



# Understanding the host inflammatory response to wound infection: An in vivo study of *Klebsiella pneumoniae* in a rabbit ear wound model

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Manuscript received: September 13, 2011  
Accepted in final form: December 19, 2011

DOI:10.1111/j.1524-475X.2012.00764.x

## ABSTRACT

Wound infection development is critically dependent on the complex interactions between bacteria and host. *Klebsiella pneumoniae* has become an increasingly common wound pathogen, but its natural history within wounds has never been studied. Using a validated, in vivo rabbit ear model, wounds were inoculated with *K. pneumoniae* at different concentrations ( $10^2$ – $10^7$  colony-forming units) with measurement of viable and nonviable bacterial counts, histological wound-healing parameters, and host inflammatory gene expression at multiple time points postinoculation (48, 96, and 240 hours). Bacteria and wound morphologies were evaluated with scanning electron microscopy. Comparable experiments were performed in ischemic ears to model immune response impairment. All wounds, despite different inoculants, equilibrated to similar bacterial concentrations by 96 hours. With a  $10^6$  colony-forming units inoculant, wounds at 240 hours showed decreased bacterial counts ( $p < 0.01$ ), with a corresponding improvement in healing ( $p < 0.01$ ) and a decrease in inflammatory response ( $p < 0.05$ ). In contrast, ischemic wounds revealed impaired inflammatory gene expression ( $p < 0.05$ ) resulting in higher steady-state bacterial concentrations ( $p < 0.01$ ), impaired healing ( $p < 0.05$ ), and biofilm formation on scanning electron microscopy. We conclude that a normal inflammatory response can effectively stabilize and overcome a *K. pneumoniae* wound infection. An impaired host cannot control this bacterial burden, preventing adequate healing while allowing bacteria to establish a chronic presence. Our novel study quantitatively validates the host immune response as integral to wound infection dynamics.

The natural history of bacterial wound infections remains a complex and poorly understood process. The ultimate consequence of this bacterial infiltration is a profound impairment in wound healing, with both a delay in keratinocyte migration and granulation tissue formation.<sup>1</sup> Several factors influence the magnitude of bacterial proliferation and persistence that occurs, including how the wound is managed,<sup>2–4</sup> virulence and survival mechanisms specific to the bacterial pathogen,<sup>5–9</sup> and the ability of the host organism to control and eliminate the infection.<sup>10–12</sup> In particular, the interplay between bacteria and the inflammatory cascade of their host continues to be an area of significant and intense investigation.<sup>13–17</sup> Following tissue damage and/or bacterial invasion, local mast cells and neutrophils release several chemokines and proinflammatory cytokines, such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), which increase local blood vessel permeability and recruitment of neutrophils and macrophages. These macrophages, which mature from the infiltrating monocytes, perpetuate the response with the release of TNF- $\alpha$  and interleukins (IL) (e.g., IL-1, IL-8), whose release are further augmented by the presence of bacterial endotoxins. In addition, macrophages and neutrophils react with and eliminate wound bacteria

directly or indirectly through antibodies while clearing other cellular debris in preparation for fibroblast-based wound remodeling.<sup>11,12,14,18</sup> However, these complex pathways are compromised in the impaired host (e.g., arterial insufficiency or ischemia, diabetic microvascular disease, reperfusion injury), where bacteria trigger an elevated, but ineffective, inflammatory response.<sup>10,15</sup> In particular, the hypoxic conditions associated with ischemia have been shown to limit the bacterial killing capacity of isolated human neutrophils.<sup>19</sup>

Although host immunity is critical in limiting the magnitude of a wound infection, the protective strategies employed by bacteria also influence the equilibrium that is achieved within an infected wound bed. Beyond the secretion of species-specific toxins or the expression of resistance genes, the survival advantage associated with bacterial biofilm formation has transformed the entire field of microbiology.<sup>7,10,20–22</sup> Bacterial biofilm can be defined as a complex community of aggregated bacteria embedded within an extracellular matrix, or extracellular polymeric substance (EPS).<sup>7,9,10</sup> This morphology, thought to be the preferred state of bacteria in their “natural” habitats, creates a physical barrier that prevents effective phagocytosis by inflammatory

Report Documentation Page				Form Approved OMB No. 0704-0188	
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1. REPORT DATE <b>01 MAR 2012</b>		2. REPORT TYPE <b>N/A</b>		3. DATES COVERED <b>-</b>	
4. TITLE AND SUBTITLE <b>Understanding the host inflammatory response to wound infection: An in vivo study of Klebsiella pneumoniae in a rabbit ear wound model</b>				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) <b>Seth A. K., Geringer M. R., Gurjala A. N., Abercrombie J. A., Chen P., You T., Hong S. J., Galiano R. D., Mustoe T. A., Leung K. P.,</b>				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) <b>United States Army Institute of Surgical Research, JBSA Fort Sam Houston, TX</b>				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION/AVAILABILITY STATEMENT <b>Approved for public release, distribution unlimited</b>					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT					
15. SUBJECT TERMS					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT <b>UU</b>	18. NUMBER OF PAGES <b>12</b>	19a. NAME OF RESPONSIBLE PERSON
a REPORT <b>unclassified</b>	b ABSTRACT <b>unclassified</b>	c THIS PAGE <b>unclassified</b>			

cells, inhibits complement cascade activation,<sup>23</sup> and inhibits the activity of antibiotics.<sup>9,24</sup> In addition, biofilm-phase bacteria possess a decreased growth rate that leaves them less susceptible to antibiotics and use cell-cell signaling (quorum sensing) to enhance their virulence and survival.<sup>2,6,25</sup> Although biofilms are harbored on surfaces throughout the body, the moist, nutritionally supportive microenvironment of the wound bed matrix is an ideal setting for bacterial biofilm formation, creating a destructive and sustainable interaction that impairs wound healing.<sup>10</sup> Furthermore, chronic wound infections have been directly linked to the presence of biofilm-phase bacteria, as validated by both imaging and sophisticated molecular sampling techniques.<sup>7,21,22,25–28</sup> This association between chronic wounds and biofilm is particularly evident in those with underlying pathology that has compromised their host defense mechanisms.<sup>10,20,21,26</sup>

To date, the majority of research aimed at defining the interaction between bacteria and their host has involved more common wound pathogens such as *Staphylococcus aureus* and *Pseudomonas aeruginosa*.<sup>14,16,17,29</sup> *Klebsiella pneumoniae*, classically an opportunistic, respiratory pathogen, triggers a destructive pneumonia in hospitalized patients or individuals with weakened immune systems.<sup>30</sup> Given this association, no studies have investigated the natural development of *K. pneumoniae* wound infections, in particular their progression in the face of an intact immune response. However, numerous studies have recently reported on the prevalence of drug-resistant strains of *K. pneumoniae* in the infected war wounds of soldiers returning from Iraq.<sup>31–33</sup> In addition, there has been an increasing amount of literature on nosocomial wound infections associated with Gram-negative bacteria such as *K. pneumoniae*, particularly in burn wounds.<sup>31,34,35</sup> These reports indicate a growing need to further understand this relatively uncommon wound pathogen, thus providing an impetus for this study.

The purpose of this study was to study the natural history of *K. pneumoniae* wound infections using an adaptation of the quantitative, in vivo rabbit dermal ulcer model.<sup>36</sup> In particular, we have identified and defined the role of the host inflammatory response in the progression of such infections. We have demonstrated that the immune response is critical to the stabilization and eventual clearance of these bacterial wound infections. In addition, the host's ability to control the level of bacterial burden within the wound directly correlates with the amount of wound healing that is achievable. We further support this hypothesis by comparing these outcomes with those in an impaired host, which is unable to mount an appropriate or effective response to bacteria. In this compromised state, bacteria are allowed to transition into a more protected, biofilm state. Through these experiments, we have developed a greater understanding of the intricate interactions between bacteria and their hosts' defenses, setting the foundation for future mechanistic investigation.

## METHODS

### Animals

Under an approved protocol by the Animal Care and Use Committee at Northwestern University, adult New Zealand white rabbits (3–6 months, ~3 kg) were acclimated to standard housing and fed ad libitum. All animals were housed in

individual cages under constant temperature and humidity with a 12-hour light-dark cycle.

### Bacterial culture and wound infection

*K. pneumoniae* BAMC 07–18 (kindly provided by LTC Clinton Murray of Brooke Army Medical Center, Fort Sam Houston) was used to study its effect on wound healing. The strain was originally isolated from the wounds of an injured soldier returned from Iraq during the war. To prepare bacterial culture for the wound infection study, a single colony of *K. pneumoniae* BAMC 07–18 was picked from an overnight culture grown on a blood agar plate (VWR, West Chester, PA) and inoculated into 10 mL of tryptic soy broth (TSB). After overnight incubation at 37 °C, 0.5 mL of the *K. pneumoniae* culture was transferred to 9.5 mL of fresh sterile TSB and incubated at 37 °C until the culture reached the log phase. Bacteria were harvested and washed in phosphate buffered saline (PBS) once by centrifugation at 4,000 r.p.m. for 15 minutes at 4 °C. The resultant pellet was resuspended in PBS and adjusted to an optical density of 0.5 at the wavelength of 600 nm (OD<sub>600</sub>). In our earlier experiments (data not shown), *K. pneumoniae* BAMC 07–18 at OD<sub>600</sub> 0.5 was equivalent to 10<sup>8</sup> colony-forming units (CFU) per mL. Ten microliters of bacteria suspensions at OD<sub>600</sub> 0.5 and its 10-fold serial dilutions were used as inoculum for infecting the rabbit ear wounds.

### Wound protocol and infection model

Rabbits were anesthetized with intramuscular injection of ketamine (22.5 mg/kg) and xylazine (3.5 mg/kg) mixture prior to surgery. Ears were shaved, sterilized with 70% ethanol, and injected intradermally with 1% lidocaine/1:100,000 epinephrine at the planned wound sites. Six, 6-mm diameter, full-thickness dermal wounds were created on the ventral ear down to perichondrium and dressed with Tegaderm (3 M Health Care, St. Paul, MN), a semiocclusive transparent film. Individual wounds were inoculated with different concentrations (using previously described 10-fold serial dilutions) of *K. pneumoniae* on postoperative day (POD) 3, as dictated by the experiment being performed. Bacteria were allowed to proliferate under the Tegaderm dressing. All dressings were checked daily throughout the protocol.

### Ischemic rabbit ear model

Ischemic wounds were created using the well-established ischemic rabbit ear model.<sup>36</sup> In brief, for each ischemic ear, an incision was made at the ear base in a circumferential manner down to bare cartilage to completely disrupt dermal blood flow to the ear. The caudal and central arteries were then dissected and ligated with 6-0 nylon suture while leaving their respective veins intact to ensure adequate venous outflow. The rostral artery and vein were kept intact as the sole pedicle supplying the ear. The dermal incision was closed and 6-mm punch wounds were created on the ventral surface of the ear as previously described.

### Harvesting of wounds

After euthanizing the animals by an intracardiac euthasol injection, wounds were harvested for various analyses.

Wounds were excised, utilizing a 10-mm biopsy punch (Acuderm Inc., Fort Lauderdale, FL). For viable bacterial count and real-time quantitative polymerase chain reaction (RT-qPCR) for viable and nonviable bacterial counts, nonischemic wounds were harvested at 24-hour intervals between 0 and 96 hours postinoculation and at 240 hours postinoculation, while ischemic wounds were harvested at 0, 48, and 96 hours. Nonischemic wounds were harvested at 48, 96, and 240 hours, and ischemic wounds at 48 and 96 hours, postinoculation for reverse transcriptase qPCR analysis to measure mRNA levels of inflammatory cytokines and scanning electron microscopy (SEM) to visualize presence of bacteria. For wound-healing experiments, ischemic and nonischemic wounds were harvested at 96 hours, with additional nonischemic wounds harvested at 240 hours. The wounds from a total of 40 rabbits were used to complete this project.

### Bacterial count measurements

The dorsal side of wounds used for bacterial counts were removed to eliminate the inclusion of bacteria outside of the infected wound surface. To recover bacteria, infected wound samples harvested at different time points were placed in tubes prefilled with homogenizer beads (Roche, Indianapolis, IN). One milliliter of PBS was added to the tube and was homogenized for 90 seconds at 5,000 r.p.m. in a MagNA Lyser homogenizer (Roche). Upon completion of the homogenization, 0.5 and 0.2 mL of the homogenate was removed for viable counts and for isolating bacterial genomic DNA, respectively.

For viable counts, 0.5 mL of wound homogenate was placed in a 1.5-mL Eppendorf tube and sonicated for 2 minutes in a Microson™ Ultrasonic Cell Disruptor set at a low-power level to break up any bacterial clumps recovered from the tissue. The power setting was predetermined empirically to ensure that the process would not cause bacterial cell death. The bacterial suspension was 10-fold serially diluted, and 50  $\mu$ L of each dilution was plated in triplicate onto blood agar plates using a WASP spiral plater (DW Scientific, Frederick, MD). Bacterial viable counts were determined using a ProtoCOL colony counter (Microbiology International, Frederick, MD) after overnight incubation of plates at 37 °C.

To recover bacterial DNA, bacteria were isolated by centrifugation of 200  $\mu$ L of the homogenate at 14,000 r.p.m. (Eppendorf 5417R) for 3 minutes at 4 °C. Bacterial DNA was isolated using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA), according to the manufacturer's instructions with a slight modification on the elution process. DNA from each sample was eluted twice in 200- $\mu$ L molecular biology grade H<sub>2</sub>O. Five microliters of DNA sample was used as the template for each of the RT-qPCRs.

### RT-qPCR

The sequences of primers and probe set used for the quantification of *K. pneumoniae* by PCR of infected wound samples were designed based on the sequence of the hemolysin gene (*khe*) associated with this organism. This gene is highly conserved among *K. pneumoniae* isolates.<sup>37</sup> Primers and probe were synthesized by Applied Biosystems (Carlsbad, CA). The sequences of the primers and probe set were: forward primer, 5'-AGCGATGAGGAAGAGTTCATCTA-3';

reversed primer, 5'-AGACGAACCTCCTGCTCGGTGTTA-3'; and probe, 6FAMCTATCCGGAAGTGTGGATAAACG GCTAMGBNFQ.

The specificity of the primers and probe set for *K. pneumoniae* was confirmed by their lack of amplification when genomic DNA from other bacteria was used as the template in the PCR reactions. Test organisms used to test the specificity of the primers and probe set included *S. aureus*, *S. epidermidis*, *P. aeruginosa*, and *Acinetobacter baumannii*. *K. pneumoniae* cultures, obtained from the midlog growth phase, were used to isolate genomic DNA (as described above) to establish a standard curve for the bacterial quantification of wound samples. The concentration of isolated genomic DNA was determined using a Quant-iT ds DNA BR Assay Kit (Invitrogen, Carlsbad, CA) and confirmed by gel electrophoresis.

All RT-qPCR reactions were performed with a StepOne Plus RT-qPCR System (Applied Biosystems) using optical grade 96-well plates. All samples were run in duplicates. Each of the PCR reaction was performed in a total volume of 20  $\mu$ L of TaqMan Gene Expression Master Mix (Applied Biosystems) containing 100 nM of each of the forward, reverse primers, and the TaqMan MGB probe, and 5- $\mu$ L of the sample template. The reaction conditions for the amplification of the DNA sample were 95 °C for 5 minutes, 40 cycles of 95 °C for 15 seconds, and 60 °C for 1 minute. Data were analyzed using the StepOne software provided by Applied Biosystems. The amount of *K. pneumoniae* genomic DNA in each sample was converted into genome copy number and compared with the number of CFU obtained by the plating method.

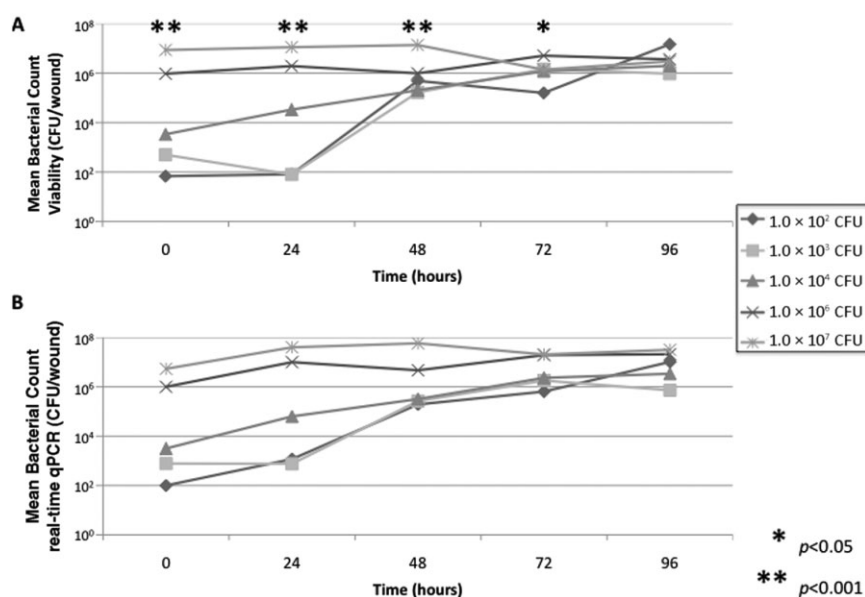
### Histological analysis

Wounds excised for histological analysis were bisected at their largest diameter for hematoxylin and eosin (H&E) staining. Tissues were fixed in formalin, embedded in paraffin, and cut into 4- $\mu$ m sections. Paraffin was removed with a xylene wash, followed by a standard H&E staining protocol to prepare samples for analysis under a light microscope. Slides were examined for quantification of epithelial and granulation gaps, and total epithelial and granulation areas, using a digital analysis system (NIS-Elements Basic Research, Nikon Instech Co., Kanagawa, Japan), as previously described.<sup>38</sup> Two blinded independent observers evaluated all histological sections. The results of both examiners were averaged. Slides were omitted if results differed more than 30% among examiners.

### SEM

To determine biofilm structure, wound samples were fixed in 2.5% glutaraldehyde in 0.1 M PBS (pH 7.2), washed 3 $\times$  in PBS, and dehydrated through an ethanol series and hexamethyldisilazane. Samples were mounted by double-sided tape to specimen stubs, followed by gold-platinum (50 : 50) ion coating (108 Auto Sputter Coater, TedPella, Inc, Redding, CA). Wounds for SEM had their dorsal sides removed prior to preparation to allow for better mounting for visualization. Samples were visualized using a Carl Zeiss EVO-40 scanning electron microscope (Oberkochen, Germany) operated at the scanning voltage of 10-kV.





**Figure 1.** Mean bacterial counts of *Klebsiella pneumoniae* nonischemic wound infections over time following different bacterial inoculant concentrations. Wounds inoculated with five different concentrations of *K. pneumoniae* demonstrated significantly different viable bacterial count (A) and real-time quantitative polymerase chain reaction (RT-qPCR) bacterial count (B) measurements from 0 to 72 hours. The bacterial counts became similar at 96 hours postinoculation as all wounds equilibrated at approximately  $10^6$ – $10^7$  colony-forming units (CFU)/wound ( $n = 8$ – $10$  wounds/inoculant) (\* $p < 0.05$ , \*\* $p < 0.001$ ).

### Total mRNA extraction and reverse transcription-qPCR

Wounds were harvested for mRNA extraction and subsequent cDNA conversion as part of reverse transcription-qPCR. The dermal layer on the dorsal side of the ear was removed, and the wound bed was punched out and immediately snap-frozen in liquid nitrogen. Wound samples were homogenized using a Mini-bead beater-8 equipment (Biospec Products, Inc., Bartlesville, OK) using Zirconia beads (2.0-mm diameter, Biospec Products, Inc.) in the presence of Trizol Reagent (Sigma-Aldrich, St. Louis, MO). Total RNA was isolated according to the manufacturer's protocol. Contaminating genomic DNA during RNA preparation was removed using the Turbo DNA-free kit (Ambion, Austin, TX). Five micrograms of total RNA was used to prepare cDNA using superscript II (Invitrogen) with 100 ng of random primers (Invitrogen).

For quantitative analysis of the level of mRNAs, reverse transcription-qPCR analyses using SYBR green 1 were performed, utilizing an ABI prism 7,000 sequence detection system (Applied Biosystems). PCR primers were designed using the Primer 3 program (<http://frodo.wi.mit.edu/>). Expression of each gene was normalized to the level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), the house-keeping gene, to get the delta cycle threshold ( $\Delta Ct$ ). The  $2^{-\Delta Ct}$  method was used to calculate gene expression of IL-1 $\beta$  and TNF- $\alpha$  within the wounds of interest. Expression of genes was detected by PCR with the following oligonucleotides: IL-1 $\beta$  (5'-CCACAGTGGCAATGAAAATG-3' and 5'-AGA AAGTTCTCAGGCCGTCA-3', accession number D21835), TNF- $\alpha$  (5'-CCAGATGGTCACCCTCAGAT-3' and 5'-TGT TCTGAGAGGCGTGATTG-3', accession number M12845), and GAPDH (5'-AGGTCATCCACGACCACTTC-3' and 5'-GTGAGTTTCCCGTTCAGCTC-3', accession number NM\_001082253).

### Statistical analysis

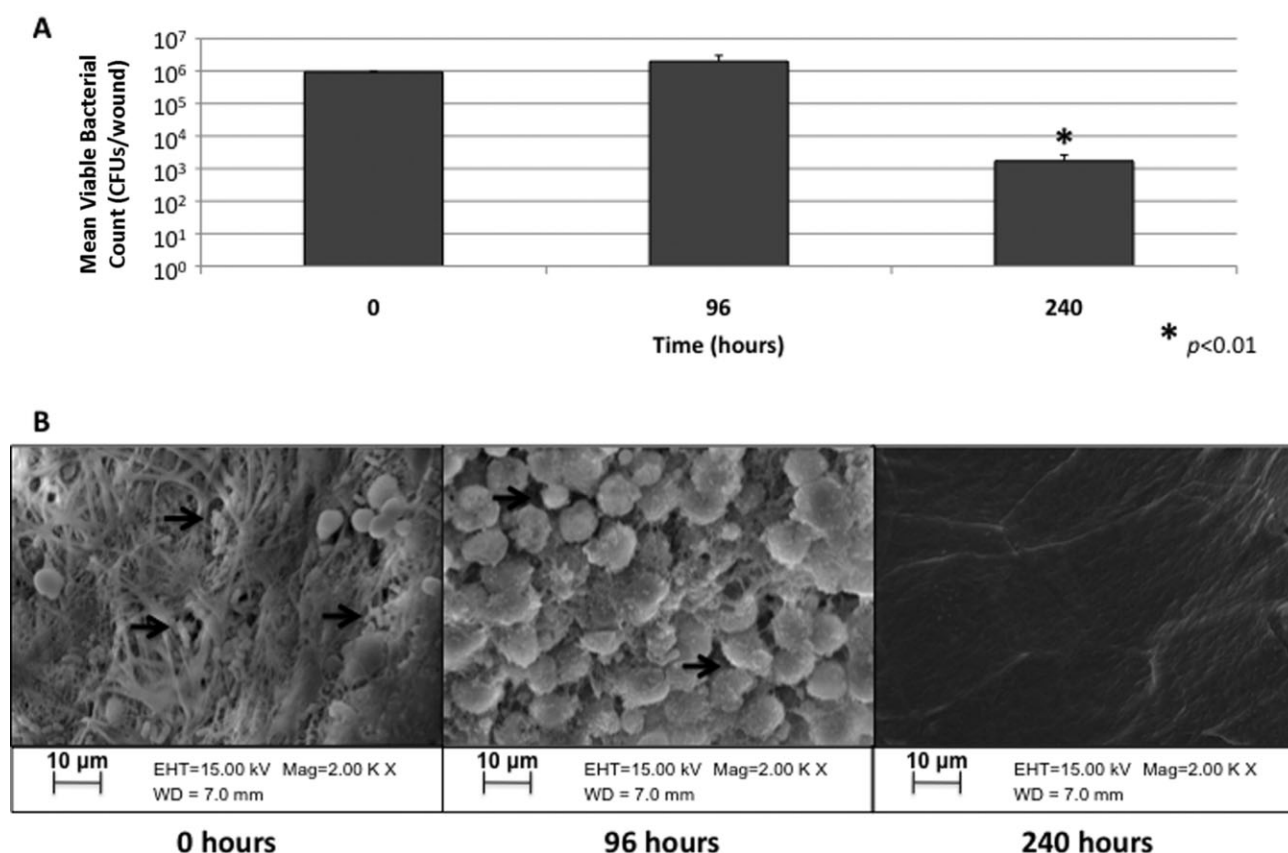
Data are presented in graphical form as mean  $\pm$  standard errors when applicable. Statistical analyses were performed

using the Student's *t*-test (two-tailed, unpaired) when comparing two study groups and the Kruskal–Wallis one-way analysis of variance when comparing the means of multiple groups. The level of significance was set at  $p < 0.05$ .

## RESULTS

Using an adaptation of the in vivo rabbit dermal ulcer model, initial experiments were performed to characterize the natural history of a developing *K. pneumoniae* wound infection. In particular, wounds exposed to different inoculant concentrations of *K. pneumoniae* were followed over time to examine the kinetics of a developing in vivo wound infection (Figure 1). Wounds exposed to a range of bacteria concentrations ( $10^2$ – $10^7$  CFU) demonstrated significant differences in bacterial viable count within the wound from 0 to 48 hours postinoculation. ( $p < 0.001$ ) (Figure 1A). However, at 72 hours, the extent of this difference was reduced ( $p < 0.05$ ), as wounds with lower inoculant concentrations exhibited an increased rate of proliferation relative to higher inoculant wounds. By 96 hours postinoculation, there was no significant difference in wound bacterial burden ( $p = 0.38$ ) between any of the wounds despite the wide range of initial size of bacterial inoculum used. This trend toward a steady-state level of viable bacterial infection, settling at approximately  $10^6$ – $10^7$  CFU/wound, was also validated through RT-qPCR analysis (Figure 1B). These initial results demonstrated an ability by the host to stabilize a proliferating *K. pneumoniae* infection presumably through its local inflammatory response.

Having demonstrated equivalent bacterial concentrations at 96 hours despite the magnitude of inoculum, all additional experiments were performed with a standard inoculant concentration of  $10^6$  CFU/wound. With their short-term dynamics established, wounds were followed for an additional 6 days (total of 240 hours postinoculation) to determine the fate of a stabilized *K. pneumoniae* infection. Viable bacterial count measurement at 240 hours demonstrated a significantly lower level of bacteria ( $p < 0.01$ ) as compared with 0 and 96 hours (Figure 2A). Wounds were also examined using SEM at these



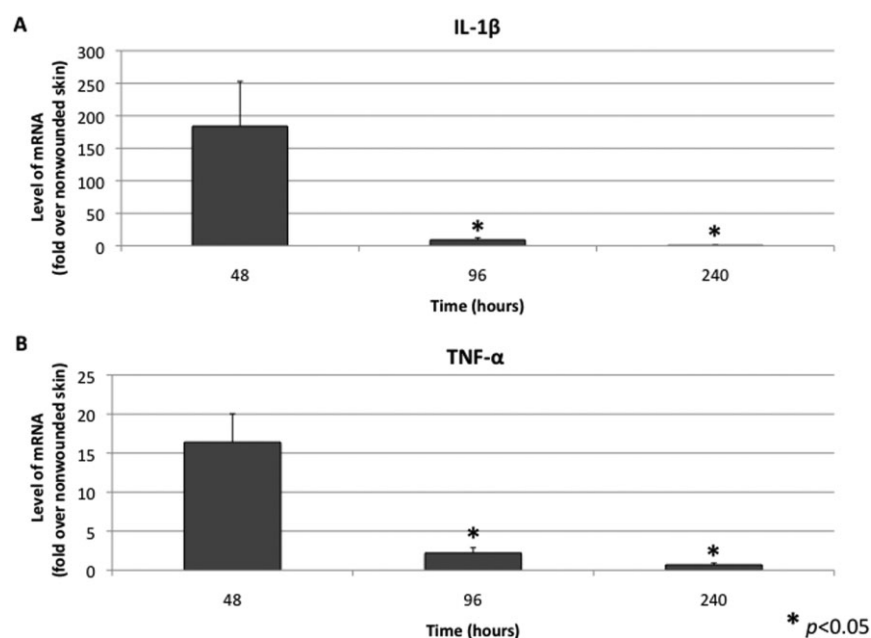
**Figure 2.** Progression of *Klebsiella pneumoniae* wound infection over time in nonischemic wounds. Viable bacterial counts (A) in wounds inoculated with  $10^6$  colony-forming units (CFU) *K. pneumoniae* demonstrated a significant decrease in bacterial burden by 240 hours postinoculation to approximately  $10^3$  CFU ( $n = 8-10$  wounds/group) (\* $p < 0.01$ ). Scanning electron microscopy (B) revealed the presence of bacteria at 0 hours (arrows), a large influx of inflammatory cells (predominantly neutrophils) by 96 hours (arrows), followed by minimal evidence of bacteria with an epithelialized wound bed at 240 hours, correlating with the measured bacterial counts. EHT, extra high tension; WD, working distance.

same time points (Figure 2B), verifying the presence of individual bacteria within the wound bed at time 0 hours. At 96 hours, a substantial host immune response characterized by a large number of inflammatory cells seen on SEM obscured the visualization of bacterial cells. However, by 240 hours, the wound bed appeared drastically different, with no discernable bacteria or inflammatory cells in an otherwise epithelialized wound bed. These images directly correlated with the observed decrease in wound bacterial burden at 240 hours, indicating that the equilibrium attained between *K. pneumoniae*, and the wound bed matrix at 96 hours was only temporary.

To specifically examine the host inflammatory response during this natural progression of *K. pneumoniae* wound infection, the level of mRNA of inflammatory cytokines IL-1 $\beta$  and TNF- $\alpha$  within the wound bed was measured through reverse transcription-qPCR (Figure 3). An initial surge in cytokine release at 48 hours is followed by a significant decrease at 96 hours, with an eventual return to baseline by 240 hours postinoculation ( $p < 0.05$ ). This progression in immune response parallels both the decrease in bacterial concentrations and the changing appearance of the wound microscopically over time. The ultimate effect of these simul-

taneous processes on wound healing is displayed in Figure 4. Wounds harvested at 240 hours visually exhibit an increased amount of epithelial and granulation tissue (Figure 4A and B) as compared with 96-hour wounds. These differences are significant when quantified through measurements of both epithelial and granulation tissue gaps and areas ( $p < 0.01$ ) (Figure 4C and D).

Further validation of the observed relationship between host inflammatory response and *K. pneumoniae* was performed through direct comparison with wounds subjected to ischemia. To verify a difference in host response secondary to ischemia, reverse transcription-qPCR was performed at 48 and 96 hours, following bacterial inoculation (Figure 5). Nonischemic wounds demonstrated a distinctly higher level of inflammatory cytokine mRNA levels at both time points ( $p < 0.05$ ), confirming that ischemia was an appropriate method to model a compromised host. Ischemic and nonischemic wounds were then inoculated with  $10^6$  CFU/wound and harvested at 48 and 96 hours postinoculation. Despite the same inoculum size, ischemic wounds had a consistently larger number of viable bacteria at both time points ( $p < 0.01$ ) (Figure 6), indicating that the impaired inflammatory



**Figure 3.** Measurement of host inflammatory cytokine mRNA levels over time in nonischemic wounds. Interleukin (IL)-1 $\beta$  (A) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) (B) mRNA levels within *Klebsiella pneumoniae*-infected wounds demonstrated an initial surge in cytokine levels at 48 hours postinoculation, followed by a significant decrease over time at 96 and 240 hours postinoculation. Note the return of cytokine levels approximately to baseline by 240 hours postinoculation ( $n = 8-10$  wounds/group) ( $p < 0.05$ ).

response of ischemia had a direct and detrimental impact on bacterial burden stabilization. Consequently, with a distinctly more severe wound infection, ischemic wounds also demonstrated marked deficits across all quantitative measures of wound healing as compared with nonischemic wounds ( $p < 0.05$ ) (Figure 7).

The phenotypic differences in host immune response between ischemic and nonischemic wounds also translated into clear morphologic differences on SEM (Figure 8). As previously noted, imaging of noncompromised wounds revealed a considerable influx of inflammatory cells, preventing the visualization of individual bacteria at 48 and 96 hours postinoculation (Figure 8A and C). In contrast, SEM revealed evidence of what appeared to be bacterial biofilm structure within ischemic wounds, in particular the clustering of individual bacterial cells within a surrounding of extracellular matrix components (Figure 8B and D). In addition, the lack of inflammatory cells seen at both time points, as compared with nonischemic wounds, further validated the magnitude of host impairment associated with ischemia.

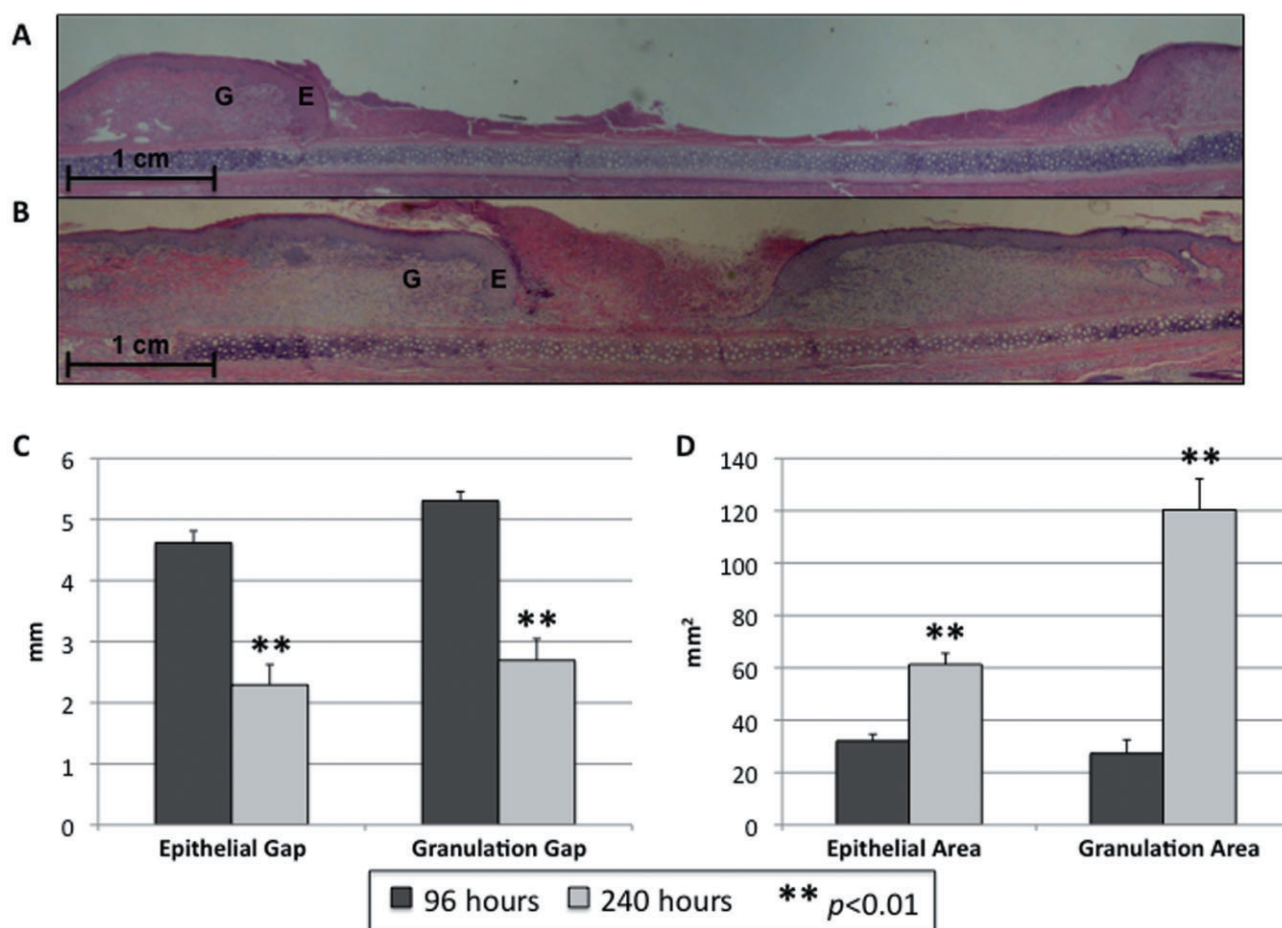
## DISCUSSION

The interaction between bacteria and their host organism is critical to the natural progression of an infection.<sup>13-17</sup> This is particularly evident within a wound bed, where a complex inflammatory response impacts not only the level of bacterial burden but also the extent of wound healing. As a result, in a host compromised by underlying systemic disease, the ability to heal an infected wound is significantly impaired.<sup>1</sup> Simultaneously, the protective mechanisms inherent to bacteria allow them to establish a chronic presence within wounds, particularly through the formation of biofilm.<sup>7,10,20-24</sup> Although the dynamic balance between bacterial infiltration and host response has primarily been studied using *S. aureus* and *P. aeruginosa*,<sup>14,16,17,29</sup> *K. pneumoniae* has now been recog-

nized as an important wound pathogen, particularly among wounded soldiers returning from combat<sup>31-33</sup> and perhaps in the settings of mixed-species wound infections. Using a quantitative, in vivo rabbit ear model, we investigated the properties of a *K. pneumoniae* wound infection over time, particularly the role of the host immune response in combating and controlling such an infection.

Previous work with *K. pneumoniae* has primarily involved its role as an opportunistic respiratory pathogen,<sup>30</sup> with few groups addressing its impact within a wound. Petersen et al.<sup>32</sup> and Yun et al.<sup>33</sup> both isolated *K. pneumoniae* from the infected wounds of several soldiers fighting in Iraq, emphasizing the importance of continued research on Gram-negative wound pathogens. Reports such as these have formed the rationale for performing this study. The prevalence of *K. pneumoniae* within diabetic foot wounds, a major cause of chronic wounds, has also been investigated, with as many as 60% of wounds being infected with an antibiotic resistant strain of the bacteria.<sup>35</sup> The growing literature on *K. pneumoniae* wound infections has prompted other groups to explore various therapeutic options. Kumari et al.<sup>39</sup> have utilized a murine burn wound infection model to test the efficacy of a bacteriophage against *K. pneumoniae*, which can be multidrug resistant in this setting. Meanwhile, another group has reported success using the antibiotic tigecycline in vitro against a number of drug-resistant pathogens that were isolated from diabetic foot wounds, including *K. pneumoniae*.<sup>40</sup> However, although the host response to wound infections is well-described,<sup>11</sup> there are currently no studies that specifically address the interactions between immune cells and *K. pneumoniae*. Furthermore, no groups have investigated this critical aspect of inflammation, and ultimately wound healing, in a quantitative and in vivo manner.

Through this study, we have demonstrated that the host inflammatory response is critical to the natural history of *K. pneumoniae* wound infections. In particular, despite exposure to a variety of different inoculant concentrations, an

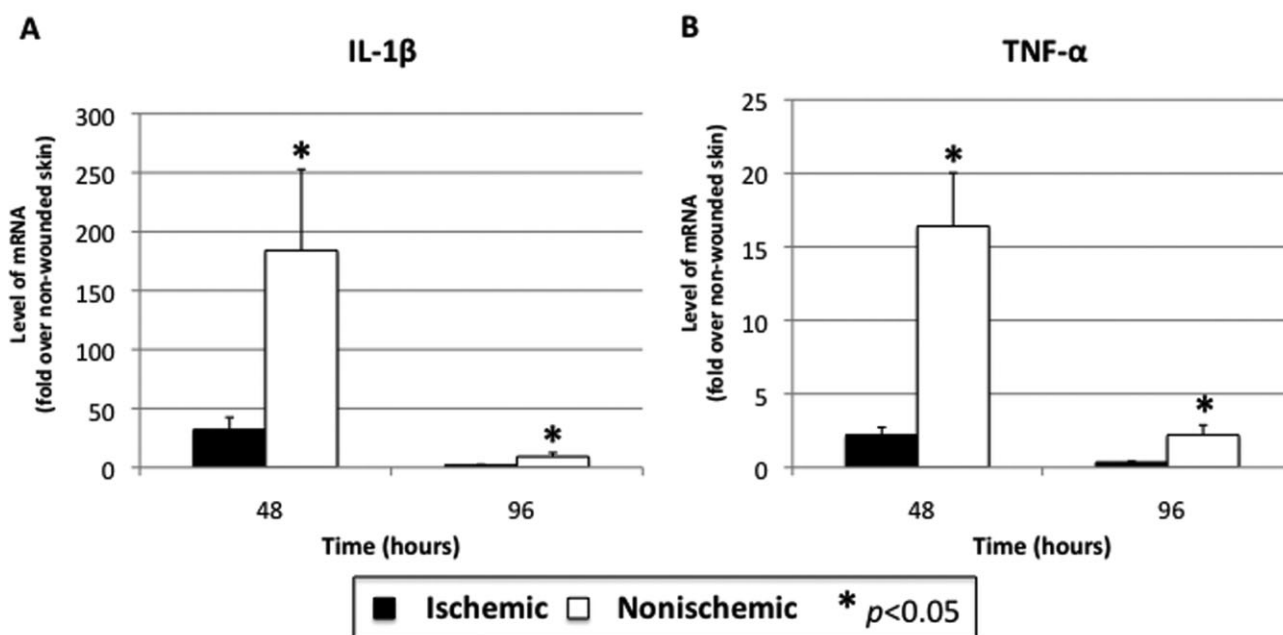


**Figure 4.** Extent of wound healing in *Klebsiella pneumoniae*-infected nonischemic wounds over time. Histological sections stained with hematoxylin and eosin at 96 hours (A) and 240 hours (B) postinoculation showed a significantly increased level of epithelialization and granulation tissue at 240 hours. These differences are shown quantitatively through histological parameter measurement (C and D). (Magnification  $\times 20$ ) ( $n = 15$  wounds/group) (\*\* $p < 0.01$ ). E, epithelial tissue; G, granulation tissue.

intact host is capable of stabilizing the bacterial burden within an active infection over time. As seen in our results, there was a tendency for bacteria within the wound to plateau at approximately  $10^6$ – $10^7$  CFU. Unlike the in vitro setting where bacteria are subjected to a limited and controlled growth environment, an in vivo wound is a complex milieu with several inherent factors that can influence the survival of bacteria. We concluded that one of these factors was the host inflammatory response, which was preventing growth in high inoculant concentration wounds, while slowing it in those with a low inoculant concentration. We validated this hypothesis by performing a similar time-course experiment in an ischemic, i.e., compromised, host. After verifying that inflammatory cytokine mRNA levels were consistently reduced secondary to ischemia, we demonstrated that ischemic viable bacterial counts were significantly higher than those in normal wounds at two separate time points. These findings taken together provide direct evidence that an inadequate immune response can detrimentally affect the dynamics of a bacterial wound infection.

The initial interaction between inflammatory cells and infiltrating bacteria within an infected wound bed precede and dictate the extent of wound healing that occurs.<sup>12</sup> Our results with *K. pneumoniae* serve to validate our understanding of this complex process. With an initial surge of inflammatory cytokines at 48 hours postinoculation, there was a subsequent stabilization in bacterial burden by 96 hours, as previously described. Although there was only minimal healing at this time point, as time progressed, we visualized a substantial increase in epithelialization and new granulation tissue formation, which correlated with a large decrease in bacterial counts by 240 hours postinoculation, and an expected return of inflammatory cytokine levels to baseline. However, the in vivo dynamics of these parallel, but intersecting, pathways are modified in a compromised host. Our data indicate that ischemic conditions directly reduced the presence of inflammatory cytokines within the wound, which in turn would prevent the effective recruitment of inflammatory cells to the wound bed. Without the appropriate inflammatory conditions, the level of bacterial burden at 48 and 96 hours is elevated



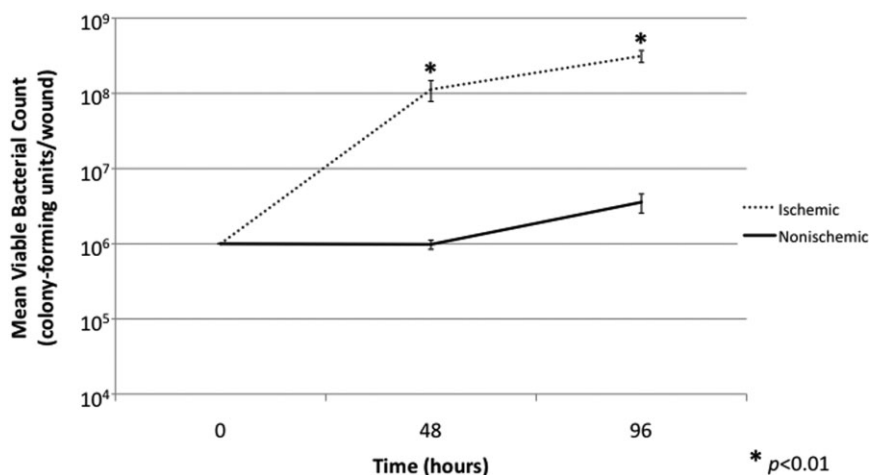


**Figure 5.** Comparison of inflammatory cytokine mRNA levels in *Klebsiella pneumoniae*-infected nonischemic and ischemic wounds over time. Ischemic wounds demonstrated significantly lower levels of interleukin (IL)-1 $\beta$  (A) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) (B) mRNA as compared with nonischemic wounds at both 48 hours and 96 hours postinoculation, verifying that ischemia was an adequate model of a compromised host inflammatory response. ( $n = 8$ –10 wounds/group) (\* $p < 0.05$ ).

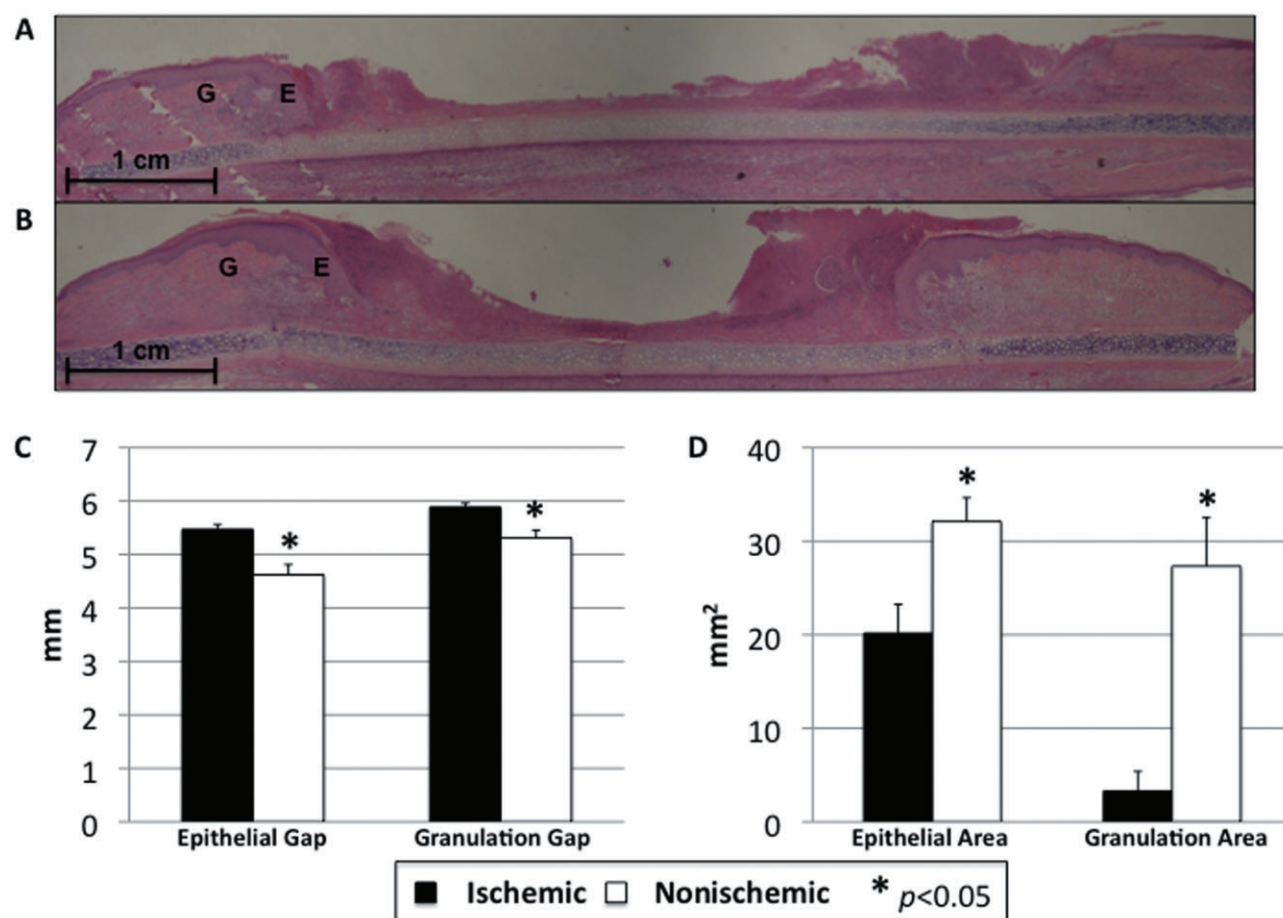
resulting in a significant impairment in wound healing. While indicating the importance of the host response in wound healing, our results also emphasize the difficulty of treating infected, nonhealing wounds in patients with compromising comorbidities, such as diabetes, obesity, and vascular insufficiency.<sup>15</sup> With a prolonged and ineffective inflammatory response, these individuals develop chronic wounds because of poorly controlled bacterial burden and the inhibition of normal wound-healing pathways.<sup>10</sup>

As initially described, *K. pneumoniae* has become a more prevalent and difficult organism to treat in the war wounds of

soldiers from Iraq.<sup>31–33</sup> This growing concern serves as the foundation for this study in which we have used a *K. pneumoniae* strain isolated from an injured soldier. Given its reputation as an opportunistic, destructive respiratory pathogen, one might assume that *K. pneumoniae* possesses a similar level of virulence within wounds. However, our *K. pneumoniae* wound infections showed evidence of resolution over time without intervention. Following a stabilization of the infection by 96 hours postinoculation, bacterial counts reduced by approximately 3 log over the course of the next 6 days. This was paralleled by a substantial decrease in the



**Figure 6.** Mean viable bacterial counts in nonischemic and ischemic *Klebsiella pneumoniae*-infected wounds over time. At 48 hours and 96 hours postinoculation, ischemic wounds revealed a markedly higher concentration of bacteria (approximately 2 log difference) than nonischemic wounds. ( $n = 8$ –10 wounds/group) (\* $p < 0.01$ ).

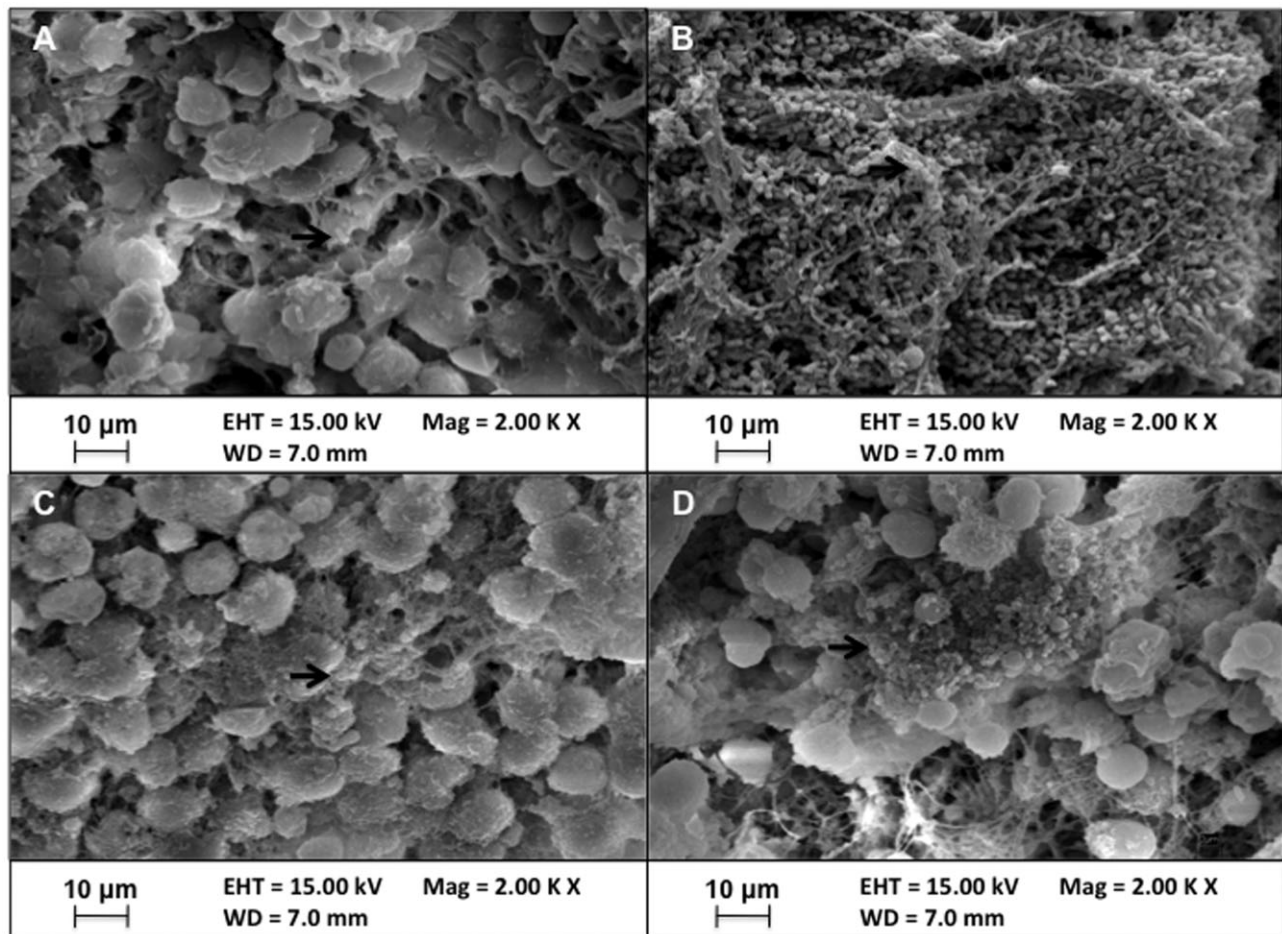


**Figure 7.** Comparison of extent of wound healing in nonischemic and ischemic *Klebsiella pneumoniae*-infected wounds at 96 hours postinoculation. Histological sections reveal a decreased level of epithelial and granulation tissue in ischemic wounds (A) as compared with nonischemic wounds (B). These differences are quantified through measurement of standard histological parameters, revealing a significant impairment of wound healing in ischemic wounds infected with *K. pneumoniae* (C and D). ( $n = 12$ – $15$  wounds/group) (\* $p < 0.05$ ). E, epithelial tissue; G, granulation tissue.

wound inflammatory response during this time, indicating a shift in wound equilibrium toward clearance of the infection. Several conclusions can be inferred from this lack of pathogenicity seen in our infection model. First, as evidenced by the multiple bacterial species seen within soldiers' wounds,<sup>31–33</sup> *K. pneumoniae* may act as an opportunistic pathogen that superinfects open wounds infiltrated by other bacterial species. Therefore, the difficulty in treating these wounds may be secondary to their polybacterial nature rather than the pathogenicity of *K. pneumoniae* alone. Second, and of potentially greater interest, is the concept that compromising the host response directly impacts the measured pathogenicity of an organism. The pathogenicity of *K. pneumoniae* is generally observed among critically ill hospital patients, whose immune defenses are often temporarily compromised.<sup>30</sup> Similarly, wounds that were compromised via ischemia, impairing their natural response to bacteria, demonstrated higher bacterial counts over time with significantly less healing. However, these findings come despite using the same bacterial strain and inoculant concentration that a

normal host was able to overcome. Eliminating the host's ability to contain an infection appears to indirectly enhance the capabilities of the causative bacteria, allowing it to have a detrimental impact on the wound bed without resistance. The end result is an increased incidence of uncommon wound pathogens, such as *K. pneumoniae*, causing severe wound infections despite their minimal impact on normal hosts.

Along with an increase in their perceived pathogenicity, this study reveals that bacteria residing within an impaired wound bed have the ability to form protective biofilms on the wound surface. SEM imaging from ischemic wounds as early as 48 hours postinoculation shows the distinct structure of bacterial biofilm throughout the wound surface. Without an effective immune response, bacteria can not only proliferate without external resistance but also cluster and secrete a protective matrix of polymeric substance, i.e., EPS. This transition to biofilm, given its intimate association with nonhealing chronic wounds,<sup>7,21,22,25–28</sup> may have an additive effect on wound-healing impairment. In contrast, imaging of noncompromised wounds revealed an extensive influx of inflamma-



**Figure 8.** Scanning electron microscopy imaging of nonischemic and ischemic *Klebsiella pneumoniae*-infected wounds at 48 hours and 96 hours postinoculation. Ischemic wounds reveal a clustering of bacterial cells within a surrounding structural matrix, i.e., biofilm, at 48 hours (B) and 96 hours (D) postinoculation (arrows). In comparison, several host inflammatory cells (predominantly neutrophils) with only a small number of bacteria are visualized in nonischemic wounds at 48 hours (A) and 96 hours (C) (arrows). Note the relative lack of inflammatory cells in ischemic wounds (B and D) as compared with nonischemic wounds (A and C) at both time points, correlating with the decreased inflammatory cytokine mRNA levels seen previously. EHT, extra high tension; WD, working distance.

tory cells without the visualization of biofilm. It is unclear whether biofilm has formed beneath the large groups of neutrophils and macrophages that are seen. The observation also raises the question whether biofilms are present in acute and active infections. Given the protective mechanisms inherent to biofilm EPS, one could presume that these wound infections would not have stabilized and resolved if biofilm was the predominant presence. Therefore, the impact of the host inflammatory response may be to trigger long-term bacterial clearance, while in the short-term preventing the formation of biofilm. Furthermore, these results imply that biofilm formation may not strictly be a phenomenon of chronic wounds but rather one of compromised hosts regardless of the acuity of the infection.

Although our study contributes toward our understanding of host–bacteria interactions, we acknowledge the limitations of our model and study design. The creation of ischemic conditions using the rabbit ear dermal ulcer model has been

well established within the literature.<sup>36,38</sup> However, despite disruption of superficial and deep vascular supply, our group's experience has shown evidence of neovascularization within the ear as early as POD 8. As a result, the inconsistency of the ischemic background beyond day 7, which translates to 96 hours postinoculation, limits the use of the model after this time point. With a model that maintains long-term ischemia, an additional ischemic time point at 240 hours postinoculation could have been performed to further supplement our findings. In addition, given the interest of our group in the treatment of wounded soldiers, we chose to perform our experiments with a strain of *K. pneumoniae* isolated from a war wound. However, we recognize that *K. pneumoniae* is an uncommon wound pathogen when compared with more well-known wound flora such as *S. aureus* and *P. aeruginosa*. Nevertheless, the principles outlined and validated by this study are primarily inherent to the host immune system. Additional studies with different bacterial species would help verify our



ability to extrapolate from this study's results. We also acknowledge that our study does not specifically address the molecular interactions between the host inflammatory pathway and bacteria. Rather, our results help establish the dynamics of this interaction while demonstrating the presence of a biofilm state within an impaired host. These results have implications for all of clinical wound care.

The interplay between inflammatory cells and infiltrating bacteria remains an essential, but poorly understood, part of microbiology research. In the infected wound, the adequacy of the immune response helps dictate the extent of both bacterial burden and wound healing through sophisticated molecular pathways. By describing and validating the dynamics of these pathways, we hope to use this work as a foundation for the future study and therapeutic modulation of the host response to bacterial infection.

## ACKNOWLEDGMENTS

This work was supported by the US Army Medical Research and Materiel Command. The authors would like to thank Jordan P. Steinberg for his assistance in performing the rabbit ischemic ear surgeries.

## DEPARTMENT OF DEFENSE DISCLAIMER

The opinions or assertions contained herein are the private views of the author and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

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