5-ethyl-2'-deoxyuridine (EdU) incorporation in keratinocytes in the presence of full serum medium or indicated concentrations of PCTR1 for 24 hours. **D:** Measurement of cAMP accumulation in keratinocytes stimulated with PCTR1 (10 nmol/L). **E:** Assessment of closure in wounded keratinocytes stimulated with PCTR1 in the presence or absence of PKA inhibitor, H89 (5 μmol/L). Data are expressed as means ± SEM. *n* = 5 independent experiments (**A**); *n* = 10 to 13 replicates from 2 independent experiments (**C**); *n* = 4 replicates from 2 independent assays (**D**); *n* = 15 to 17 replicates from 3 independent experiments (**E**). *P < 0.05 by one-way analysis of variance followed by Dunnett’s post hoc tests (**A, C, and E**), or unpaired *t*-test (**D**).
Collagen area was measured by converting each image to grayscale and selecting an upper threshold value to minimize the background on the green channel using ImageJ software version 1.52s. Collagen fiber alignment analysis was performed on wound tissue sections stained with Picrorosius Red using an algorithm previously described in detail by Cash et al.

Infection of Dorsal Wounds with *S. aureus*

*S. aureus* (serotype (b)c1; ATCC 27217) was grown in sterile lysogeny broth (LB) at 37°C with shaking (220 rpm) overnight (Innova 4000 Incubator Shaker, New Brunswick Scientific Co., Edison, NJ). On the following day, the bacteria were subcultured at dilution of 1:100 in fresh LB Broth medium to reach mid-logarithmic phase (OD600 = 0.5 to 0.8). They were then washed twice with PBS, centrifuged (4000 × g, 10 minutes, 4°C), and resuspended in cold PBS at the desired concentration of approximately 50,000 colony-forming units (CFU.; per mL). The final bacterial concentration was determined using serial 10-fold dilutions on LB agar plates (37°C, overnight). Each dorsal wound was infected with 20 μL of diluted bacterial solution (approximately 50,000 CFU/mL) to achieve an initial bacterial burden of 1 × 10^5 CFU/wound. Either PCTR1 (100 ng/wound per day in 0.9% saline solution) or sterile 0.9% saline solution was topically applied to the wound. Animals were euthanized at the indicated time points after wounding. The wound tissues were collected using a 12-mm biopsy punch (Acuderm, Fort Lauderdale, FL) 2 hours following the final treatment and kept in sterile LB Broth medium (1 mL, ice-cold). Wound tissues were further minced, and serial 10-fold dilutions were prepared on LB agar plates. The colonies were counted, and the results were described as CFU per wound.

Gene Expression Analysis

Reverse transcription and quantitative PCR was used to evaluate the effect of PCTR1 on gene expression during wound healing after *S. aureus* infection. Murine cutaneous wounds were infected as described above and treated topically with PCTR1 (100 ng/wound per day) or saline at days 5 to 7 after wounding. Wound biopsies were collected at day 7 (2 hours after treatment) and stored at −80°C until RNA extraction. The RNeasy Fibrous Tissue Mini Kit protocol (QIAGEN, Hilden, Germany) was used for processing the tissue samples. Briefly, tissues were lysed in cold Buffer RLT (containing beta-mercaptoethanol) in a glass tissue grinder and homogenized with Qiashredder columns (QIAGEN). Following incubation with Proteinase K at 55°C for 10 minutes, the lysates were passed through RNeasy Mini Columns with on-column DNase I digestion, as described in the manufacturer’s protocol. Total RNA was quantified by NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific). Reverse transcription was performed using Applied Biosystems High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific), followed by quantitative PCR using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA) on custom-made PCR arrays (Bio-Rad) containing validated primer assays for a selected panel of genes, which included: i) cytokines, chemokines, and their receptors; ii) antimicrobial peptides and related genes; iii) adhesion receptors and structural genes; iv) growth factors and matrix remodeling genes; and v) epithelial repair genes. Data are presented as relative expression using the 2^(-ΔΔCt) method after internal normalization to B2m.

Targeted LC-MS/MS

Wounds were collected and minced using surgical scissors on ice in methanol containing internal synthetic isotope standards that are commercially available (d5-5S-HETE, d5-LTB4, d5-PGE2, d5-LXA4, d5-RvD2, d5-LTC4, and d5-LTD4; Cayman Chemical) or that were synthesized via total organic synthesis ([13C]3-N-MCTR1, [13C]2-N-PCTR1, [13C]3-N-PCTR2, and [13C]3-N-PCTR3). The minced wounds were then subjected to solid phase extraction prior to liquid chromatography-tandem mass spectrometry (LC-MS/MS) as detailed in Dalli et al.

Briefly, supernatants were collected following centrifugation (17,136 x g for 10 minutes at 4°C) then acidified (pH 3.5) prior to solid phase extraction via Biotage C18 cartridges (Uppsala, Sweden). Lipid mediators eluted in sequential methyl formate, and methanol fractions were then collected and exposed to a constant gentle stream of nitrogen gas to facilitate solvent evaporation. The samples were resuspended in methanol/water (50:50), injected, and then analyzed using an LC-MS/MS system equipped with a Poroshell reverse-phase C18 column (100 mm × 4.6 mm × 2.7 μm; Agilent Technologies, Santa Clara, CA), and a high performance liquid chromatography system (Shimadzu, Kyoto, Japan) coupled to a QTrap 5500 mass spectrometer operating with Analyst software version 1.6.2 (Sciex, Framingham, MA). For products eluted in methyl formate (ie, resolvins, lipoxins, leukotrienes, prostaglandins, protectins, and maresins), the QTrap was operated in negative ionization mode. A second system was used for products eluted in methanol from solid-phase extraction (ie, cys-SPM
Figure 2. PCTR1 stimulates wound closure. A: Schematic of the treatment protocol. B: Representative images (day 4; upper panels) and quantification (lower panel) of wound re-epithelialization in saline control or PCTR1-treated (100 ng per day; topical) wounds (scale in millimeters). The black dotted line denotes the initial leading wound edge (upper panels). Dotted black line indicates T50 of control; dotted blue line indicates T50 of PCTR1-treated (lower panel). C: Representative hematoxylin and eosin staining of wound cross sections at day 7 after wounding. The arrows indicate the initial wound edge. The epidermis (e) and granulation tissue (GT) are indicated, with GT area quantified in the lower panel. D: Assessment of wound collagen by Picrosirius Red staining with grayscale conversion (representative images in upper panel; day 7) and quantification indicated (lower panel). The arrows indicate the initial wound edge. E: Representative images and assessment of collagen fiber alignment in wound sections after Picrosirius Red staining. Grayscale images were used for analysis. Here, parallel collagen fiber alignment = 1, whereas randomly oriented collagen fibers yield a lower alignment score. Data are expressed as means ± SEM. n = 10 to 13 per group (B); n = 3 to 4 wounds per group (C); n = 4 to 5 wounds per group (D); n = 4 wounds per group (E). *P < 0.05 by two-way analysis of variance followed by Sidak’s post hoc test (B). Scale bars: 200 µm (C and D); 50 µm (E). T50, time to 50% closure.
enzymatically digested in PBS with collagenase II (250 U/ML). Using scheduled multiple reaction monitoring transitions coupled with information-dependent acquisition and enhanced product ion-scanning, specific lipid mediators were identified by matching retention time and at least six diagnostic MS/MS ions, compared with authentic standards. Mediator abundance was quantified using multiple reaction monitoring peak areas as compared with an external standard curve generated with authentic standards for each mediator and after normalization of extraction recovery based on internal isotope standards (see above). To assess global changes in lipid mediator biosynthesis over the course of wound infection, as well as the actions of PCTR1 administration on wound lipid mediator levels, open source software was used to perform statistical analyses (MetaboAnalyst version 4.0, www.Metaboanalyst.ca) and pathway visualization (Cytoscape version 3.5, www.Cytoscape.org). For inclusion in these analyses, mediators had to be identified in >50% of samples of at least one experimental group. Next, for individual samples where mediators were not identified, a missing value imputation was performed using one-fifth of the minimum positive value for that specific mediator. For both the partial least squares discriminant analysis and heatmap, the data were subjected to a generalized log transformation followed by autoscaling (mean-centered and divided by the standard deviation for each mediator). A heatmap illustrating the relative change in abundance of individual mediators across each experimental group was constructed. For pathway visualization, lipid mediators are displayed as nodes in their respective metabolomes. The size of the node is reflective of mediator abundance in the PCTR1-treated wounds, and the color indicates the fold change from untreated wounds.

Flow Cytometry and Cell Isolation

Populations of leukocytes in digested wound tissue were assessed using flow cytometry. After washing with cold PBS, the wound tissue was cut into several pieces and enzymatically digested in PBS with collagenase II (250 U/mL) containing 2.5 nmol/L calcium chloride at 37°C for 30 minutes. Following incubation, the tissue was further minced into small pieces and washed twice with cold PBS following centrifugation (400 x g, 5 minutes). The pellet was resuspended in PBS with dispase II (1.5 U/mL) and collagenase D (2.4 U/mL) with 2.5 mmol/L calcium chloride for 30 to 60 minutes at 37°C with vortexing for 5 seconds at 15-minute intervals. Finally, the digested wound was filtered using a 40-µm cell strainer. Single cells were washed with flow cytometry staining buffer, incubated in Fc-block, and stained with primary-conjugated fluorescent antibodies (eBioscience; PerCP anti-CD45, APC anti-F4/80, PE anti-Ly6G, and PE/Cy7 anti-Ly6C) for 30 minutes. After washing with flow cytometry staining buffer, cells were analyzed using a BD FACS-Canto flow cytometer (BD Biosciences, San Jose, CA) and FlowJo software version 10.6 (Becton, Dickinson and Company, Franklin Lakes, NJ).

Statistical Analysis

Data are presented as means ± SEM. Statistical comparisons between two groups were conducted using an unpaired two-tailed t-test, whereas comparisons of multiple groups were performed using a one-way analysis of variance with Tukey’s or Dunnett’s multiple comparison post hoc tests as indicated. When assessing multiple groups over time, a two-way analysis of variance with Sidak’s multiple comparison post hoc test was used. In all cases, significance was considered to be P < 0.05. Statistical outliers were determined using the ROUT test. GraphPad Prism software version 7.0 was used for all statistical analyses.

Results

PCTR1 Enhances Human Keratinocyte Migration in a cAMP/PKA-Dependent Manner

To determine whether cys-SPM could potentially stimulate tissue repair, migration of human primary keratinocytes was assessed because this is a key event in re-establishing the disrupted epithelial layer in skin wounds. Migration was evaluated using a scratch wound and the cells were stimulated with physiological concentrations (1 nmol/L or 10 nmol/L) of structurally distinct cys-SPM comprising the PCTR (PCTR1, PCTR2, PCTR3), RCTR (RCTR1, RCTR2, RCTR3), and the MCTR (MCTR1, MCTR2, MCTR3) families (Figure 1A). In comparison with untreated cells in which approximately 25% of the initially wounded area was covered by cells 24 hours after wounding, PCTR1 (10 nmol/L) significantly enhanced migration such that approximately 46% of the wound area was covered by cells at 24 hours after wounding (Figure 1A). Representative images of keratinocytes at baseline and 24 hours after wounding in the absence or presence of PCTR1 are shown in Figure 1B. This enhanced migration of human keratinocytes was similar in extent to EGF, and no statistical difference was observed between cells treated with PCTR1 and those treated with EGF. By contrast, neither PCTR2 nor PCTR3 significantly enhanced the rate of migration. Similarly, neither the MCTRs nor the RCTRs shared the stimulatory properties of PCTR1 in enhancing keratinocyte migration (Figure 1A). Focusing on PCTR1, the time course of wound closure was assessed and PCTR1 was found to significantly enhance migration of keratinocytes beginning at 12 hours after wounding and persisted until 24 hours after wounding (Supplemental Figure S2). In this context, the enhanced wound closure is attributed to cell migration, because the proliferation of human keratinocytes assessed by DNA incorporation of the thymidine ortholog, EdU, was not affected by PCTR1 (0.1 nmol/L to 10 nmol/L) as compared with full serum-containing medium (Figure 1C).
Figure 3  Wound infection with *Staphylococcus aureus* increases PCTR1 and shifts the lipid mediator metabolome. A: Schematic of the experimental protocol. B: Wound bacteria colony-forming units (CFU) at indicated times after wounding. C and D: Representative MS/MS spectra of synthetic PCTR1 (C) and PCTR1 identified in infected wounds (D: day 11). E: Quantification of PCTR1 in control and infected wounds at 1, 5, or 11 days post-wounding (DPW). F: Partial least squares discriminant analysis of global lipid mediator profiles of uninfected control (Ctrl) or *S. aureus* (S.A.) infected wounds at day 1, 5, or 11 (D1, D5, D11) after wounding, as determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Data are expressed as means ± SEM. *n* = 8 to 14 wounds per time point (B); *n* = 4 wounds per group per time point (D–F). *P* < 0.05 day 5 versus day 1 by one-way analysis of variance followed by Tukey’s post-test (B) or two-way analysis of variance followed by Sidak’s post hoc test (E); †*P* < 0.05 day 11 versus day 5 by one-way analysis of variance followed by Tukey’s post hoc test (B).
Because other SPM bind specific G-protein coupled receptors to elicit cellular responses, it was questioned whether PCTR1 would modulate the levels of second messenger cAMP in human keratinocytes. Stimulation of keratinocytes with PCTR1 (10 nmol/L; 15 minutes) significantly elevated levels of cAMP (Figure 1D). To determine whether activation of the cAMP/PKA pathway plays a causal role in the enhanced migration observed with PCTR1 treatment, migration in the presence of PKA inhibitor, H89, was evaluated. As shown in Figure 1E, the enhanced migration observed with PCTR1 was significantly and completely blocked in cells pretreated with H89. Collectively, these results demonstrate that PCTR1 is an agonist of human keratinocyte migration and has unique structural features that impart this agonist activity.

**PCTR1 Enhances Wound Closure**

To determine whether the enhancement of keratinocyte migration by PCTR1 observed in vitro translates to accelerated wound closure in vivo, full-thickness dorsal skin wounds in mice were treated topically with saline control or PCTR1 (100 ng/day) (Figure 2A). In this model, wounds were splinted to reduce contraction and allow for evaluation of the initial phases of external wound closure. In control wounds, the time to 50% closure occurred by day 5 after wounding (Figure 2B). Topical application of PCTR1 significantly reduced the time to 50% closure by nearly 1 day. An early formation of the neo-epidermis was visible in PCTR1-treated wounds by day 4 after wounding (see images in Figure 2B). By day 7 after wounding, a neo-epidermal layer was evident in the wounds of both saline and PCTR1-treated mice by hematoxylin and eosin staining of bisected wound cross sections, with no quantitative difference in the amount of granulation tissue formed at this time point (Figure 2C). There were no differences observed between saline and PCTR1 treatment in the total amount of fibrosis in the wounds at this early time point, as assessed by Picrosirius Red staining (Figure 2D). However, collagen alignment reduced in wounds of PCTR1-treated mice, which is indicative of reduced scar formation (Figure 2E). Collectively, the results demonstrate that PCTR1 enhances the early phases of wound closure, which is consistent with accelerated keratinocyte migration.

**Wound Infection with S. aureus Increases PCTR1**

Rapid re-establishment of the epidermal barrier after injury is important for reducing exposure of underlying tissue to microbes present on the skin surface. Because wound infection is a clinical manifestation of altered wound healing that has high morbidity and that PCTR1 has previously been shown to enhance host defense against bacterial pathogens, whether PCTR1 is produced in wounds infected with the common skin pathogen, *S. aureus* was assessed. For this, skin wounds of mice were infected with *S. aureus* (1 × 10^3 CFU), and the kinetics of wound infection were monitored over a period of 11 days (Figure 3A). The amount of bacteria in the wounds increased significantly from day 1 to day 5 after infection, indicating bacterial proliferation (Figure 3B). From the peak at day 5 to day 11, levels of wound bacteria significantly decreased, indicating host containment. Levels of PCTR1 in the wounds at each time point were assessed using targeted LC-MS/MS. As indicated by comparison with authentic PCTR1 standard prepared by total organic synthesis (Figure 3C), PCTR1 was detected in infected wounds (Figure 3D). Diagnostic ions present in the MS/MS spectra used for identification included m/z 650 (M+H), 632 (M+H-H2O), 503 (521-H2O), 325 (343-H2O), 308, 290 (308-H2O), 257 (275-H2O), 245, 231, and 227 (245-H2O). PCTR1 was not readily detected in uninjured wounds (12-mm biopsy) or during initial stages of infection, but increased significantly by day 11 after infection, the time point at which bacterial levels were decreasing in the wounds (Figure 3E).

Modulation of the temporal dynamics of other lipid mediator pathways comprising metabolomes of arachidonic acid (AA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) by wound infection was next assessed using targeted LC-MS/MS profiling. This analysis included identification of SPM (eg, lipoxins, resolvins, protectins, and maresins) and their pathway markers (eg, 17-hydroxydocosahexaenoic acid; 17-HDHA, 14-HDHA, and 18-hydroxyeicosapentaenoic acid; 18-HEPE), as well as proinflammatory eicosanoids such as the prostaglandins (eg, PGE2, PGD2) and leukotrienes (eg, LTD4, LTE4). Comparison of the global lipid mediator profiles using partial least squares discriminant analysis indicated divergence based on both time after wounding and infection with *S. aureus* (Figure 3F). At day 1 after injury, infection with *S. aureus* suppressed wound levels of several lipid mediators, including PGD2, 17R-RvD1, LXB4, 15R-LXA4, and PGF2α, as well as SPM pathway markers 17-HDHA, 14-HDHA, and 18-HEPE (Supplemental Figure S3). By contrast, RvE1 increased in infected wounds at day 1 after injury. Levels of some lipid mediators rebounded at day 5 after infection, whereas a substantial increase in almost all lipid mediators was observed in infected wounds relative to uninfected wounds by day 11 (Supplemental Figure S3). These results suggest that infection shifts the time course of lipid mediator production in wounds, with a suppression at early time points that coincides with bacterial proliferation, and a subsequent elevation in lipid mediators that coincides with bacterial clearance.

**PCTR1 Diminishes Wound Bacteria and Levels of Both Proinflammatory and Pro-Resolving Lipid Mediators in Infected Skin Wounds**

PCTR1 reduces levels of proinflammatory lipid mediators, such as prostaglandins, in other models of acute infectious inflammation. Given that infection of wounds shifted the
Figure 4  PCTR1 diminishes wound infection and proinflammatory and pro-resolving lipid mediators. A: Schematic of the treatment of S. aureus—infected wounds with PCTR1 (100 ng; topical) or saline control. B–D: Representative MS/MS spectra of PCTR1 (B), PCTR2 (C), and PCTR3 (D) identified in wounds of mice treated with PCTR1 (day 7). E: Quantification of PCTR1, PCTR2, and PCTR3 in infected wounds of mice treated with PCTR1 or saline control. F: Interaction network pathway of bioactive metabolomes of arachidonic acid (AA), docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA), in PCTR1-treated infected wounds as determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS). G: Schematic of the treatment of S. aureus—infected wounds with PCTR1 (100 ng; topical) or saline control. H: Quantification of bacteria levels in S. aureus—infected wounds of mice treated with control or PCTR1. I: Assessment of bacterial killing in the disk diffusion assay; A: ampicillin (10 μg), B, C: PCTR1 10 ng and 100 ng, respectively. Data are expressed as means ± SEM. n = 4 wounds per group (F); n = 8 to 10 wounds per group (H); n = 3 independent experiments (I). *P < 0.05 by unpaired t-test (E and H). CFU, colony-forming units; SA, S. aureus.
Figure 5  PCTR1 modulates monocyte/macrophage phenotype and augments expression of genes involved in immunity, antimicrobial responses, and tissue repair in infected wounds. A: Representative flow cytometry dot plots of CD45^+ Ly6G^+ F4/80^- neutrophils, CD45^+ Ly6G^-/C0^+ F4/80^+ macrophages, and CD45^+ Ly6ChiLy6G^- monocyte/macrophage populations in infected wounds treated with PCTR1 and collected at 7 days post-wounding (DPW). B: Quantification of leukocyte populations in infected wounds treated with PCTR1. Dotted black lines denote the levels of each population at day 5 after wounding. C–H: Expression of a selected panel of genes was determined at day 7 after wounding by reverse transcription and quantitative PCR. C and D: Cytokines, chemokines, and their receptors. E: Antimicrobial peptides and related genes. F: Adhesion receptors and structural genes. G: Growth factors and matrix remodeling genes. H: Genes involved in epithelial repair. Data are expressed as means ± SEM. n = 4 wounds per group (B–H). *P < 0.05 versus day 5 by one-way analysis of variance followed by Tukey’s post-test; †P < 0.05 control versus PCTR1 by one-way analysis of variance followed by Tukey’s post-test.)

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lipid mediator profiles and that PCTR1, as well as other lipid mediators, was increased as bacterial clearance ensued, whether PCTR1 modulates levels of other lipid mediators was questioned. For this, wounds were infected with *S. aureus* and began topical treatment of infected wounds at day 5 after injury (Figure 4A), the time point at which bacteria levels peaked (refer to Figure 3). Wounds were collected 2 days after treatment with PCTR1, and levels of PCTRs and other lipid mediators were quantified by LC-MS/MS. In wounds treated with PCTR1, PCTR1, PCTR2, and PCTR3 were identified using diagnostic ion assignments as compared with authentic standards prepared by total organic synthesis, as well as internal isotopic standards [ie, (13C)2-N-PCTR1, (13C)3-N-PCTR2, (13C)4-N-PCTR3] (Supplemental Figure S4). Representative MS/MS spectra for PCTR1, PCTR2, and PCTR3 identified in wounds are shown in Figure 4, B–D. Quantification of the PCTRs indicated that PCTR1 treatment significantly increased wound levels of PCTR1 and PCTR2, whereas PCTR3 was marginally elevated at this time point (Figure 4E). Using interaction network pathway analysis of lipid mediators comprising DHA, AA, and EPA bioactive metabolomes and constructed from their established biosynthetic pathways, a marked decrease in several lipid mediator pathways was observed in infected skin wounds of mice treated with PCTR1 (Figure 4F).

Given that PCTR1 treatment of infected wounds diminished levels of other lipid mediators that facilitate host defense, whether PCTR1 was sufficient to enhance bacterial containment was questioned. Wounds were treated with PCTR1 at the peak of bacterial burden and collected at day 7 to assess bacteria levels (Figure 4G). In this analysis, it was determined that PCTR1 significantly reduced the number of bacteria in the wounds at day 7 after injury, indicating an enhancement of host defense despite diminishing levels of other host-protective lipid mediator pathways (Figure 4H). Whether PCTR1 has direct antibacterial actions using the disk diffusion assay *in vitro* was studied next. PCTR1 at two different doses (10 ng and 100 ng) did not directly kill *S. aureus* as compared with ampicillin, which markedly diminished bacteria levels (Figure 4I).

**PCTR1 Enhances Tissue Reparative Immune Cell Phenotypes**

Because PCTR1 diminished wound bacteria levels without possessing direct antibacterial actions, whether innate immune cell populations that facilitate bacterial clearance were affected by PCTR1 was investigated. For this, biopsies of infected wounds were collected from mice at 5 days post-wounding (DPW), as well as those treated with saline control or PCTR1 at 6 or 7 DPW. Using single-cell suspensions of digested wounds, levels of CD45+Ly6G+F4/80−neutrophils, CD45+Ly6G−F4/80+macrophages, and inflammatory (CD45+Ly6C+Ly6G−) monocytes/macrophages were assessed by flow cytometry (Figure 5A). This analysis revealed that Ly6G+ neutrophils did not significantly differ between 5, 6, or 7 DPW in either control or PCTR1-treated wounds (Figure 5B). Levels of wound macrophages increased significantly by 7 DPW compared with 5 DPW, but were not different between control and PCTR1-treated mice. By contrast, the percentage of Ly6C+ monocytes/macrophages increased significantly from 5 to 7 DPW in control wounds, but not in wounds of PCTR1-treated mice (Figure 5B). These results demonstrate that PCTR1 enhances bacterial containment without altering neutrophil levels and is associated with a reduction of inflammatory monocytes/macrophages on the basis of Ly6C expression.

**PCTR1 Augments Expression of Genes Involved in Immunity, Antimicrobial Responses, and Tissue Repair in Infected Wounds**

Because PCTR1 treatment of infected wounds decreased bacterial levels without altering neutrophils and was associated with a shift in the Ly6C+ monocyte/macrophage population, whether genes involved in inflammatory versus tissue reparative immune phenotypes, as well as host defense, were modulated by PCTR1 was studied. To this end, *S. aureus*–infected wounds were treated as described above and wound tissue collected at 7 DPW to assess gene expression by reverse transcription and quantitative PCR. When assessing a
panel of genes encoding chemokines, cytokines, and their receptors, a trend was observed toward an increase in type 2 cytokine *Il4*, accompanied by a decrease in proinflammatory cytokine, *Il1b*, in wounds of PCTR1-treated mice (Figure 5C). An increase in IL-10 family cytokines including *Il10, Il19,* and *Il22,* as well as *Il6,* in PCTR1-treated wounds compared with saline control was also observed. Most chemokine receptors (eg, *Cxcl10, Ccl3*) were down-regulated in the PCTR1-treatment group, except for *Cxcl5* (Figure 5D). Interestingly, this cytokine signature was associated with an increase in expression of several genes involved in host defense, including *Reg3g, Defb1, S100a8,* and *S100a9* (Figure 5E). In addition to modulating immunity and host defense, PCTR1 treatment also led to increases in structural genes (eg, *Tagln*), as well as several growth factors that play key roles in epidermal tissue repair, angiogenesis, and lymphangiogenesis (eg, *Pdgfb, Vegfc, Vegfa, Tgfr1*) (Figure 5, F and G). As further evidence of enhanced tissue repair, several genes involved in epidermal formation/maturation were also modestly increased, including keratins *Krt14, Krt16, Krt6a,* and *Krt2* (Figure 5H). Collectively, these results indicate that PCTR1 shifts the mediator profile in infected wounds away from proinflammatory mediators and toward a tissue reparative and enhanced host-defense profile. These findings are consistent with the significant reduction in bacterial levels in wounds of PCTR1-treated mice.

**Discussion**

The results of the present study document a new role for the pro-resolving mediator PCTR1 in actively engaging tissue repair of damaged epithelial barriers. These actions were coincident with enhanced clearance of wound bacteria, which suggests that this SPM plays multiple beneficial roles in the wound microenvironment that may be amenable to the development of novel therapeutic strategies.

The cys-SPM comprise a novel series in each SPM family that actively engage innate immune cells to resolve inflammation. The first members of this family uncovered were MCTR1 and MCTR2, which were identified in self-resolving inflammatory exudates, human macrophages, human milk, and injured planaria.18,19 These mediators resolve acute infectious inflammation by limiting neutrophil infiltration, enhancing bacterial phagocytosis, and stimulating macrophage efferocytosis—key actions in the resolution of inflammation.18

In addition, they markedly enhance regeneration of planaria undergoing head resection.18 The subsequent structural elucidation of the PCTRs and RCTRs indicate that they are formed via novel allylcysteine intermediates involved in protectin and resolvin biosynthesis, respectively.18,20 Like the MCTR family, PCTRs and RCTRs have inflammation-resolving actions on immune cells, including stimulation of macrophage efferocytosis.18,20,31 The PCTRs stimulate tissue regeneration in planaria, indicating similar roles to the MCTR and RCTR families. Analysis of human keratinocyte migration indicated that PCTR1 uniquely engaged these cells to migrate at an accelerated rate. These results indicate that although the MCTR, PCTR, and RCTR families have similar roles in resolution of inflammation, they may have distinct roles and cellular targets in injured tissue that are just beginning to be appreciated.

Structure-activity results with human keratinocyte migration indicate that both the 16R-glutathionyl group and the 17S-hydroxy group are likely required structural features for the biological activity of PCTR1 in this context. This is because neither PCTR2 (16R-cysteinylglycylnor PCTR3 (16R-cysteiny1) was as effective as PCTR1 in promoting keratinocyte migration. Along these lines, PCTR1 enhances macrophage migration in a manner that is not altered by inhibition of γ-glutamyl transferase, suggesting that conversion to PCTR2 is not necessary for this stimulatory action on leukocytes.32 Moreover, neither MCTR1 nor RCTR1, which share the glutathione group with PCTR1, but have distinct positional hydroxyl groups on the DHA backbone, as well as distinct double-bond geometry, modulated keratinocyte migration. As an in vivo correlate to enhanced keratinocyte migration, PCTR1 was found to markedly accelerate wound closure. PCTR2 and PCTR3 were increased in wounds treated with PCTR1. Although these products did not directly enhance keratinocyte migration, it is possible that they could play additional roles in injured tissue and contribute to the enhanced tissue repair observed in PCTR1-treated wounds. Indeed, PCTR2 and PCTR3 stimulate key functions in resolution, including macrophage phagocytosis and efferocytosis.31 Likewise, the current results do not exclude potential roles of MCTRs or RCTRs in tissue repair, which are also likely to engage immune cells to facilitate resolution and thereby indirectly modulate the tissue repair program in the skin and potentially other organs (eg, liver). Along these lines, recent results independently confirm the potent organ-protective activities of the cysSPM.31–25

Wound infection is a prominent clinical manifestation of delayed tissue repair and one of the key biological actions of SPM is that they enhance host defense against exogenous bacterial pathogens, including *E. coli* and *S. aureus.* In wounds infected with *S. aureus,* a robust increase in bacteria levels was observed that were subsequently diminished, indicating host containment. During this time frame, PCTR1 was increased, and add back of PCTR1 significantly diminished wound bacteria levels. These results are consistent with prior studies demonstrating that PCTR1 increases during resolution of *E. coli* peritonitis and enhances macrophage phagocytosis of *E. coli.*32 Moreover, PCTR1 resolves acute lung inflammation induced by lipopolysaccharide.22,25 PCTR1 was not readily identified in noninfected wounds at the time points sampled, although it cannot be ruled out that it may be produced at alternate time points during the dynamic wound repair response. These results indicate that bacterial infection may be a stimulus for immune cell—dependent PCTR1 production in wounds. Along these
lines, PCTR1 is produced during infectious peritonitis and in M2-polarized macrophages in the presence of bacteria.\textsuperscript{32,33} Interestingly, in infected wounds of PCTR1-treated mice, there was an increase in the expression of host-derived antimicrobial peptides, including Reg\textsuperscript{3g}. Prior studies have demonstrated that Reg\textsuperscript{3g} is critical for host-defense against Gram-positive bacteria such as \textit{S. aureus} in the intestine and lung.\textsuperscript{34,35} Mechanistically, it is induced by cytokines including IL-22 and IL-6 that converge on STAT3 signaling in epithelial cells.\textsuperscript{34,35} Wounds of PCTR1-treated mice also had higher levels of IIL22 and IIL6, consistent with engagement of this host-protective axis. IL-22 can be produced by multiple T-cell subsets, as well as innate lymphoid cells (ILC) including ILC3.\textsuperscript{36} Moreover, PCTR1 biosynthesis can be initiated by ILC3 during bacterial peritonitis and subsequently enhances host defense in part by promoting macrophage phagocytosis.\textsuperscript{37} Along these lines, ILC3 are required for epithelial repair during skin wounding, and these actions are largely dependent upon regulation of macrophage recruitment.\textsuperscript{38} Thus, the containment of bacteria by PCTR1 in wounds is likely to be multifactorial and could involve both macrophage phagocytosis and stimulation of antimicrobial peptides. Future studies will be required to elucidate fully these diverse roles and cellular targets, as well as the predominant cellular sources of PCTR1 during wound infection.

Consistent with a role for PCTR1 in controlling wound macrophages, a significant time-dependent shift in monocyte/macrophage phenotype was observed in wounds of PCTR1-treated mice. Specifically, the levels of Ly6\textsuperscript{ch} monocytes/macrophages were decreased by PCTR1, which is consistent with a shift to a less proinflammatory/more tissue reparative phenotype. In support of this view, this shift was coincident with a decrease in proinflammatory cytokines (eg, IIL1b) and a reciprocal increase in cytokines that engage tissue repair programs in macrophages (eg, IIL4, IIL10, IIL6). Other SPM promote this tissue reparative macrophage phenotype, and resolin D2 promotes a shift in the ratio of Ly6\textsuperscript{ch} to Ly6\textsuperscript{col} macrophages and facilitates regeneration of injured skeletal muscle.\textsuperscript{17,39} In addition to regulation of macrophage phenotype by SPM, IL-4 polarized human macrophages also produce higher amounts of PCTR1, PCTR2, and PCTR3 than proinflammatory, lipopolysaccharide/interferon-\gamma—stimulated macrophages, suggesting that PCTR biosynthesis is intimately linked with the macrophage-dependent tissue repair program.\textsuperscript{32,33} Importantly, alterations in the shift from inflammatory to reparative macrophages is associated with delayed tissue repair observed in chronic inflammatory diseases, such as type 2 diabetes.\textsuperscript{40,41} Given that several studies have demonstrated deficits in SPM in type 2 diabetes, including skin wounds, uncovering their specific roles in the normal tissue repair program could illuminate novel agonist-based approaches to rescuing tissue repair in these contexts.\textsuperscript{26,31}

In summary, using an unbiased screening of nine structurally distinct natural cys-SPM on human keratinocytes, a new role of PCTR1 was identified in the engagement of epithelial repair. These actions were confirmed in a murine model of cutaneous injury, where PCTR1 significantly increased the initial phases of wound closure. In the context of wound infection, PCTR1 additionally facilitated bacterial containment, which was associated with engagement of host-defense pathways and monocyte phenotype transitions associated with tissue repair. The present results are consistent with and go beyond earlier studies,\textsuperscript{18} suggesting that PCTR1 engages both immune cells and epithelial cells to directly promote tissue repair and host defense (Figure 6). Elucidation of these novel functions of PCTR1 could potentially inform development of agonist-based therapeutics for conditions in which chronic infection and delayed tissue repair are prominent features.

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Author Contributions

B.E.S., X.L., C.O.R., and A.E.S. performed experiments, analyzed data, and wrote the manuscript. R.N. and N.A.P. prepared synthetic CTRs by total organic synthesis and wrote the manuscript. C.N.S. designed experiments, supervised research, and wrote the manuscript. M.S. conceived and planned the project, supervised research, analyzed data, and wrote the manuscript. M.S. is the guarantor of this work and, as such, had full access to the data and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Supplemental Data

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References

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Control of Infection and Repair by PCTR1


