INTRODUCTION
Chronic wounds, which include pressure sores and venous and diabetic foot ulcers (DFUs), are a global problem leading to substantial morbidity and mortality (Gottrup, 2004). After injury, skin-resident microbiota and pathogenic species may colonize the wound and proliferate (Eming et al., 2014). Hence, understanding the role of bacteria, both pathogenic and commensal, in the context of skin wounding is important, yet comparatively little research attention has been focused on this area (Loesche et al., 2017; Misic et al., 2014).

Poor progression of chronic wounds is often associated with infection and the presence of recalcitrant microbial biofilms comprising *Staphylococcus*, *Pseudomonas*, and *Corynebacterium* species and a variety of other organisms (Attinger and Wolcott, 2012; James et al., 2008; Mancl et al., 2013; Rhoads et al., 2012). The innate immune system detects infection and injury via pattern recognition receptors (PRRs) such as the Nod-like receptors. PRRs respond to highly conserved microbial structures: pathogen-associated molecular patterns that can trigger inflammatory and defense responses such as keratinocyte-mediated production of antimicrobial peptides (AMPs). AMPs provide rapid and efficient antimicrobial activity against a wide range of pathogens (Dutta and Das, 2016; Harder et al., 2013). The skin has many AMPs, including cathelicidins, β-defensins, S100A15, RNase-7, and histones (Buchau et al., 2007; Doroschner et al., 2001; Gallo and Hooper, 2012; Halverson et al., 2015; Simanski et al., 2010; Sorensen et al., 2006; Yang et al., 2017) and induces members of the β-defensin family under conditions of inflammation, infection, and wound healing (Mangoni et al., 2016; Schneider et al., 2005).

Several pivotal studies have provided insight into the host response during cutaneous wound repair (Campbell et al., 2013; Grice et al., 2010), yet relatively little is known about the skin microbiota and whether they have detrimental or beneficial impacts on repair. Here, we show an association between the bacterial profile of noninfected human DFUs and healing outcome, correlating with up-regulated expression of the PRR NOD2. Using both NOD2 stimulated and *Defb14*−/− mice we show they had a global delay in healing in vivo, associated with alterations in wound microbiota. Taken together, these studies suggest a key role for NOD2-mediated regulation of local skin microbiota, which in turn affects chronic wound etiology.
bacterial burden correlates with DFU healing outcome. Among the groups (Figure 1f and g). Collectively, these data was assessed by direct Gram stain of DFU biopsy tissue, online). Finally, the overall presence of bacteria in wounds (Supplementary Materialsonline (see Supplementary Table S1 all mapped reads at the genus level can be found in the Supplementary Materials online)).

cory) (P < 0.05) (Figure 1e). The taxonomic information for all mapped reads at the genus level can be found in the Supplementary Materials online (see Supplementary Table S1 online). Finally, the overall presence of bacteria in wounds was assessed by direct Gram stain of DFU biopsy tissue, which showed no significant difference in bacterial numbers between the groups (Figure 1f and g). Collectively, these data suggest that bacterial community diversity rather than overall bacterial burden correlates with DFU healing outcome.

NOD2 is up-regulated in human chronic wounds that fail to heal

We next assessed whether PRR expression was altered, because PRRs have been implicated in skin microbiome regulation (Campbell et al., 2013; Dasu et al., 2010; Lai et al., 2009; Lin et al., 2012). Several TLRs trended toward increased expression in nonhealing wounds (Figure 2a–e), but only the intracellular PRR NOD2 was significantly increased (P < 0.05) (Figure 2f). NOD2 is implicated in barrier function, epithelial turnover, and repair (Cruickshank et al., 2008); therefore, we investigated NOD2 function in keratinocytes. Keratinocyte scratch wound closure was significantly reduced after treatment with the NOD2 ligand muramyl dipeptide (MDP) (P < 0.05) (Figure 2g and h). Scratch closure was also inhibited by a range of toll-like receptor (TLR) ligands (see Supplementary Figure S1a online); however, TLR2 ligands did not affect closure. The addition of mitomycin C to inhibit proliferation (Figure 2h) showed no difference in migration between MDP treatment and control, implicating NOD2 signaling in the proliferative component of scratch wound closure. Quantitative real-time PCR confirmed that MDP treatment significantly increased keratinocyte mRNA expression of NOD2 (P < 0.05) (Figure 2i).

Experimental stimulation of the NOD2 pathway delays cutaneous wound healing

We next investigated the impact of NOD2 activation using C57BL/6 mice subcutaneously injected with MDP or vehicle control before incisional wounding. MDP treatment up-regulated Nod2 mRNA in the wound (see Supplementary Figure S1b) and showed a trend for up-regulation of the Nod2-associated downstream signaling molecules Rip2 but not Tak1 (see Supplementary Figure S1c and d). MDP treatment significantly delayed wound closure (Figure 3a), shown by increased histological wound area (P < 0.001) (Figure 3b) and reduced re-epithelialization (P < 0.01) (Figure 3c). MDP-treated wounds had increased local wound recruitment of both neutrophils (P < 0.001) and macrophages (P < 0.01) (Figure 3d–f), and we observed an extended keratinocyte activation response (extension of keratin 6 staining from the wound edge compared with control, P < 0.01) (Figure 3g and h). In line with these results, Ki67 staining in MDP-treated wounds showed significantly increased wound edge proliferation in MDP-treated wounds (Figure 3i and j). Collectively, these results show that MDP-mediated activation of NOD2 significantly delays repair.

NOD2 stimulation induces an antimicrobial response in cutaneous wound healing

NOD2 has a known role in gut and lung epithelial AMP production, specifically defensins (Rohrl et al., 2008; Tan et al., 2015). MDP-treated wounds had significantly up-regulated levels of mBD3 (P < 0.05) and mBD14 (P < 0.05) mRNA compared with control wounds (Figure 4a). Similarly, in vitro, MDP-stimulated normal human epidermal keratinocytes significantly induced human β-defensin (hBD) genes hBD1, hBD2 (the human ortholog to mBD3), and particularly hBD3 (the human ortholog to mBD14) (P < 0.05) (Figure 4b). We further explored the effect of MBD14 on wound healing, focusing on the keratinocyte response. We used an mBD14 peptide (Reynolds et al., 2010), which we confirmed as biologically active because it inhibited Pseudomonas aeruginosa growth (see Supplementary Figure S2a online), and scratch-wounded primary mouse keratinocyte monolayers were treated with 1, 10, or 25 µg/ml of mBD14 peptide. Keratinocyte migration was significantly decreased in a dose-dependent manner (P < 0.01) (Figure 4c and d). Cell viability was unaffected by the peptide as determined by examination of morphological features, suggesting that mBD14 directly influences epidermal migration. The sequence homology between mBD14 and hBD3 is approximately 69% ( Hinrichsen et al., 2008; Rohrl et al., 2008); therefore, we tested mBD14 peptide on human keratinocytes with similar results (see Supplementary Figure S2b). We also investigated the impact of hBD3 on keratinocyte function using hBD3-transfected cells; however, we saw no effect on keratinocyte scratch closure (see Supplementary Figure S2c).

Defb14—null mice had delayed wound healing

To further clarify the role of mBD14, we investigated excisional wound healing in mice that lack BD14 (Defb14−/−) and wild-type (WT) littermate controls. Histological analysis showed delayed wound repair in Defb14−/− mice (Figure 5a), with significantly increased wound area (P < 0.01) (Figure 5b) and delayed re-epithelialization (P < 0.05)
Figure 1. The microbiome profile of human DFUs is an indicator of healing outcome. DFU samples were collected at baseline, and their wound microbial communities were sequenced by 16S PCR-DGGE or 16S RNA Illumina (San Diego, CA) high-throughput sequencing. Longitudinal healing was measured over the subsequent 12 weeks to define healing outcome. (a) UPGMA dendrogram of DFU DGGE fingerprints for healed (green) and nonhealed (purple) wound tissue showed clustering based on time to heal, ≥60% intrapersonal variation versus ≤30% interpersonal variation. (b) NMDS plot showing differences in clustering of microbial communities from 16S RNA Illumina high-throughput sequencing and (c) diversity, which was calculated using Shannon Weiner diversity index. (d, e) Taxonomic classification of the skin microbiome showing proportion of bacteria in each treatment group and at the phylum level and genus level. Individual taxa with abundances too low to visualize clearly and unassigned reads are grouped into the “other” category, composed of 12 additional phyla plus unassigned reads at the phylum level and 225 additional genera plus unassigned reads at the genus level. (f) Representative Gram-stained histological sections (arrows indicate stained bacteria) and (g) quantification of numbers of bacteria per field of view. All data are representative of two independent experiments, with n = 19 patients for a and n = 25 for b–g. *P < 0.05. P-values were determined by one-way analysis of variance in b and c, two-way analysis of variance with Tukey post hoc test in d and e, or by a paired two-tailed Student t test in g. Mean ± standard error of the mean. Scale bar in f = 20 μm. DFU, diabetic foot ulcer; DGGE, density gradient gel electrophoresis; NMDS, nonmetric multidimensional analysis; UPGMA, unweighted pair group method with arithmetic mean.
(Figure 5c) at 3 days after wounding. There was a significant reduction in neo-epidermal area although no difference in the distance contribution of keratin 6-expressing cells ($P < 0.05$) (Figure 5d–f). Defb14−/− wounds had an extended epidermal proliferative response compared with control, with increased numbers of Ki67-expressing basal keratinocytes at the periwound edge ($P < 0.05$) (Figure 5g and h). Examination of the immune cells showed no change in wound neutrophils (Figure 5i) but significantly increased macrophages, suggesting altered immune response dynamics ($P < 0.01$) (Figure 5j). Defb14−/− wounds had increased wound granulation tissue inducible nitric oxide synthase+ cells (associated with classically activated macrophages) at 3 days after wounding ($P < 0.01$) (Figure 5k) and a concomitant trend toward a decrease in Arg1+ cells (expressed by alternatively activated macrophages) compared with controls (Figure 5l). Collectively, these data suggest an altered epidermal response and a highly proinflammatory local wound environment in the absence of Defb14.

**Defb14-null mice have an altered wound bacterial profile**

Chronic wounds had altered communities of bacteria compared with wounds that healed well, and we had shown that mBD14 peptide inhibited the growth of *P. aeruginosa*.
Therefore, we assessed bacterial abundance in Defb14−/− mice. Total eubacterial abundance was significantly increased in Defb14−/− mice compared with controls as shown by Gram staining (P < 0.01) Figure 6a and b) and 16S quantitative real-time PCR (P < 0.05) (Figure 6c). Quantitative real-time PCR analysis of common skin bacterial species showed increased levels of P. aeruginosa (P < 0.01) and P. acnes (P < 0.05) (Figure 6d and g), implicating BD14 in a bacterial dysbiosis that is detrimental to healing.

**DISCUSSION**

Human skin is colonized by a diverse array of bacteria and microbes that generally live in harmony with the host, yet overgrowth of commensal species or pathogen infection can negatively affect healing (Grice and Segre, 2012a, 2012b).
Although the precise relationship between the microbes and healing remains unclear, diabetic wounds are thought to be colonized by distinct microbiota, including increased *Pseudomonas aeruginosa*, compared with normally healing wounds (Grice et al., 2010; Hinojosa et al., 2016; Price et al., 2011). However, not all wounds fail to heal, and it remains unclear whether an altered skin microbiota is associated with a predisposition to delayed healing. The data presented here suggest that in the absence of clinical infection, microbiome profiles (and associated host response) play an important role in determining subsequent healing outcome. Thus, bacteria present on our skin before injury could dictate how we heal.

In DFU patients, rather than the more “common” wound pathogens, we observed changes in genera abundance such as *Corynebacterium, Enterococcaceae*, and *Helcococcus* associated with nonhealing. We assessed the DFU microbiome at the time of clinical presentation before the outcome of healing was known. Previous and complementary longitudinal analysis of DFU-associated bacteria have linked poor healing to a more stable microbiome, whereas wounds that healed well had a more dynamic microbiome that transitioned between community types (Loesche et al., 2017). Similarly, our findings implicated a less diverse microbiome at the initiation of healing, such as venous leg ulcers, decubitus ulcers, and wounds that fail to heal by secondary intention. Studies do, however, suggest that neither patient demographics nor wound type exert major influence on the bacterial composition of the chronic wound microbiome (Wolcott et al., 2016).

Several previous studies have shown that TLRs are differentially regulated when comparing acute wounds with chronic wounds, and a number of PRRs, such as TLR3, are important for wound chronicity (Campbell et al., 2013; Dasu et al., 2010; Lai et al., 2009; Lin et al., 2012). By contrast, our study tested PRR levels in longitudinally evaluated healing versus nonhealing chronic wounds. In this context, the only PRR to show statistically significant alteration was NOD2. Because the expression of NOD2 can be up-regulated in response to bacterial ligation, it is plausible that the observed differential NOD2 levels in nonhealing wounds may reflect a response to the differential bacterial composition of the wound environment.

We further investigated the effect of experimentally activating NOD2 in a murine model, via the ligand MDP. Here, MDP treatment led to a significant delay in healing. Studies have linked NOD2 dysregulation to an altered innate immune response, susceptibility to inflammation and delayed healing in acute wounds from elderly subjects (Hardman and Ashcroft, 2008; Lesage et al., 2002). NOD2, but not TLR2, has an essential role during re-epithelialization after murine cutaneous injury (Campbell et al., 2013), and in the murine gut NOD2 regulates epithelial turnover and immune cell recruitment (Bowcutt et al., 2014; Cruickshank et al., 2008). In the clinical setting, mutations in NOD2 are linked to the rare inflammatory skin condition Blau syndrome and delayed wound healing (Kurokawa et al., 2003). Functional studies have shown that both loss-of-function and gain-of-function mutations in NOD2 are associated with chronic inflammation (Kobayashi et al., 2005; Watanabe et al., 2004). This apparent dichotomy is thought to be because NOD2 can directly drive proinflammatory signals and inhibit other pathways such as the TLR2-mediated pathway of inflammation (Watanabe et al., 2004). Other research suggests that the ability of NOD2 to mediate a proinflammatory or anti-inflammatory effect is dependent on the nature of accessory
factors present, such as cytokines or bacterial products (Feerick and McKernan, 2017). In this context, both NOD2 overexpression in human chronic wounds and Nod2 stimulation in murine wounds is associated with delayed wound closure.

NOD2 has a well-characterized role in the recognition and clearance of intracellular bacteria through activation of the proinflammatory pathway and other host defense pathways including AMPs (Philpott et al., 2014). In addition to antimicrobial roles (Hinrichsen et al., 2008), AMPs have been shown to modulate cytokine production (e.g., IL-1β, IL-22), keratinocyte migration and proliferation, and angiogenesis (Harder et al., 2013; Ong et al., 2002). MDP stimulation of NOD2 led to a significant up-regulation of mBD3 and -14 protein expression. Further characterization of macrophage polarization looked at the proportion of iNOS+ or Arg1+ macrophages (see Supplementary Figure S3 for illustrated method). All data are representative of two independent experiments with n = 5-6 mice/group. *P < 0.05, **P < 0.01. P-values were determined by paired, two-tailed Student t test. Mean ± standard error of the mean. Scale bar = 200 μm in a, scale bar = 100 μm in f, scale bars = 50 μm in h and i–l. IHC, immunohistochemistry; iNOS, inducible nitric oxide synthase; WT, wild type.

Figure 5. Delayed healing in Defb14-deficient mice. Defb14−/− mice and littermate controls were excisionally wounded and analyzed 3 days after wounding. (a) Representative hematoxylin and eosin stained sections of Defb14−/− excisional wounds (day 3). Arrows indicate wound margins. (b) Analysis of histological wound area and (c) re-epithelialization at day 3 after wounding. Analysis of the distance contribution from (d) the wound edge and (e) neo-epidermal area of keratin 6 expressing epidermal keratinocytes, illustrated in (f) representative images of WT and Defb14−/− wounds at 3 days after wounding; dashed outline indicates neo-epidermal area. (g) Quantification of the percentage of basal keratinocytes expressing proliferation marker Ki67. Wound edge = 0–500 μm from the wound and per-wound edge = 500–1,000 μm from the wound. (h) Representative Ki67 staining, denoting location of wound and peri-wound edge. IHC quantification of (i) neutrophils and (j) macrophages. Further characterization of macrophage polarization looked at the proportion of (k) iNOS+ or (l) Arg1+ macrophages (see Supplementary Figure S3 for illustrated method). All data are representative of two independent experiments with n = 5-6 mice/group. *P < 0.05, **P < 0.01. P-values were determined by paired, two-tailed Student t test. Mean ± standard error of the mean. Scale bar = 200 μm in a, scale bar = 100 μm in f, scale bars = 50 μm in h and i–l. IHC, immunohistochemistry; iNOS, inducible nitric oxide synthase; WT, wild type.
(mouse ortholog of human hBD2 and 3) in keratinocytes in vitro and wounded skin in vivo. Dysregulation of AMPs in the skin may be an important factor in the host susceptibility to bacterial colonization and wound repair.

Specific loss of Defb14 (mDB14) severely impaired multiple aspects of wound healing, with reduced re-epithelialization, increased inflammation, and a higher bacterial burden, including P. aeruginosa, which we have previously shown to be detrimental to the healing response (Williams et al., 2017). These findings support previous observations that AMPs have diverse functions, including modulation of the innate immune system and altering TLR responsiveness (Beaumont et al., 2014; McGlasson et al., 2017; Semple et al., 2015; Wang et al., 2017). Some AMPs, such as cathelicidin, promote neutrophil recruitment and antimicrobial activity, and indeed, Defb14<sup>−/−</sup> mouse wounds displayed limited neutrophil recruitment, despite delayed healing and a higher bacterial burden (Beaumont et al., 2014; Choi et al., 2012; Mookherjee and Hancock, 2007). The role of BD14 in keratinocytes is particularly poorly understood. Here, we showed that treatment of in vitro keratinocyte scratch assays with mBD14 impaired scratch closure, although it remains unclear whether this is a direct effect or the result of activating other keratinocyte pro-repair pathways, such as local cytokine production (Wang et al., 2017).

Collectively, our work suggests that a greater knowledge of host microbial interactions is essential to understand wound healing progression. Bacterial ligands and antimicrobial factors are almost invariably multifactorial in function, conveying both beneficial and detrimental impacts on healing. Specifically, understanding the dynamics of host-microbial interactions will be key for better managing the treatment of patients with chronic wounds. In the future, simple diagnostic tests to rapidly stratify healing potential based on wound bacterial composition will likely be coupled with bacteria-selective treatments and/or selective manipulation of the microbiome to promote healing.

**MATERIALS AND METHODS**

**Human chronic wounds**

Local ethical committee approval was obtained for all human studies, with informed consent obtained in accordance with the Declaration of Helsinki. All patients were given information about the study before consenting, and they also signed an informed consent form for use of their samples. A total of 25 wound biopsy samples from patients (mixed sex, age ≥ 40 years) with chronic DFUs (defined as distal to medial and lateral malleoli; with a known duration ≥ 4 weeks; grade A1/B1, University of Texas ulcer classification; no infection or ischemia) were obtained at the time of presentation (week 0). All patients received standard of care treatment, including regular debridement, non-antimicrobial dressing, and offloading. No local anesthetic was used at any time during treatment. At week 0, wound biopsy samples were collected from the margin of DFUs before debridement using aseptic technique. Photographs of patients’ wounds were taken weekly over 12 weeks to determine longitudinal healing outcome. DFUs were then separated into two groups, those that healed (full wound closure at ≤7 weeks, 10 patients) and those that failed to heal (wound not closed at 12 weeks, 9 patients) following current best practice treatment.

**Generation of hBD3-expressing HaCaT cell line**

A hBD3—stably overexpressing HaCaT cell line was constructed by transfecting cells with a plasmid containing hBD3 cloned into pcDNA3.1 (kind gift of Julia Dorin, University of Edinburgh). Lipofectamine 2000 (Life Technologies, Wallingford, MA) was used for transfection per the manufacturer’s guidelines. Stably transfected cells were selected for by addition of 500 μg/ml G418 (Life Technologies). Overexpression of hBD3 in the stable cell line

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H Williams et al. 
β-Defensin 14 and Cutaneous Wound Healing in Mice and Humans

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**Figure 6. Bacterial dysbiosis in Defb14-deficient mice.** (a) Gram stain of representative histological sections and (b) quantification shows altered bacterial burden in Defb14<sup>−/−</sup> day 3 wounds compared with control. (c) This is confirmed through reverse transcriptase quantitative real-time PCR (eubacterial 16S) of total bacterial abundance, which shows a significant increase compared with WT littermate controls. These differences are associated with a significant increase of (d) Pseudomonas aeruginosa and (e) Propionibacterium acnes, but there was no difference in (f) Staphylococcus aureus or (g) Staphylococcus epidermidis as shown by species-specific quantitative real-time transcriptase-PCR. All data are representative of two independent experiments with n = 5–6 mice per group. *P < 0.05, **P < 0.01. P-values were determined by paired two-tailed Student t-test. Mean ± standard error of the mean. Scale bar = 20 μm in a. WT, wild type.
compared with control vector transfected line was confirmed by real-time PCR, using TaqMan primer probe (Applied Biosystems, Fisher Scientific UK, Loughborough, UK) to the coding region of hBD3 (assay ID Hs04194486_g1; Applied Biosystems, Foster City, CA).

**Cell culture and scratch migration assay**
HaCaT cells (established human keratinocyte cell line) were cultured in DMEM plus 5% fetal bovine serum. Normal human epithelial keratinocytes (PromoCell, Heidelberg, UK) were cultured in Keratinocyte Growth Medium 2 (C-20011; PromoCell) plus supplements (PromoCell). Primary murine keratinocytes were isolated and cultured (Hager et al., 1999) with collagen IV-coated plates and Cnt-PR medium (CELLnTEC, Bern, Switzerland). Confluent keratinocyte sheets seeded in 24-well plates were scratch wounded and cultured in CnT-PR medium (CELLnTEC, Bern, Switzerland). The primer sequences for quantitative real-time PCR are listed in Supplementary Table S2 online.

**RNA isolation and quantitative real-time PCR**
Total host RNA was isolated using the Purelink RNA kit (Invitrogen by Life Technologies Ltd., Paisley, UK). cDNA was transcribed from 1 μg total RNA using a high capacity cDNA reverse transcriptase; Roche, West Sussex, UK) and quantitative real-time PCR performed using the SYBR Green 1 Kit (Eurogentec, Hampshire, UK) and an iCycler iQ quantitative PCR thermal cycler (Bio-Rad, Hertfordshire, UK). The primer sequences for quantitative real-time PCR are listed in Supplementary Table S2 online.

**DNA extraction from tissue samples and manipulation**
All tissue samples were incubated in enzymatic lysis buffer (20 mmol/L Tris at pH 8.0, 0.2 mmol/L EDTA, 1.2% Triton X-100) and lysed by lysozyme (20 mg/mL) for 30 minutes at 37°C. DNA was extracted using a Qiagen DNeasy blood and tissue kit (Qiagen, West Sussex, UK).

**PCR amplification, purification, and denaturing gradient gel electrophoresis**
The V3 variable region of the 16S rRNA gene was amplified from purified DNA by PCR using guanine-cytosine-rich eubacterium-specific primers P3_GC-341F and 518R (see Supplementary Table S1) (Walter et al., 2000) using a PTC-100 DNA Engine thermal cycler (Bio-Rad). Samples were purified using a Qiagen MinElute purification kit (Qiagen). Polyclonal antibodies were used to detect the D-CODE Universal Mutation Detection System (Bio-Rad). Denaturing gradient gels of 10% (weight/volume) acrylamide-bisacrylamide (37:5:1 acrylamide, formamide, dH2O) were made as described previously (Walter et al., 2000). Density gradient gel electrophoresis gel images were aligned and analyzed with BioNumerics software, version 4.6 (Applied Maths, Sint-Martens-Latem, Belgium), and profiles were used to produce an unweighted pair group method with arithmetic mean dendrogram.

**16S rRNA gene sequencing analysis**
16S amplicon sequencing targeting the V3 and V4 variable regions of the 16S rRNA gene (see Supplementary Table S1) was performed on the Illumina MiSeq platform. The raw amplicon data were processed using quantitative insights into microbial ecology (QIIME), version 1.9.0 (Caporaso et al., 2010), and R, version 3.3.1 (R Core Team, 2016). The nonmetric multidimensional analysis plot was created using the isoMDS function in the MASS package (Venables and Ripley, 2002) in R, and statistical analysis was performed using the adonis function in the vegan package in R. The Shannon Wiener diversity index was also calculated in R, using the diversity function in the vegan package (Okansen et al., 2016).

**Hucker-Twort Gram stain**
The Hucker-Twort Gram stain was used to distinguish Gram-positive and Gram-negative bacteria in formalin-fixed tissue. Slides were stained using a 3D-Histech Pannoramic-250 Flash Slide Scanner (3D Histech, Budapest, Hungary), using a x20/0.25 Plan Apochromat objective (Zeiss, Oberkochen, Germany). All tissue was blinded before analysis. The sum of scores for relative amounts of Gram-positive and Gram-negative bacteria in the wound bed tissue was quantified based on clinical microbiology proficiency guidelines (score 0–4+); zero (score 0); rare or scant (score 1+); few (score 2+); moderate (score 3+); and many, numerous, or heavy (score 4+) with regard to the number of organisms present per oil immersion field (×100).

**Animals and wounding**
Following local ethics committee approval, all animal studies were conducted in accordance with UK Home Office regulations. Mice were housed in isolator cages with ad libitum food and water. WT (C57BL/6) mice were bred from WT×WT matings, and Dxeb-14-null mice (C57BL/6) were bred from heterozygous matings and have been described previously (Navid et al., 2012). Eight-week-old female WT mice were anesthetized and injected subcutaneously with 10 μg MDP (MurNAc-L-Ala-D-isoGln) (Bachem, UK, G-1055) or vehicle (phosphate buffered saline) at 24 hours and again at 2 hours before wounding (n = 10 mice/group). Mice were anesthetized and wounded following our established protocol (Ansell et al., 2014). Briefly, two equidistant, 1-cm, full-thickness incisional or 6-mm excisional wounds were made through both skin and panniculus carnosus muscle at the injection site and left to heal by secondary intention.

**Histology and immunohistochemistry**
Histological sections were prepared from tissue fixed in 10% buffered formalin saline and embedded in paraffin. Next, 5-μm sections were stained with hematoxylin and eosin or subjected to immunohistochemical analysis using keratin 6 and keratin 14 (PRB-169P and PRB-155P, respectively; Covance, Maidenhead, UK), anti-Ki67 (ab16667; Abcam, Cambridge, UK), anti-neutrophil (MA1-40038; Thermo Scientific, Roncum, UK), anti-Mac-3 (553322; BD Biosciences, Oxford, UK), NOS2 (SC-651; Santa Cruz Biotechnology, Heidelberg, Germany), and arginase-1 (SC-18354; Santa Cruz Biotechnology). Primary antibodies were detected using the appropriate biotinylated secondary antibody followed by ABC-peroxidase reagent (PK-6104 or PK-6101; Vector Laboratories, Peterborough, UK) with NovaRed substrate (SK-4800; Vector Laboratories) and counterstained with hematoxylin. Images were captured using a Nikon Eclipse E600 microscope (Nikon) and a SPOT insight camera (Image solutions, Inc). Total immune cell numbers (quantification is
illustrated in Supplementary Figure S3 online), granulation tissue wound area, and percentage re-epithelialization were quantified using Image Pro Plus software (Media Cybernetics).

Minimum inhibitory concentrations
Minimum inhibitory concentrations were determined using the microdilution method (Moore et al., 2008). Briefly, an overnight culture of *P. aeruginosa* (NCTC 10781) was diluted in sterile Mueller-Hinton broth (Oxoid, Basingstoke, UK) to an optical density of 0.5. The biologically active form of the mBD14 peptide (Reynolds et al., 2010), FP2KLRFKCCIRGRCGAVLNCGLKEE-QIGRCSNSGRKCCRRKK (oxidized cysteines to form 3 disulfides) (Cambridge Peptides, Cambridge, UK), was serially diluted in inoculated media and incubated at 37°C for 24 hours with agitation. Growth was measured as light absorbance (495 nm) relative to uninoculated and was detected using a microtiter plate reader (Powerwave XS; Bio Tek Instruments, Pottown, UK).

Statistical analysis
Normal distribution and statistical comparisons between groups were determined using Shapiro-Wilk test, Student t test (two tailed), one- or two-way analysis of variance with Tukey post hoc test where appropriate using GraphPad Prism 7, version 7.01 (GraphPad Software, Inc.; La Jolla, CA) with the exception of the analysis for 16S rRNA gene sequencing analysis. For all statistical tests, the variance between each group was determined, and probability values of P less than 0.05 were considered statistically significant.

CONFLICT OF INTEREST
The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://10.1016/j.jid.2018.04.014.

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H Williams et al.
β-Defensin 14 and Cutaneous Wound Healing in Mice and Humans

www.jidonline.org 2273
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