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Title

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<https://escholarship.org/uc/item/3t500866>

Journal

Federation proceedings, 36(1)

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Publication Date

2022

DOI

10.1096/fj.202101019R

Peer reviewed



Published in final edited form as:

FASEB J. 2022 January ; 36(1): e22090. doi:10.1096/fj.202101019R.

Therapeutic Evaluation of Immunomodulators in Reducing Surgical Wound Infection

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Abstract

Despite many advances in infection control practices, including prophylactic antibiotics, surgical site infections (SSIs) remain a significant cause of morbidity, prolonged hospitalization, and death worldwide. Our innate immune system possesses a multitude of powerful antimicrobial strategies which make it highly effective in combating bacterial, fungal, and viral infections. However, pathogens use various stealth mechanisms to avoid innate immune system, which in turn buy them time to colonize wounds and damage tissues at surgical sites. We hypothesized that immunomodulators that can jumpstart and activate innate immune responses at surgical sites, would likely reduce infection at surgical sites. We used three immunomodulators; fMLP (formyl-Methionine-Lysine-Proline), CCL3 (MIP-1 α), and LPS (Lipopolysaccharide), based on their documented ability to elicit strong inflammatory responses; in a surgical wound infection model with *Pseudomonas aeruginosa* to evaluate our hypothesis. Our data indicate that one-time topical treatment with these immunomodulators at low doses significantly increased proinflammatory responses in infected and uninfected surgical wounds and were as effective, (or even better),

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AUTHOR CONTRIBUTIONS: S. H. S. conceived and coordinated all aspects of the studies and wrote the paper. F.M conducted and contributed to Figures 1-7. R.R conducted and contributed to Figures 1-5 and 7. M.F.M. contributed Figures 1, 4, 6, and 7. A.A., contributed to Figures 4 & 6. M.M., conducted statistical analyses for all figures. S.K.G., contributed to statistical analysis in Figure 4. M.B., contributed to data analyses and research design, and T.M.K. and J.R. contributed to data analyses and research design, and reagents.

CONFLICT OF INTEREST: Dr. Sasha Shafikhani is the inventor on an approved patent (PCT/US2019041112), filed by Rush University Medical Center.

than a potent prophylactic antibiotic (Tobramycin) in reducing *P. aeruginosa* infection in wounds. Our data further show that immunomodulators did not have adverse effects on tissue repair and wound healing processes. Rather, they enhanced healing in both infected and uninfected wounds. Collectively, our data demonstrate that harnessing the power of innate immune system by immunomodulators can significantly boost infection control and potentially stimulate healing. We propose that topical treatment with these immunomodulators at the time of surgery may have therapeutic potential in combating SSI, alone or in combination with prophylactic antibiotics.

Keywords

Wound infection; Wound healing; Surgical Site Infection (SSI); *Pseudomonas aeruginosa*; Immunomodulators; fMLP (fMLF); CCL3 (MIP-1 α); Lipopolysaccharides (LPS); Innate Immune System; Leukocytes; Neutrophils

INTRODUCTION

Various infection prevention measures have been used to reduce surgical site infection (SSI), including surgical hand asepsis, reduction of foot traffic in and out of the operating room, use of intraoperative skin antiseptic agents, perioperative high inspired oxygen, perioperative glycemic control, appropriate selection of surgical dressings, and perioperative antibiotic prophylaxis (1-9). Despite these preventive measures, SSI remains one of the most common and important healthcare-associated infections, accounting for 17-20% of all hospital-acquired infections (10-14). The Centers for Disease Control and Prevention (CDC) estimates that approximately 500,000 SSIs occur annually in the United States (US). Each SSI case is associated with approximately 7-11 additional postoperative hospital days (15-17), and 2-11-times higher risk of death compared with the operative patients without SSI (15, 18-22). The annual costs for Healthcare Associated Infections (HAIs) are astronomical and increasing every year, with SSIs contributing the most (~33.7%) to the overall costs, approximately \$3.4 billion in US and between \$3.5 to \$10 billion in Europe (15, 16). Without question, SSI remains an important public health threat. It is not surprising that the US Department of Health and Human Services has identified combatting SSI as a top national priority.

Administration of antibiotic prophylaxis in the perioperative period, (~1 hour before surgery for most antibiotics), is the standard of care for most surgical procedures - although in some cases, (e.g., cardiac surgeries), post-surgical antibiotic for up to 3 days is recommended (18, 21-23). While antibiotics have saved millions of lives over the past 9 decades, their use is not without its problems. Excessive antibiotic use can lead to emergence of antibiotic resistance, increased risk for *Clostridium difficile* infection, cytotoxicity, allergic reactions, and immunological and neurological diseases - many of which have been attributed to dysbiosis in the gut flora (24-29). These limitations highlight the need for new approaches (preferably antibiotic-free) to enhance infection control at surgical sites.

We have evolved a remarkable and powerful innate immune system that recognizes invading pathogens as “none-self” and mobilizes its plethora of antimicrobial defenses to protect us against infection (30-36). Germline-encoded Pattern Recognition Receptors (PRRs) are at

the heart of innate immune system sensory and processing centers. Recognition of microbial pathogens by PRRs, such as toll-like receptors (TLRs), sets in motion a signaling cascade that culminates in the production of proinflammatory cytokines, such as TNF- α and IL-1 β (30-35). These inflammatory cytokines function as powerful chemoattractants for effector innate immune leukocytes which destroy invading pathogens by various direct or indirect mechanisms (30-36). Neutrophils are the first effector inflammatory leukocytes infiltrating at the site of injury. In addition to their antimicrobial functions through phagocytosis, reactive oxygen species (ROS), neutrophil extracellular trap (NET) production, and antimicrobial peptides (AMPs) (37, 38), they also express various proinflammatory cytokines, such as IL-1 β , which set the stage for the subsequent inflammatory responses, including monocytes recruitment and their differentiation into M1 classical macrophage phenotype which further contributes to microbial killing and infection control (39-43). Although, there is a high degree of redundancy in innate immune system's ability to sense and respond to invading pathogens, critical components of the innate immune system must function in an orderly fashion to eventually clear infection. For instance, mutations in PRRs, inflammasomes, or even individual inflammatory cytokines can render humans or animals vulnerable to infection (44-55). Further highlighting the importance of neutrophils and innate immune system in combating infection, Granulocyte Colony Stimulating Factor (G-CSF) and Granulocyte/Macrophage Colony Stimulating Factor (GM-CSF) cytokines - which belong to a group of growth factors termed "Colony Stimulating Factors" which support survival, clonal expansion, and differentiation of hematopoietic myeloid progenitor cells (56-59) - have been approved for systemic use and shown to be effective in boosting infection control in patients suffering from neutropenia (reviewed in (60)).

Although neutrophil response begins immediately after injury and/or in response to infection and healthy immune system is effective in controlling infection for the most part, neutrophil and inflammatory responses reach their peak at 1-3 days after injury, depending on presence or absence of infection (61-63). This lag period in reaching the peak innate immune responses could potentially render wound tissues at surgical sites vulnerable to bacterial colonization at least early after surgery. In addition, pathogens have evolved many stealth virulence strategies that allow them to establish infection by dampening host's immune responses even in immunocompetent healthy individuals (64-68). These virulence stealth strategies utilized by pathogens, could further delay immune responses from reaching their peaks, thus extending the vulnerability period to infection in wounded tissues after surgery.

We hypothesized that immunomodulators that can jumpstart and activate innate immune responses at surgical sites, would likely enhance tissue's ability to fight off infection at surgical sites. SSIs include superficial incisional wound infections, infections of the deep incision space, and infections of organ space (22, 69, 70). We assessed the efficacy of immunomodulator-based approaches in reducing SSI in a surgical full-thickness incisional wound infection model against *Pseudomonas aeruginosa*, which is one of the most common and serious causes of wound and surgical site infections, both in healthy and immunocompromised individuals (71-75). We selected 3 immunomodulators with broad proinflammatory activities to evaluate their effectiveness in reducing *P. aeruginosa* infection in this model. We chose fMLP (*N*-formyl-Methionine-Leucyl-Phenylalanine; a.k.a., fMLF) because it primarily functions as a potent activator of phagocytic leukocytes (particularly

neutrophils) through its interaction with formyl peptide chemokine receptors (FPRs), although it has also been implicated in triggering the production of proinflammatory cytokines through activation of TLR2 and TLR4 (76-80). Of note, fMLP is a natural immunomodulator which is released from injured tissues, although it can also be released from invading bacterial pathogens (63, 81). CCL3 (a.k.a., MIP-1 α) was chosen because it is an important proinflammatory cytokine which has been shown to recruit and activate phagocytic leukocytes (e.g., neutrophils), maintain the effector immune responses, and stimulate wound healing by engaging multiple chemokine receptors, such as CCR1, CCR4, and CCR5 (82-86). We also chose LPS (Lipopolysaccharide) because it is a bacterial ligand and a potent immunomodulator that triggers inflammatory responses primarily through TLR4, but it has also been implicated in triggering inflammatory responses through TLR2 and non-canonical caspase-11 inflammasome (80, 87). Our data indicate that one-time topical treatment with these immunomodulators at low doses significantly increased proinflammatory responses in infected and uninfected surgical wounds and were as effective, (or even better), than a potent prophylactic antibiotic (Tobramycin) in reducing *P. aeruginosa* infection in wounds. Moreover, treatment with these immunomodulators did not adversely impact wound healing processes in infected or uninfected wounds.

MATERIALS AND METHODS

CONTACT FOR REAGENT AND RESOURCE SHARING:

Further information and requests for reagents may be directed to, and will be fulfilled by, the Lead Contact, Sasha Shafikhani (Sasha_Shafikhani@rush.edu).

PROCEDURES RELATED TO ANIMAL STUDIES:

We have approval from the Rush University Medical Center Institutional Animal Care and Use Committee (IACUC No: 18-037 & 20-042) to conduct the research as indicated in these studies. All procedures complied strictly with the standards for care and use of animal subjects as stated in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Academy of Sciences, Bethesda, MD, USA). We obtained 6-7-week-old C57BL/6 and TLR4^{-/-} knockout mice (Stock #029015) from the Jackson Laboratories (Bar Harbor, ME). These Mice were allowed to acclimate to the environment for 1 week prior to experimentation. Wounding and wound infection were carried out as we described previously (88) (71, 89, 90). We used *Pseudomonas aeruginosa* PA103 for this study, which has been described previously (91, 92) and we have shown that it causes massive infection and exacerbates wound damage in diabetic wounds (71). Immunomodulators were added at indicated levels right after wounding and prior to infection. Bacteria were prepared overnight as we described (92, 93). Infection levels in wounds were evaluated by determining the number of bacteria, colony forming unit (CFU) per gram of wound tissues, as described (6, 7, 71, 94, 95).

WOUNDING AND SURGICAL SITE INFECTION/TREATMENTS:

Full-thickness excisional wounding was performed, using sterile biopsy punches (5-mm diameter, AcudermH Inc. Lauterdale, FL), as we had described previously (71, 89, 90). Each animal received 4 equidistant wounds on its back below the shoulder blades.

fMLP (a.k.a., fMLF), CCL3 (a.k.a., MIP-1 α), and LPS immunomodulators (at indicated concentrations) were added once topically to the wound, right after wounding surgical procedure and prior to infection. Wound tissues from wound edges (~1 mm) were collected and analyzed in these studies as we described previously (89, 90, 95). For antibiotic prophylaxis, Tobramycin solution (0.35 mg/mL) or saline control were administered intraperitoneally (i.p.) in 0.2 mL saline, 1 hour before starting surgery, as described (96, 97).

HISTOPATHOLOGICAL EVALUATION:

Wound healing was assessed by digital photography, as described (71, 89, 90). Briefly, wound areas were determined by ImageJ at indicated timepoints. Wound closure rates were assessed by dividing the wound area at indicated timepoints to wound area at Day 0 (day of wound surgery). Leukocytes' infiltration in the wound bed were performed using hematoxylin and eosin (H&E) staining as described previously (6, 71). The number of leukocytes in wounds were assessed by determining the number of polymorphonuclear and mononuclear round cells which stained positive with eosin as described (71, 89). Neutrophil contents in wounds were assessed by anti-Ly6G histological analysis (6) and activated neutrophil contents in wounds were assessed by Myeloperoxidase (MPO) measurements, using ELISA (98). Macrophage levels in wounds were assessed by anti-CD68 histological analysis (89, 90).

REAGENTS:

Hematoxylin & Eosin Staining (Richard Allan Scientific Hematoxylin, Eosin Y, and Bluing Reagent Cat. Numbers: 7111L, 7211L, and 7301L from Thermo Fisher; anti-CD68 (Cat. No. NBP2-33337) and anti-Ly-6G (Cat. No. NBP2-00441) antibodies were purchased from Novus biologicals, CO. Myeloperoxidase (MPO) Mouse ELISA Kit (Cat. No., EMMPO) was obtained from Invitrogen; Lipopolysaccharides (LPS) was obtained from Sigma (Cat. No. L3012); CCL3 (rhCCL3/MIP-1 α isoform LD78a; Cat. No., 450-MA/CF) was obtained from R&D; *N*-formyl-Met-Leu-Phe (fMLP, a.k.a., fMLF), Cat. No. 59880-97-6 from Sigma; Mouse IL-1 β uncoated ELISA kit (Cat. No., 88-7013-88) and Mouse TNF- α uncoated ELISA kit (Cat. No., 88-7324-88) (ThermoFisher). Collagenase D, CAS No. 9001-12-1 (Sigma Aldrich).

STATISTICAL ANALYSES:

All variables were evaluated for violations of model assumptions. Distributions were evaluated descriptively using box plots, histograms, and quantile-quantile plots. If substantial skewness was discovered appropriate transformations were applied (i.e., square root). If outliers were identified, then sensitivity analyses were performed (with and without the outlier) and the more conservative result selected. Non-parametric methods were used if violations could not be appropriately compensated. T-tests were used for simple two group tests with homoscedasticity evaluated and (Satterthwaite approximation) adjustments made if necessary. Multi-group comparisons were performed using General Linear Models with Dunnett adjustment to compensate for error inflation for the contrasts between variables. Time varying data were analyzed using repeated measures (Proc Mixed) models with "Day" as the time varying factor with an autoregressive lag one covariance structure. Models were

evaluated for fit and multiple contrasts were adjusted for error inflation using the step-down Bonferroni (Holm) method. Data are represented using Mean \pm SEM, figures plots were produced using GraphPad Prism version 5.0 and all statistical analyses were performed using SAS version 9.4 (SAS Institute, Cary, NC). P-values less than or equal to 0.05 were considered as significant.

RESULTS

Treatment with immunomodulators boost innate immune responses in wound.

Full-thickness incisional wounds were generated on the back of C57B mice, as described (71, 89), and immediately treated with PBS (control) or PBS containing fMLP (5ng or 50ng per wound), CCL3 (1 μ g per wound), or LPS (10ng or 100ng per wound), prior to infection with *P. aeruginosa strain* PA103, which we have shown to establish robust infection and cause damage in diabetic wounds (71). Wound tissues were harvested at 24h after treatment and infection and assessed for their IL-1 β and TNF- α proinflammatory cytokines by Enzyme-Linked Immunosorbent Assay (ELISA). Consistent with our hypothesis, immunomodulators were able to significantly boost the production of these proinflammatory cytokines in PA103-infected wounds, particularly when applied at higher concentrations (Fig. 1A-B).

We next evaluated the impact of immunomodulators (fMLP at 50ng, CCL3 at 1 μ g, and LPS at 100ng per wound) on inflammatory leukocytes' migration into wounds, 24h after treatment and infection by histological analysis, using hematoxylin and eosin (H&E) staining, as described (6, 89). In line with increased proinflammatory cytokines (Fig. 1), CCL3 and LPS immunomodulators also significantly enhanced leukocytes migration into wounds (Fig. 2A-B). fMLP treatment also increased leukocytes numbers in wound but the differences did not reach statistical significance. Phagocytic leukocytes (namely macrophages and neutrophils) play critical roles in combating invading pathogens in wound and at surgical sites (42, 99-101). We evaluated the impact of immunomodulators on macrophage and neutrophil responses in these wound by histological analyses using their specific markers CD68 and Ly6G respectively (89, 102). Data indicated that treatments with CCL3 and LPS immunomodulators significantly increased both macrophage and neutrophil contents of these wounds (Fig. 2C-F). Consistent with the leukocyte response, fMLP treatment also increased neutrophil and macrophage contents in infected wounds but the differences did not reach statistical significance. To further corroborate these data, we assessed neutrophil activation in wound using myeloperoxidase (MPO) - a marker primarily used for activated neutrophils (6, 103) - by ELISA. Data indicated that treatment with all 3 immunomodulators significantly increased activated neutrophils in infected wounds (Fig. 2G). Collectively, these data indicated that topical treatment with these immunomodulators enhanced inflammatory responses in infected wounds, albeit to different degree.

Immunomodulators enhance infection control in wound.

We next assessed the efficacy of Tobramycin antibiotic prophylaxis in controlling *P. aeruginosa* infection in this wound infection model, as a way to evaluate how immunomodulator-based therapies may compare in their ability to control infection with

a conventional antibiotic prophylaxis therapy. Tobramycin is a powerful antibiotic against *P. aeruginosa* infections, and it has been shown to be effective even against Gentamycin resistant *P. aeruginosa* clinical strains (104-106). We administered Tobramycin at 3.5 mg/kg by intraperitoneal injection (i.p), prophylactically at 1 hour prior to wounding and infection with PA103 strain at 10^3 or 10^6 bacteria/wound and assessed infection burden in wounds by colony forming unit (CFU) and bacteria counts determination as described (6, 7, 71). Dosing of Tobramycin was determined from previous publications, based on its efficacy to control infection without causing adverse side effects, such as nephrotoxicity or ototoxicity (97, 107-109). Tobramycin (Tob) prophylaxis therapy was very effective and reduced *P. aeruginosa* infection significantly by ~ 1 log-order, when 10^3 PA103 was used to infect (Fig. 3A), and by ~ 1.3 log order, when 10^6 PA103 was used to infect (Fig. 3E). Of note, no bacteria was detected in PBS-treated wounds, indicating that wound environment is effective in preventing low level infection with environmental bacteria (Fig. 3A, E).

We next assessed the effectiveness of immunomodulators in controlling infection by treating wounds with the aforementioned immunomodulators (fMLP at 50ng, CCL3 at 1 μ g, and LPS at 100ng per wound) prior to infection with 10^3 or 10^6 PA103. Data indicated that immunomodulators reduced infection by 1-1.3 log order, when 10^3 PA103 was used to infect (Fig. 3B-C), and by ~ 2 -2.8 log order, when 10^6 PA103 was used to infect (Fig. 3F-H). These data demonstrated that these immunomodulators are at least as effective as systemic Tobramycin prophylactic antibiotic in reducing infection in wound.

Immunotherapy-induced enhancement in infection control is dependent on immune responses.

We reasoned that if our hypothesis is correct that immunomodulators enhance infection control by boosting innate immune responses in wound tissue, in situations where innate immune responses are not available and cannot be enhanced, immunomodulators should lose their effectiveness. Unlike fMLP and CCL3 which can stimulate inflammatory responses by engaging multiple receptors (76-80, 82-86), LPS primarily activates immune responses by engaging toll-like receptor 4 (TLR4) (80). To assess the dependence of LPS therapy on TLR4-mediated inflammatory responses, we evaluated the impact of LPS treatment on proinflammatory cytokines production, neutrophil activation, and infection control in TLR4^{-/-} knockout mice. Consistent with our hypothesis, LPS treatment did not boost TNF- α and IL-1 β production, or neutrophil activation in TLR4^{-/-} infected wounds (Fig. 4A-C). Importantly, LPS-treated TLR4^{-/-} wounds contained ~ 2.4 log-order more bacteria than LPS-treated C57B normal wounds, indicating that LPS-induced enhanced infection control in wound is primarily dependent on its ability to trigger inflammatory responses by engaging TLR4 receptor (Fig. 4D). Of note, TLR4^{-/-} wounds contained significantly more bacteria (~ 0.8 log-order) than C57B normal wounds, indicating that TLR4 plays an important role in the recognition and inflammatory responses to *P. aeruginosa* infection (Fig. 4E).

Immunomodulators do not adversely affect healing in infected wounds.

Exuberant and persistent inflammation has been shown to be a major impediment to tissue repair and wound healing in chronic wounds (110-112). Although these immunomodulators

were effective in reducing infection, there remained a possibility that their use could lead to exuberant inflammation, which could harm tissue repair and adversely impact healing processes. To assess the potential long-term harmful side-effects of immunomodulator-based therapies on tissue repair, we assessed healing in wounds treated with immunomodulators or PBS and infected with PA103 (10^6) by digital photography, as described (71, 89). Data indicated that not only treatment with immunomodulators did not harm healing processes, they modestly but significantly improved healing in infected wounds (Fig. 5A-F).

Immunomodulators enhance proinflammatory responses in uninfected wound without harming healing processes.

Majority of surgical sites do not become infected (113, 114). Therefore, for immunomodulators to have therapeutic value, they must not adversely affect healing processes in uninfected wounds. To assess the impact of immunomodulators on healing processes in uninfected wounds, we first assessed inflammatory responses in uninfected wounds treated with fMLP (50ng/wound), CCL3 (1 μ g/wound), or LPS (100ng/wound). Data indicated that treatment with CCL3 and LPS significantly increased IL-1 β , TNF- α , and MPO proinflammatory markers in uninfected wounds (Fig. 6A-C). fMLP-treated uninfected wounds showed trends toward higher IL-1 β and TNF- α contents but the differences did not reach statistical significance, as compared to PBS treated wounds (Fig. 6A-B). Of note, fMLP treatment significantly increased activated neutrophil contents (MPO) in uninfected wounds (Fig. 6C). Corroborating these data, CCL3 and LPS immunomodulators also increased leukocytes contents in uninfected wounds and similar to infected wounds, fMLP treatment increased leukocyte contents in uninfected wounds, although the differences did not reach statistical significance (Fig. 6D-E). These data indicated that these immunomodulators can boost inflammatory responses in uninfected wounds, albeit to different degree. We next assessed the impact of immunomodulators on wound healing in uninfected wounds. Similar to infected wounds, treatment with these immunomodulators did not harm healing processes, rather, they also modestly improved healing in uninfected wounds (Fig. 7).

DISCUSSION

We set out to examine whether treatment with immunomodulators - that can boost and direct innate immune responses at wound surgical sites - would be able to reduce infection at surgical wound site. For this purpose, we chose three proinflammatory immunomodulators; namely, fMLP, CCL3, and LPS, based on their documented ability to elicit strong inflammatory responses (63, 76-81, 87). Our data show that one-time topical treatment with these immunomodulators at very low doses were as effective, (if not better), as prophylactic Tobramycin in reducing *P. aeruginosa* infection in wound. Encouragingly, our data indicate that these immunomodulators not only did not adversely affect tissue repair and healing processes in infected or uninfected wounds, but they also improved wound healing, albeit modestly. Given that inflammation, aside from its role in combating invading pathogens, plays a critical role in wound healing processes (39, 115), our data suggest that immunomodulators may be able to accelerate healing processes even in the absence of infection by jumpstarting inflammatory responses.

Harnessing host innate immune powers by immunomodulators to control SSI has several advantages over prophylactic antibiotics, although they could also potentially be administered in combination with antibiotics to further enhance their effectiveness. First, it is highly unlikely for a pathogen to develop resistance to all antimicrobial weapons that our immune system has at its disposal, including; phagocytosis, bursts of reactive oxygen species (ROS), hypochlorous acid (HOCl), neutrophil extracellular traps (NET), and antimicrobial peptides (AMPs) which are our own natural antibiotics with diverse structures and activities against viral, fungal, and bacterial pathogens (37-43, 116-119). Second, the choice of antibiotic prophylaxis is empirical and is determined based on the most probable cause of infection at the particular surgical site (21, 120). Prophylactic antibiotics could fail if the patient encounters a different pathogen or a pathogen that is resistant to the administered antibiotic (121). In contrast, immunomodulator-based therapies would not be empirically based because they mobilize innate immune system at surgical sites which in theory should be effective against majority of infections regardless of their origin (bacterial, fungal, or viral), due to the plethora of antimicrobial defenses at the disposal of innate immune system as discussed above. Third, immunomodulators likely have fewer undesirable side effects and may be safer than prophylactic antibiotics. For example, it is unlikely that topical use of immunomodulators would result in development of resistance, akin to antibiotic resistance, or lead to gut dysbiosis, as many antibiotics do (24-29).

Healthy people are expected to have normal immune responses, which for the most part are effective in protecting us against infection. Therefore, one might question the therapeutic value of using immunomodulators in healthy people with intact immune system. We posit that normal immune function does not necessarily mean optimal immune function which is needed to repel infection after surgery for the following reasons. First, inflammatory leukocytes' migration (such as neutrophil influx) reach their peak between 1-3 days after injury, depending on whether or not infection is present, although they begin migrating into the wound site immediately after injury (61-63). This lag period before inflammatory leukocytes reach their peak can potentially leave tissues at surgical sites unprotected and vulnerable to infection. In addition, many pathogens have evolved stealth virulence strategies that dampen host's immune responses, further delaying the immune function from reaching its optimal peak and allowing these pathogens to establish infection even in immunocompetent healthy individuals (64-68). Immunomodulators could potentially overcome at least some of these stealth strategies and shorten the period needed for immune function to reach its peak, thus fortifying tissue's defenses against invading pathogens.

In conclusion, we provide evidence that immunomodulators with the ability to mobilize and direct inflammatory responses at surgical site in wound, can enhance surgical site tissue defenses against infection without adversely impacting healing processes and tissue repair. Future studies should look to optimize immunomodulator-based therapies by increasing their levels without harmful side-effects, and by combining them with each other or with prophylactic antibiotics.

ACKNOWLEDGEMENTS:

We would like to thank the rest of Shafikhani lab members for their valued opinions on these studies. This work was supported by the National Institutes of Health (NIH) grants R01AI150668 and R01DK107713, both to (S.H.S.).

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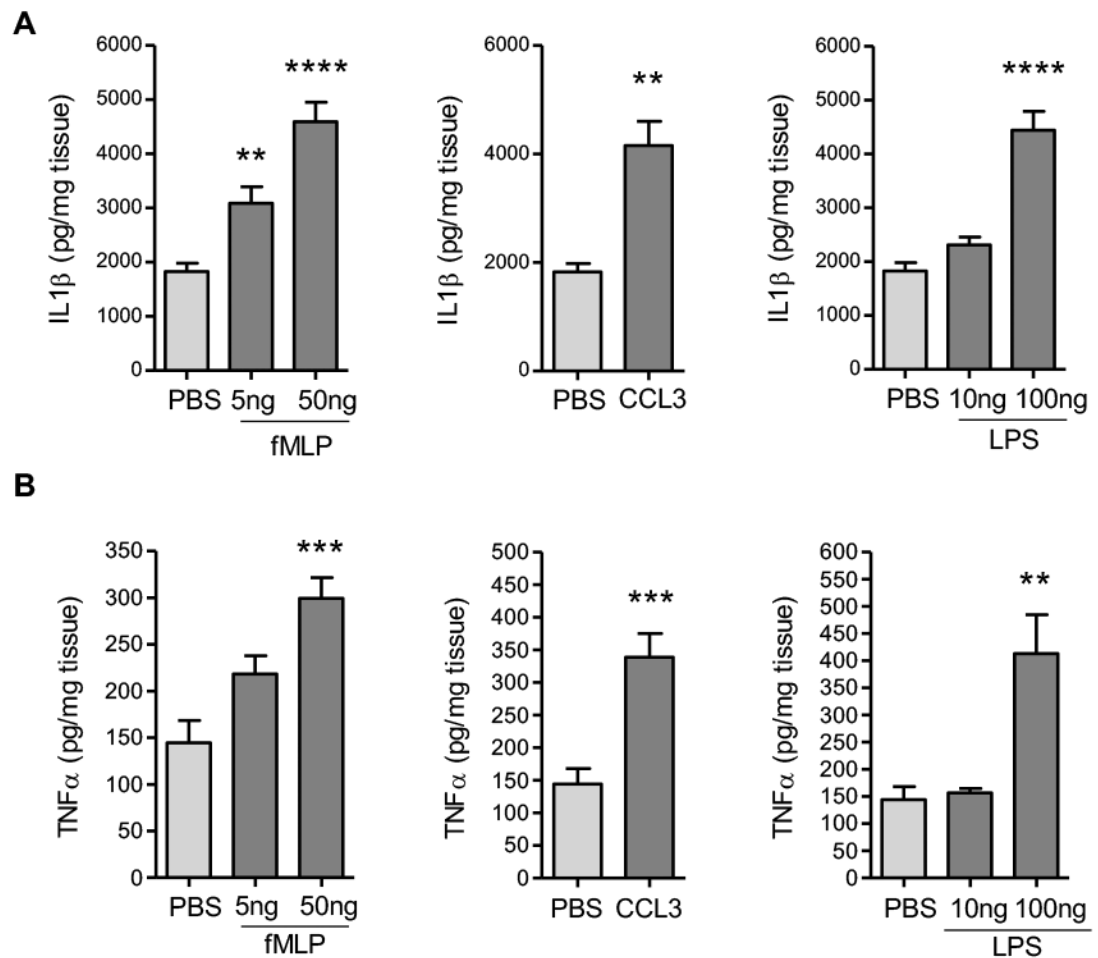


Figure 1. Immunomodulators increase proinflammatory cytokines production in wounds.

Wounds on C57B mice were topically treated with the indicated immunomodulators (fMLP at 5ng or 50ng/wound; CCL3 at 1 μ g/wound; and LPS at 10ng or 100ng/wound) or PBS control after wounding. Wounds were then infected with PA103 *P. aeruginosa* strain (at 10⁶ bacteria/wound). 24 hours after infection, the levels of IL-1 β (A) and TNF- α (B) in wounds were determined by ELISA after normalization with tissue weight. The corresponding data are shown as Mean \pm SEM. (7 mice/group were used in this study except for fMLP at 5ng/wound and LPS at 10ng/wound where n=3 mice/groups. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

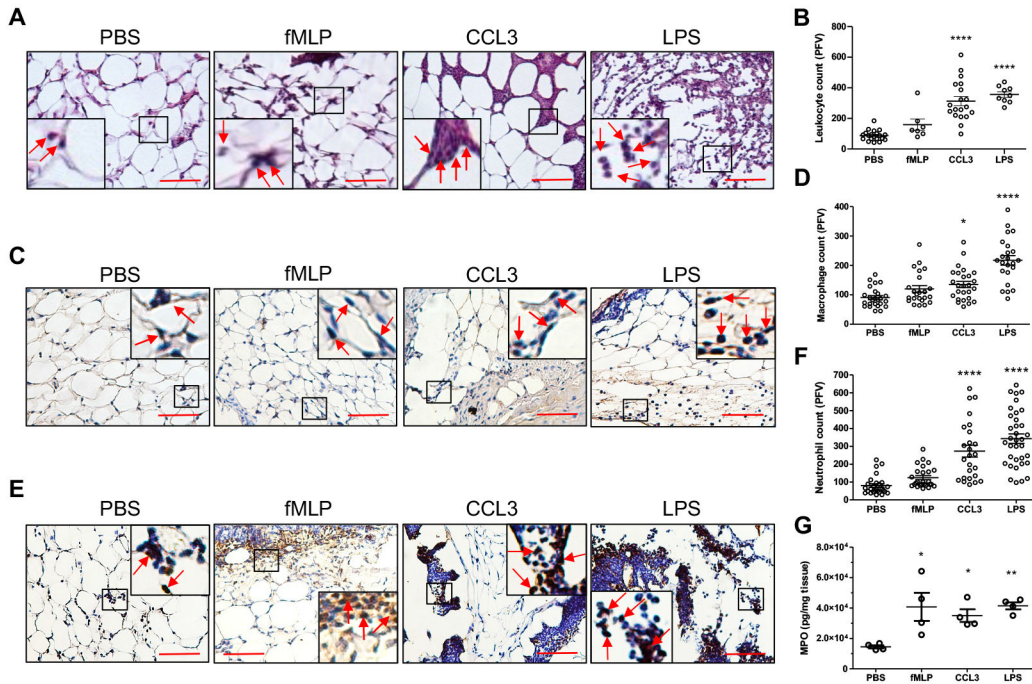


Figure 2. Immunomodulators enhance leukocytes influx and neutrophil activation in infected wound.

C57BL/6 wounds were treated with PBS or received topical treatments with fMLP, CCL3, and LPS, (at 50ng, 1µg, and 100ng per wound, respectively), before infection with 10^6 PA103. 24 hours after infection, wounds were examined for their leukocytes' contents by histological analysis, using H&E staining (A & B); for their macrophage contents using anti-CD68 antibody (C & D); and for their neutrophil contents using anti-Ly6G (E & F). Representative regions from underneath the wounds extending in the dermis are shown in (A, C, & E), and the corresponding data are plotted as Mean \pm SEM and shown in (B, D, & F). (Inserts are the magnified regions indicated by smaller rectangles within the images. Red scale bar = 50µm). (G) The aforementioned wounds were assessed for their activated neutrophil contents by evaluating their MPO contents by ELISA. (N 3 mice/group, 9 random fields/wound/mouse. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

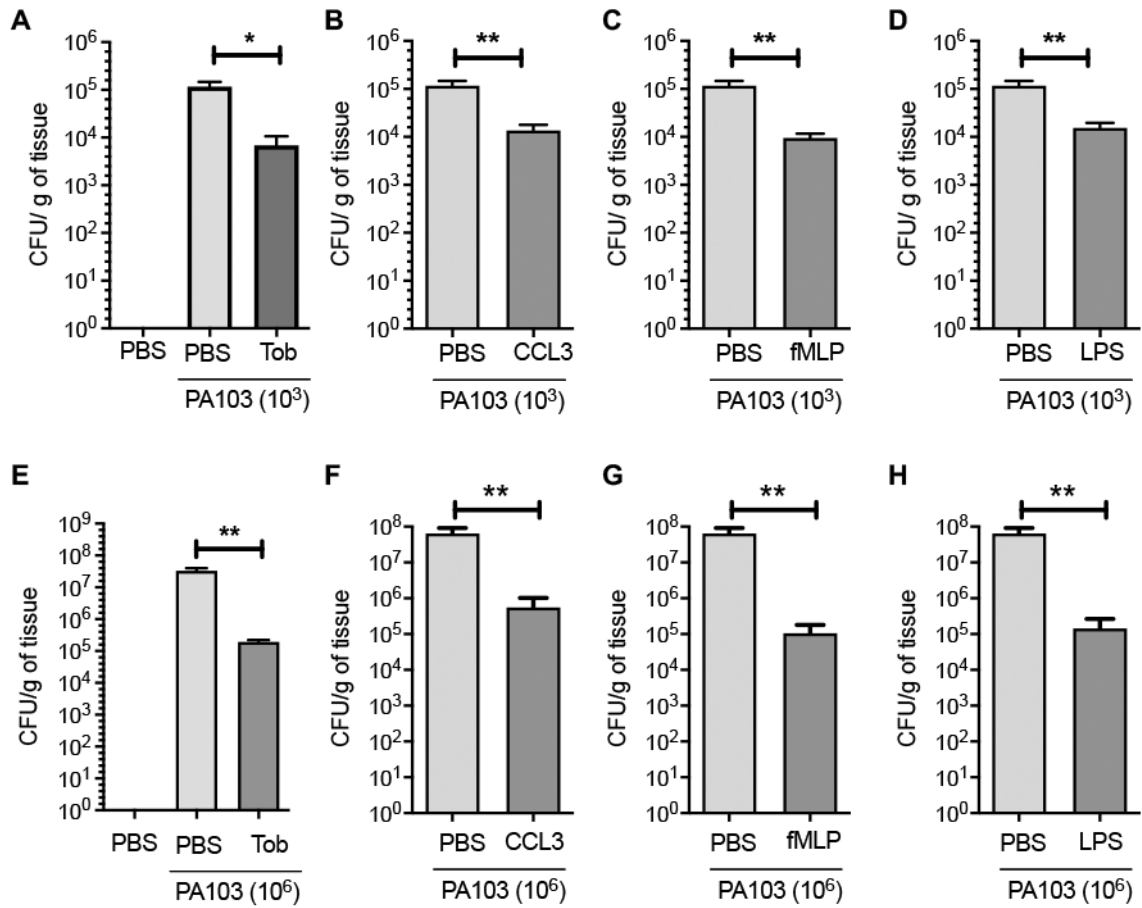


Figure 3. Immunomodulators enhance infection control in wound.

(A and E) C57BL/6 mice received systemic Tobramycin (Tob) or PBS or by i.p. injection 1h before surgical wounding. They were then infected with PA103 at 10³ (A) or 10⁶ (E) bacteria/wound. Wounds were collected at 24h after infection and assessed for their bacterial contents by bacterial colony forming unit determination (CFU). (B-D and F-H) C57BL/6 wounds were infected with either 10³ (B-D) or 10⁶ (F-H) PA103 bacteria per wound after treatment with fMLP, CCL3, and LPS immunomodulators. Wounds were then collected 24h after treatment, and infection was assessed for their bacterial contents by CFU determination. Data were normalized with their corresponding wound tissue weight. (These experiments were performed twice, each time with N=4 mice/group; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

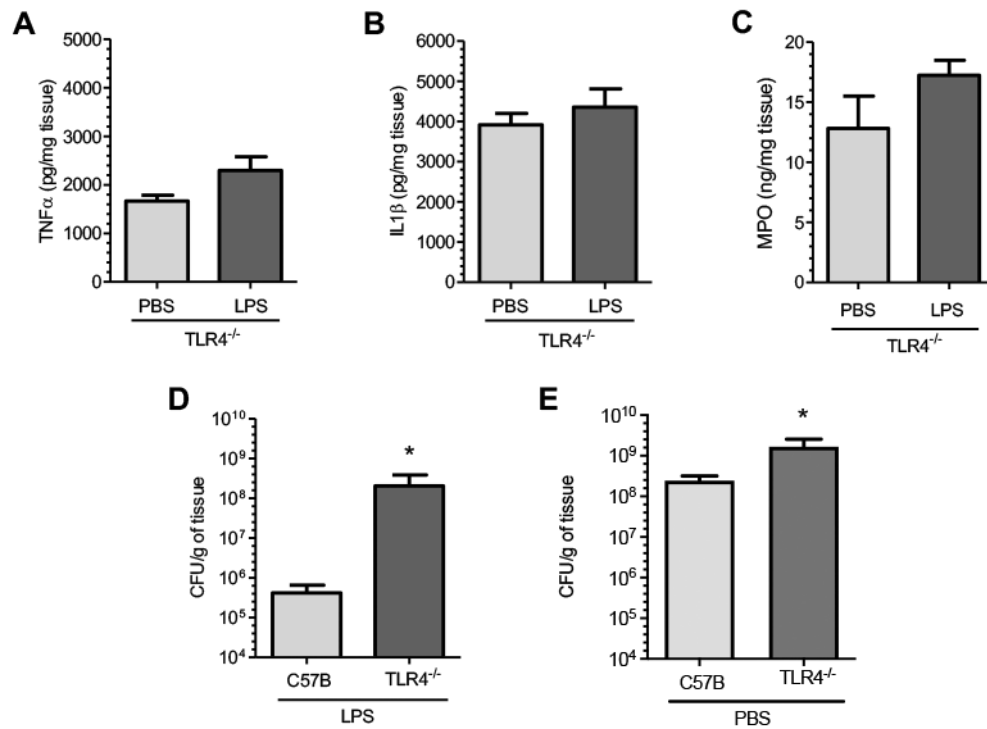


Figure 4. LPS-induced enhancement in infection control in wound is dependent on TLR4 receptor signaling.

(A-C) Wounds in TLR4^{-/-} mice were treated with PBS or received LPS (100ng/wound) topical treatment before infection with 10⁶ PA103. 24 hours after infection, wounds were examined for TNF-α (A), for IL-1β (B), for activated neutrophils, using MPO (C), all by ELISA. (D) Wounds in C57BL/6 and TLR4^{-/-} mice were treated with PBS or received LPS (100ng/wound) topical treatment before infection with 10⁶ PA103. 24 hours after infection, wounds were assessed for their bacterial counts by CFU determination. The corresponding data are plotted as Mean ± SEM. (N=4 mice/group; *p<0.05).

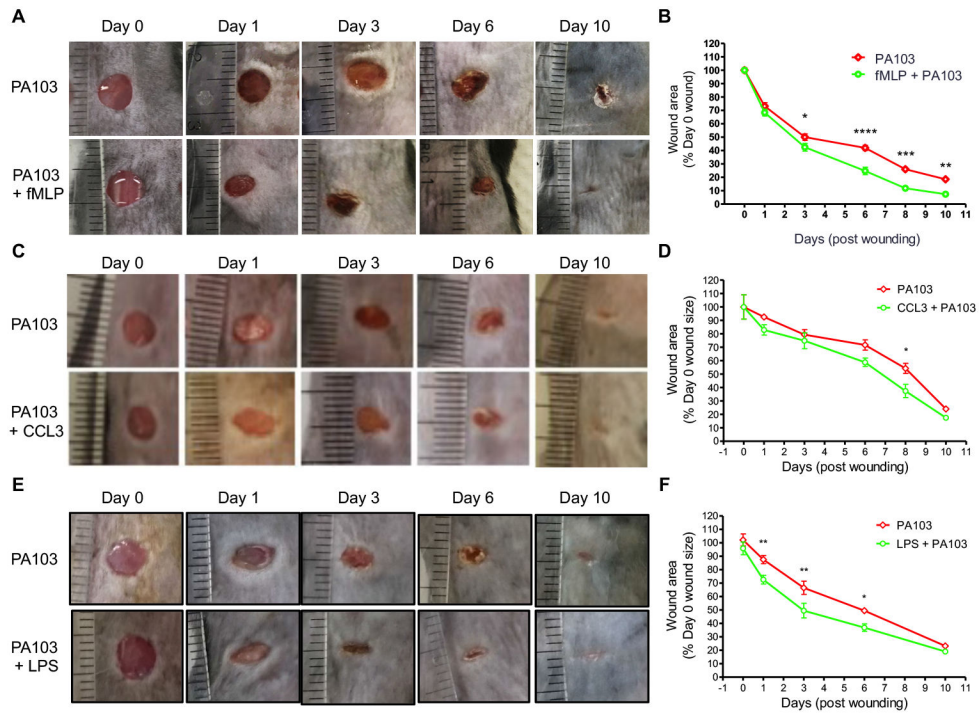


Figure 5. Immunomodulators do not adversely affect healing in infected wounds. (A-F) Wounds in C57BL/6 mice were treated with fMLP (50ng), CCL3 (1µg), or LPS (100ng) per wound prior to infection with 10⁶ PA103. Wound healing was assessed by digital photography at day 0 (at the time of wounding and treatment) and at days 1, 3, 6, and 10 post-infection. Representative wound images are shown in (A, C, & E) and the corresponding data are plotted as the Mean ± SEM and shown in (B, D, & F). (N = 4 mice/group; *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001).

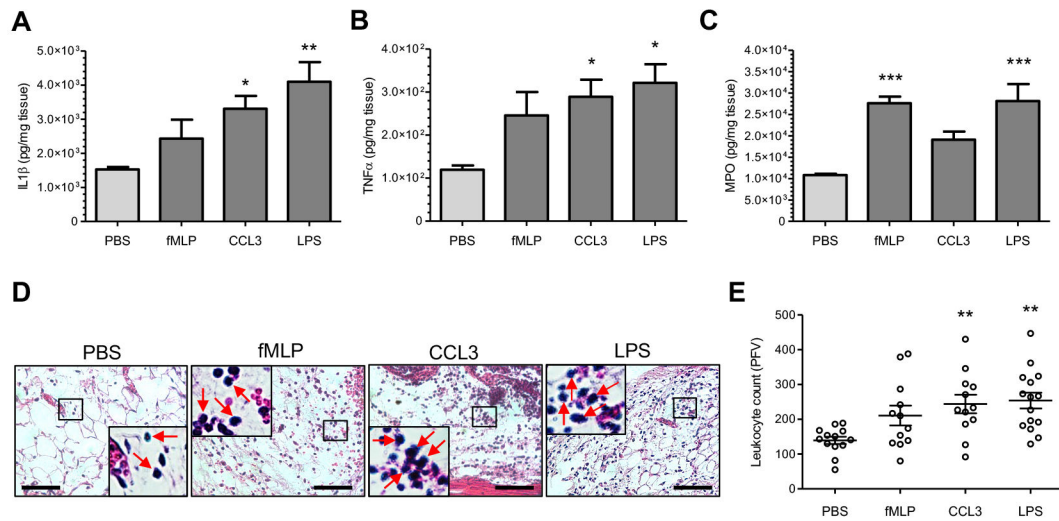


Figure 6. Immunomodulators enhance proinflammatory responses in uninfected wound. Wounds in C57BL/6 were treated with PBS or received fMLP, CCL3, or LPS (at 50ng, 1 μ g, or 100ng per wound, respectively). 24 hours after treatment, wounds were analyzed for their IL-1 β (A), for TNF- α (B), for their MPO contents (C), all by ELISA. (D-E) The aforementioned wounds were assessed for their leukocytes' contents by histological analysis, using H&E staining. Representative regions from underneath the wounds extending in the dermis are shown at indicated magnification in (D) and the corresponding data are plotted as Mean \pm SEM and shown in (E). Black scale bar = 50 μ m. (Inserts are the magnified regions indicated by smaller rectangles within the images). (N=4 mice/group; *p<0.05, **p<0.01, ***p<0.001).

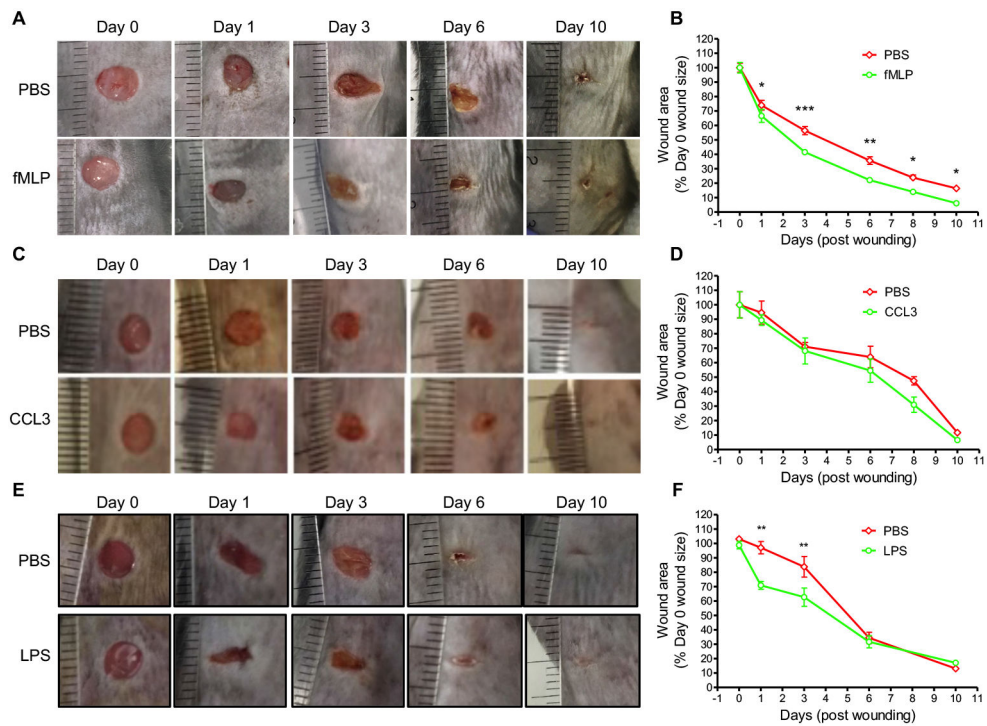


Figure 7. Treatment with immunomodulators do not adversely affect healing in uninfected wounds.

Wounds in C57BL/6 were treated with PBS or received fMLP, CCL3, or LPS at 50ng, 1µg, and 100ng per wound respectively. Wound healing was assessed by digital photography at day 0 (at the time of wounding at treatment), and at days 1, 3, 6, and 10 post-wounding and treatment. Representative wound images are shown in (A, C, & E) and the corresponding data are plotted as the Mean ± SEM and shown in (B, D, & F). (N 4 mice/group; *p<0.05, **p<0.01, ***p<0.001).