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Microbial Host Interactions and Impaired Wound Healing in Mice and Humans: Defining a Role for BD14 and NOD2

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Chronic wounds cause significant patient morbidity and mortality. A key factor in their etiology is microbial infection, yet skin host-microbiota interactions during wound repair remain poorly understood. Microbiome profiles of noninfected human chronic wounds are associated with subsequent healing outcome. Furthermore, poor clinical healing outcome was associated with increased local expression of the pattern recognition receptor NOD2. To investigate NOD2 function in the context of cutaneous healing, we treated mice with the NOD2 ligand muramyl dipeptide and analyzed wound repair parameters and expression of antimicrobial peptides. Muramyl dipeptide treatment of littermate controls significantly delayed wound repair associated with reduced re-epithelialization, heightened inflammation, and up-regulation of murine β-defensins 1, 3, and particularly 14. We postulated that although murine β-defensin 14 might affect local skin microbial communities, it may further affect other healing parameters. Indeed, exogenously administered murine β-defensin 14 directly delayed mouse primary keratinocyte scratch wound closure in vitro. To further explore the role of murine β-defensin 14 in wound repair, we used *Defb14*$^{-/-}$ mice and showed 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.

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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; Dorschner 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*-null murine models we show insights into the role of the innate defense response in controlling the skin microbiota during wound repair.

RESULTS

Human chronic wound microbiome is linked to healing outcome

Patients were recruited with chronic noninfected DFUs (grade A1/B1, no infection or ischemia at the time of

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Abbreviations: AMP, antimicrobial peptide; DFU, diabetic foot ulcer; MDP, muramyl dipeptide; PRR, pattern recognition receptor; TLR, toll-like receptor; WT, wild type

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presentation). Total eubacterial diversity was profiled using 16S PCR-density gradient gel electrophoresis on DFU punch biopsy tissues collected at clinical presentation (week 0). Patients were then separated into two groups according to their time to heal over a period of 12 weeks; DFU healed in 7 weeks or less (n = 10) versus nonhealed at 12 weeks or longer (n = 9). Eubacterial DNA profiles (unweighted pair group method with arithmetic mean dendrogram) at presentation (week 0) showed clear segregation between wounds that would heal versus those that would not (Figure 1a: wound closure at ≤7 weeks in green vs. ≥12 weeks in purple, n = 19). The 16S rRNA Illumina (San Diego, CA) high-throughput sequencing of a further set of DFU samples (n = 25) and nonmetric multidimensional analysis showed no clear separation between the microbial profiles of the healed compared with the nonhealed wounds (Figure 1b); however, nonhealing wounds were associated with significantly reduced overall phylum diversity (Figure 1c). Phylum level relative abundance was consistent between healed and nonhealed wounds (Fig 1d); however, genus-level taxonomic classification of the wound microbiome showed a significantly altered microbial community in healed versus nonhealed wounds, including relative abundance variation within common skin-associated taxa such as *Staphylococcus* (23% in healed wounds vs. 19% in nonhealing wounds), *Anaerococcus* (3% in healed wounds vs. 10% in no-healing wounds), and *Coprococcus* (classified in other genera category) ($P < 0.05$) (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 ($P < 0.05$)