Cutaneous Nod2 expression regulates the skin microbiome and wound healing in a murine model.

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ABBREVIATIONS USED: AMP, antimicrobial peptide; DGGE, density gradient gel electrophoresis; FISH, fluorescence *in situ* hybridisation; MDP, muramyl dipeptide; NLR, nod-like receptor; NOD2, nucleotide-binding oligomerisation domain-containing protein 2; PAMP, pathogen associated molecular pattern, PRR, pattern recognition receptor; TLR, toll-like receptor.

ABSTRACT

The skin microbiome exists in dynamic equilibrium with the host but when the skin is compromised, bacteria can colonise the wound and impair wound healing. Thus the interplay between normal skin-microbial interactions versus pathogenic-microbial interactions in wound repair is important. Bacteria are recognised by innate host pattern recognition receptors (PRRs) and we previously demonstrated an important role for the PRR NOD2 (nucleotide-binding oligomerisation domains-containing protein 2) in skin wound repair. NOD2 is implicated in changes in the composition of the intestinal microbiota in Crohn's disease but its role on skin microbiota is unknown. *Nod2*-deficient (*Nod2*$^{-/-}$) mice had an inherently altered skin microbiome compared with wild-type (WT) controls. Furthermore, we found *Nod2*$^{-/-}$ skin microbiome dominated and caused impaired healing, revealed in cross-fostering experiments of WT with *Nod2*$^{-/-}$ pups which then acquired altered cutaneous bacteria and delayed healing. High-throughput sequencing and qPCR revealed a significant compositional shift, specifically in the genus *Pseudomonas* in *Nod2*$^{-/-}$ mice. To confirm whether *Pseudomonas* directly impairs wound healing, WT mice were infected with *P. aeruginosa* biofilms and akin to *Nod2*$^{-/-}$ mice, were found to exhibit a significant delay in wound repair. Collectively, these studies demonstrate the importance of the microbial communities in skin wound healing outcome.

INTRODUCTION

Skin is colonised by diverse microorganisms, collectively termed the skin microbiome. Recent methodological advances in high-throughput sequencing have revealed the complexity of microorganisms associated with skin (Grice et al., 2009, Group et al., 2009) and have begun to directly implicate a microbial imbalance, a so-called dysbiosis in skin health and disease (Achermann et al., 2014). Our skin is also routinely exposed to potentially pathogenic microorganisms, such as *Staphylococcus aureus, Pseudomonas* and *Enterobacter* species (Grice et al., 2009), and has therefore evolved a tightly regulated innate immune response to actively manage the interactions with the skin microbiome.

Following injury, it is essential that the skin repairs itself effectively and rapidly. Exposed subcutaneous tissue provides a perfect niche for adventitious pathogens to override the natural microbiome colonising the wound (Siddiqui and Bernstein, 2010). Skin cells respond to bacterial invasion via cutaneous PRRs including Toll-like receptors (TLRs) and the nucleotide-binding oligomerisation domain (NOD) leucine-rich repeat-containing receptors (NLRs) (Kawai and Akira, 2011). PRRs recognise and bind to conserved, pathogen-associated molecular patterns (PAMPs) which ultimately lead to induction of pro-inflammatory cytokines and secretion of antimicrobial peptides (AMPs) (Kawai and Akira, 2011). NOD2 is an intracellular receptor, which recognises the muramyl dipeptide (MDP) motif from bacterial peptidoglycans of both Gram-positive and Gram-negative bacteria (Girardin et al., 2003). Mutations in the leucine-rich region of the NOD2/CARD15 gene are associated with the pathogenesis of several chronic inflammatory diseases of barrier organs including Crohn's disease (Lesage et al., 2002), asthma (Wong et al., 2015) and Blau syndrome (Kurokawa et al., 2003). Recognition of MDP via NOD2 leads to the activation of the NF-κB pathway inducing a variety of inflammatory and antibacterial factors. While a

number of studies have highlighted roles for PRRs during cutaneous repair, including members of the TLR and NLR families (Campbell et al., 2013, Dasu et al., 2010, Lai et al., 2009, Lin et al., 2012), the role of PRRs modulating the wound microbiome during repair remains unclear.

Although key studies have provided insight into the regulation of the host-microbiome axis (Grice et al., 2009, Oh et al., 2014), what we now must understand is how cutaneous microorganisms interact with the host and the impact on wound repair. Our previous work revealed a novel intrinsic role for murine Nod2 in cutaneous wound healing (Campbell et al., 2013). NOD2 has also been implicated in the regulation of the gut microbiome (Philpott et al., 2014). Given the potential importance of host microbiota/skin interactions during tissue repair we hypothesised a major link between the Nod2 delayed healing phenotype and the role of Nod2 in cutaneous bacteria modulation. Using a *Nod2* null murine model we reveal fundamental insights into the role of the innate host response in modulating skin bacteria, with direct effects on tissue repair.

RESULTS

Nod2-deficient mice have an altered skin microbiome

To investigate the role of the PRR Nod2 in the skin we used the murine *Nod2*$^{-/-}$ model. Histologically the skin of *Nod2*$^{-/-}$ mice was comparable to WT mice (Figure 1a). However, through density gradient gel electrophoresis (DGGE) we observed major differences in the *Nod2*$^{-/-}$ skin microbiome from birth through to adulthood (Figure 1b). 16S rDNA sequencing data of differentially expressed bands indicated enrichment in *Pseudomonas* species (Figure 1c-d), and this was confirmed by quantitative real-time PCR (qPCR) which showed increased relative abundance of *Pseudomonas aeruginosa* in *Nod2*$^{-/-}$ skin (Figure 1e) and a trend towards reduced "commensal" species, such as *Staphylococcus epidermidis* (Figure 1e).

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen. Histological Gram-staining of skin sections showed no significant difference in the total number of bacteria visualised in the epidermis or dermis. There was however, a trend towards increased overall numbers of bacterial cells in the dermis of *Nod2*$^{-/-}$ skin and a corresponding propensity towards increased abundance of Gram-negative bacteria (Figure S1a-c).

Injury exacerbates skin microbiome dysbiosis in Nod2-deficient mice

We next addressed the potential contribution of altered skin microbiome to the healing delay observed in *Nod2*$^{-/-}$ mice (Campbell et al., 2013) (Figure 2a). Injury increased the total eubacterial abundance in *Nod2*$^{-/-}$ but not WT mice (Figure 2b). Fluorescence *in situ* hybridisation (FISH) confirmed this increased total eubacterial DNA abundance (16S probe) in *Nod2*$^{-/-}$ mouse wounds (Figure 2c, quantified in Figure S2a-c). Despite this increase, the bacterial diversity induced by injury was less pronounced in *Nod2*$^{-/-}$ than that of the WT mice (≤60% versus ≤40% respective similarity score between skin and wound; Figure 2d), which agrees with recent observations from Loesche et al. that wound microbiota stability is associated with delayed healing (Loesche et al., 2017). Thus, in the absence of *Nod2*, injury leads to increased relative bacterial abundance but reduced injury induced changes in bacterial profile. qPCR showed that specific pathogenic species, such as *Pseudomonas aeruginosa* and *Propionibacterium acnes*, were increased in *Nod2*$^{-/-}$ mouse wounds (Figure 2e), which was confirmed by 16S rDNA sequencing (Figure S2d-e). Opportunistic pathogenic species of *Pseudomonas* are linked to chronic inflammation and wound infection (Fazli et al., 2009, Wu et al., 2011), and are thus clear candidates to confer delayed wound healing.

Antimicrobial peptide expression is altered in *Nod2* null skin following injury

A key component of the antimicrobial host response is the production of AMPs, predominately members of the defensin family (Gallo and Hooper, 2012). Studies in Crohn's disease patients and *Nod2*-deficient mice showed reduced α-defensin expression within the intestinal mucosa (Eckmann and Karin, 2005, Maeda et al., 2005). Although α-defensins are absent in the skin, specific AMPs including β-defensins are strongly induced in response to cutaneous injury (Ahrens et al., 2011). Unwounded skin of newborn (i.e. with minimal bacterial exposure) *Nod2*$^{-/-}$ mice had greater expression of both mBD-1 and mBD-14 than matched WT mice **(**Figure 3a). Adult injury-induced changes in defensin expression also differed between genotypes, with mBD-1 significantly upregulated in WT, while *Nod2*$^{-/-}$ wounds displayed abnormal induction of mBD-3 and mBD-14 in response to injury (Figure 3b-d). IL-22, a known regulator of mBD-14 expression (Liang et al., 2006), was strongly increased in *Nod2*$^{-/-}$ wounds. Finally, we confirmed increased mBD-14 at the protein level *in vivo,* demonstrating a greater extent of keratinocyte expression and an increased number of mBD-14 positive dermal inflammatory cells in adult wound tissue (Figure 3f-h).

Altered expression of AMPs in the absence of *Nod2* may contribute to an altered microbial community but equally it may reflect the host response to changes in the composition of the skin microbial community, overall bacterial burden or the cutaneous location of the microbes in the tissue. In experiments analysing mice born by caesarean section the data showed that cutaneous defensin expression was similar between WT and *Nod2*$^{-/-}$ mice (Figure S3) suggesting that defensin profiles change in response to microbial challenge. As *Nod2*$^{-/-}$ mice had an altered microbiome, an important question was then whether skin dysbiosis was sufficient to alter healing outcome and whether this phenotype could be transferrable.

Co-housing from birth directly links skin microbiome to healing outcome

To address causation and to investigate a potential link between bacterial dysbiosis and healing outcome we mixed newborn WT and *Nod2*$^{-/-}$ mice litters from birth with a *Nod2*$^{-/-}$ mother. WT mice reared in this mixed environment displayed a clear healing delay with significantly increased wound area (Figure 4a-b), and increased local immune cell recruitment (Figure 4c). The reverse experiment was performed whereby newborn WT and *Nod2*$^{-/-}$ litters were co-housed with WT mothers and interestingly whilst there was no rescue of delayed healing in *Nod2*$^{-/-}$ mice, the WT mice had a variable response with five mice out of eight having delayed healing (Figure S4a-b) but all showing significantly greater inflammation (Figure S4c), suggesting the maternal microbiome contribution mediated a partial rescue effect in WT mice.

Next we analysed the microbial communities in wound tissue from the co-housing experiment using 16S rRNA Illumina high-throughput sequencing. Non-metric multidimensional analysis showed statistically significant segregation (P<0.05) based on environment, i.e. separately housed mice versus co-housed mice (Figure 5a). There was also a trend towards reduced alpha diversity between each group when compared to WT, as calculated by the Shannon-Wiener index. When focusing on specific skin microbiota at the phylum level, again using the Shannon-Wiener index, there was a significant change in the diversity of *Bacteroidetes* between environment, separately housed WT versus separately housed *Nod2*$^{-/-}$, and separately housed *Nod2*$^{-/-}$ versus the co-housed mice (P<0.05, P<0.01) respectively (Figure 5c). Furthermore, phylum and genus level taxonomic classification of the wound microbiome is depicted and revealed a significantly altered microbial community in separately housed versus co-housed mice including common skin associated taxa such as *Corynebacterium* and *Brevibacterium* (Figure 5d). The taxonomic information for all mapped

reads at the genus level can be found in the supplementary material (Table S2). 16S rRNA gene sequencing showed that the alterations to the microbial community compositions are different for WT and *Nod2*$^{-/-}$ mice including the genera *Actinobacillus* and *Flavobacterium*.

Finally, to confirm these differences we also performed DGGE which revealed that mixing of pups resulted in a major shift in the skin microbiome of both genotypes (≤50% similarity versus non-mixed) establishing an intermediate skin bacterial profile (~65% similarity between genotypes; Figure 5e). qPCR confirmed that mixed WT wounds acquired increased abundance of specific bacterial species characteristic of *Nod2*$^{-/-}$ mice such as *P. aeruginosa*, accompanied by an overall increase in total eubacterial abundance (Figure 5f). Thus, these data provide compelling experimental evidence that skin microbiome directly influences healing outcome.

Direct administration of *Pseudomonas aeruginosa* to wild-type mouse wounds significantly delays healing

Although we report wide-ranging changes in bacteria in *Nod2*$^{-/-}$ mice, a common theme across experiments was increased relative abundance of *Pseudomonas* sp. To confirm a direct role for *Pseudomonas* sp. in wound repair we treated full thickness excisional wounds in WT mice with *Pseudomonas aeruginosa* biofilms, and assessed subsequent healing (Figure 6a). Significantly delayed healing was observed following direct application of *Pseudomonas aeruginosa* to mouse wounds versus non-treated controls (Figure 6b). Treated wounds were larger (Figure 6c), with delayed re-epithelialisation (Figure 6d), and increased local inflammation (Figure 6e). These data confirmed that the presence of pathogenic bacteria, similar to wound infection, directly delays murine wound healing, and establishes a link to

the *Nod2*$^{-/-}$ phenotype where a delay in wound repair is associated with an increased cutaneous presence of the genus *Pseudomonas*.

DISCUSSION

A wealth of literature has characterised the role of the host response in regulating gut microbiome, with wide-ranging implications for normal physiology and disease (Perez-Chanona et al., 2014, Philpott et al., 2014). By contrast, comparatively few studies have addressed the role of the cutaneous host response-microbiome axis in skin physiology and pathology. We hypothesised that the skin microbiome plays an important role in the cutaneous healing response. Our results show that skin bacterial profiles profoundly influence wound healing outcome. Direct experimental manipulation of the *Nod2* gene leads to bacterial dysbiosis associated with local changes in AMPs, and ultimately delays healing. Moreover, when WT mice were co-housed from birth with mice lacking *Nod2* they acquired an altered microbiome and developed delayed healing. Cutaneous dysbiosis as revealed by eubacterial DNA profiling, 16S high-throughput sequencing and qPCR implicated the genus *Pseudomonas* in murine delayed wound repair, and WT mice infected with *P. aeruginosa* biofilms confirmed this. These results suggest microbial therapy directed at bacterial manipulation of the genus *Pseudomonas,* in addition to other bacterial species previously identified to cause a delay in wound repair including *Staphylococcus aureus* and *Staphylococcus epidermidis* (Schierle et al., 2009), might be an effective strategy to treat wound healing in the future.

A growing body of literature links NOD2/CARD15 polymorphisms with a dysregulated innate immune response and susceptibility to diseases, including Crohn's disease (Lesage et al., 2002), Blau syndrome (Kurokawa et al, 2003), early-onset sarcoidosis (Kapral, 1966),

and graft-versus-host disease (Hoebe et al., 2005). In the gut, NOD2 has a well-characterised role in host recognition of bacteria and MDP, which is widely expressed by a variety of commensal and pathogenic gut bacteria (Kanneganti et al., 2007, Kawai and Akira, 2010). Studies in Crohn's patients and *Nod2*-deficient mice showed that intestinal changes in bacterial composition are associated with altered α-defensin expression within the intestinal mucosa (Philpott et al., 2014). α-defensins are not expressed in skin; however the cutaneous effects of NOD2 are associated with altered β-defensins, yet the exact role these AMPs are playing in our *Nod2* null mice remain to be elucidated. Changes in skin β-defensins have previously been linked to skin infection (e.g. *Staphylococcus aureus*; (Zanger et al., 2010) and skin disease (e.g. atopic dermatitis; (Ong et al., 2002). Thus, a picture is emerging across multiple epithelial tissues whereby a loss of NOD2-mediated surveillance activity inhibits local host responses to pathogenic challenge, resulting in aberrant inflammation and bacterial dysbiosis.

All wounds will be rapidly colonised by resident bacteria but only some wounds will become "infected". Considerable recent interest has been focused on the potential ability of these colonising bacteria to form and exist as highly-AMP resistant polymicrobial biofilms (Malone et al., 2017). A number of bacterial genera/species, such as *Streptococcus*, *Enterococcus, S. aureus*, and *P. aeruginosa*, have already been linked to infected chronic wounds (Bjarnsholt, 2013, James et al., 2016, Zhao et al., 2014). However, the clinical diagnosis for wound infection (in humans) is based on the basic criteria of heat, odour and appearance. In this study we show for the first time that similarly appearing murine acute (non-infected) wounds, display differences in wound microbiota profile that clearly influence healing outcome.

Arguably the most important finding in this study comes from the newborn mouse co-housing experiments, where passive transfer of skin bacteria from *Nod2* null to WT mice conferred a "Nod2-like" delayed healing phenotype. The concept of transferring signature bacterial profiles to closely related individuals has now been established clinically. For example, unaffected relatives of Crohn's disease patients reportedly share some features of the disease-associated microbiome composition (Joossens et al., 2011). Faecal transplants also referred to as "gut microbiome transplants", a procedure in which faecal bacteria from a healthy donor is transplanted into a patient, have shown promise in the treatment of Crohn's disease and ulcerative colitis (Young, 2016). Similarly, cross-strain murine relocation/uterine-implantation studies reveal that environmental influences dominate the gastrointestinal tract microbiome (Friswell et al., 2010). Our data now strongly suggest that the cutaneous microbiome is also highly susceptible to environmental influences, with clear functional consequences. Finally, our data suggest a potential therapeutic opportunity for the treatment of cutaneous dysbiosis in relation to wound repair, via microbial manipulation of the skin microbiome. Indeed, mounting research suggests the profound benefits of probiotic supplementation for gut microbiota in health and disease (Gareau et al., 2010, Rolfe, 2000). These may now be extended to other epithelia, including the skin (Mohammedsaeed et al., 2014).

MATERIALS & METHODS

Animals and wounding

All animal studies were performed in accordance with UK Home Office Regulations. All mice used in this study were bred in the same room under the same conditions at the University of Manchester's Biological Services Facility, where they have been housed for ≥10 generations. Mice were housed in isolator cages with *ad libitum* food and water. The

room was maintained at a constant temperature of 21°C, with 45-65% humidity on a 12 hour light-dark cycle. *Nod2* null mice (C57BL/6J background) were bred from homozygous matings and have been described previously (Campbellet al., 2013). Wild-type (WT) (C57BL/6J) mice were bred from WTxWT matings onsite to generate controls for experiments. Eight week-old female mice were anaesthetised and wounded following our established protocol (Ansell et al., 2014). Briefly, two equidistant 6-mm full-thickness excisional wounds were made through both skin and panniculus carnosus muscle and left to heal by secondary intention. For co-housing experiments, mice were marked by tattooing and then 2-3 tattooed pups (P0) of one genotype were placed in the same cage with 2-3 tattooed pups (P0) of the other genotype and fostered onto WT or *Nod2*$^{-/-}$ mothers for at least 5 weeks prior to separation (for weaning). After weaning, only mice of the same sex were housed together before wounding at 6 weeks.

Pseudomonas aeruginosa infected mouse model

An overnight broth culture of *Pseudomonas aeruginosa* (NCTC 10781) was diluted to turbidity equivalent of 0.5 McFarland Standard (O.D of 0.132 at 600 nm) in Mueller-Hinton broth (Oxoid, Hampshire, UK). 50 µL of the diluted culture was applied to 6-mm diameter sterile 0.2 µm filter membranes (Merck Millipore Ltd, Hertfordshire, UK) placed on Mueller-Hinton agar plates. These were then incubated at 37˚C for 72 hour, with transfer to a new agar plate every 24 hour. The resultant biofilms were applied to 6-mm excisional wounds and covered with a non-woven Sawabond® 4383 dressing (Sandler, Schwarzenbach/Saale, Germany).

Collection of murine tissue and contralateral skin swabs

Excisional wounds were harvested at 1, 3 and 5 days post-wounding and bisected (laterally at the midpoint), with one half placed on dry ice for DGGE analysis or fixed in formalin for histology, and the remaining half snap frozen in liquid nitrogen and stored at -80°C. Skin swabs from an area of intact contralateral skin were also collected using sterile Dual Amies transport swabs (Duo Transwab; MWE, Wiltshire, UK) and inoculated into 1 ml of transport medium and processed within 3 hours of collection.

DNA extraction from tissue samples and manipulation

All biological specimens were incubated in enzymatic lysis buffer (20 mM Tris at pH 8.0, 0.2 mM EDTA, 1.2% triton X-100) and lysozyme (20 mg/ml) for 30 min at 37°C. DNA was extracted using a Qiagen DNeasy™ blood and tissue kit (Qiagen Ltd, West Sussex, UK) in accordance with the manufacturer's instructions, but with the added step of using 0.1 mm sterile zirconial/silica beads (BioSpec, Bartlesville, USA) to homogenise the samples.

PCR amplification and purification

The V3 variable region of the 16S rRNA gene was amplified from purified DNA by PCR using GC-rich eubacterium-specific primers P3_GC-341F and 518R (see Table S1) as previously described (Walter et al., 2000) using a PTC-100 DNA Engine thermal cycler (Bio-Rad MJ Research, Hertfordshire, UK). Samples were purified using a Qiagen MinElute® purification kit (Qiagen Ltd) in accordance with manufacturer's instructions.

Density gradient gel electrophoresis (DGGE)

Polyacrylamide electrophoresis was performed using the D-CODE Universal Mutation Detection System (Bio-Rad, Hertfordshire, UK) according to the manufacturer's instructions for perpendicular DGGE. Denaturing gradient gels of 10% (wt/vol) acrylamide-bisacrylamide

(37:1:5) were made (Fisher Scientific, Loughborough, UK) containing a 30-70% linear gradient of denaturants (urea and formamide) increasing in the direction of electrophoresis as described previously (Walter et al., 2000). DGGE gel images were aligned and analysed with BioNumerics software version 4.6 (Applied Maths, Belgium) in a multistep procedure following the manufacturer's instructions. After normalisations of the gels, individual bands in each lane of the gel were detected automatically allowing matching profiles to be generated and used to produce an Unweighted Pair Group Method with Arithmetic Mean (UPGMA) dendrogram.

Excision and sequencing of DGGE bands

Selected bands were sterilely excised from the gel under UV illumination in 20 µl nanopure H_2O in nuclease-free tubes. PCR products were purified using QIAquick PCR purification kit (Qiagen Ltd) and re-amplified using the reverse 518R primer. Sequencing was performed using BigDye terminator sequencing on an ABI 3730 genetic analyser (Applied Biosystems by Life Technologies Ltd, Paisley, UK) for Sanger sequencing. Sequences obtained were compared to those in the EMBL nucleotide sequence database using BLAST searches to identify closely related gene sequences.

16S rRNA gene sequencing analysis

16S amplicon sequencing targeting the V3 and V4 variable region of the 16S rRNA gene (see Table S1) was performed on the Illumina MiSeq platform. The raw amplicon data was further 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 NMDS plot and the Shannon-Weiner index were created using the isoMDS function in the 'MASS' package (Venables and

Ripley, 2002) in R and the statistical analysis was performed using the Adonis function in the 'vegan' package (Okansen et al. 2016) in R.

Hucker-Twort Gram Stain

The Hucker-Twort Gram stain was used to distinguish Gram-positive and Gram-negative bacteria in formalin-fixed tissue. Tissue was flooded with crystal violet stain for 3 min and rinsed with running H_2O. Gram's iodine was added for 3 min and washed with H_2O. Following differentiation in pre-heated acetic alcohol at 56°C, tissue was immersed with Twort's stain for 5 min and washed with H_2O. Slides were rinsed in alcohol, cleared in xylene and mounted with DPX mountant (Sigma-Aldrich, Dorset, UK); the slides were imaged using a 3D Histech Pannoramic 250 Flash Slide Scanner (3D Histech, Budapest, Hungary).

Quantitative real-time PCR (qPCR)

Bacterial DNA and/or total RNA was isolated from frozen skin or wound tissue as previously described or by homogenising in Trizol reagent using the Purelink RNA kit (Invitrogen™ by Life Technologies Ltd, Paisley, UK) according to the manufacturer's instructions. cDNA was transcribed from 1 µg of RNA (Promega RT Kit, Hampshire, UK and AMVreverse transcriptase, (Roche, West Sussex, UK) and qPCR performed using the SYBR Green I core kit (Eurogentec, Hampshire, UK) and an Opticon quantitative PCR thermal cycler (BioRad, Hertfordshire, UK). The primer sequences for real-time qPCR are listed in Table S1.

Histology and immunohistochemistry

Histological sections were prepared from normal skin and wound tissue fixed in 10% buffered formalin saline and embedded in paraffin. 5 µM sections were stained with

haematoxylin and eosin (H+E) or subjected to immunohistochemical analysis using the following antibodies: rat anti-neutrophil polyclonal (Fisher Scientific) and chicken anti-BD-14 polyclonal (a generous gift from Professor Hehlgans, University of Regensburg). Primary antibody was detected using the appropriate biotinylated secondary antibody followed by ABC-peroxidase reagent (Vector Laboratories, Peterbourgh, UK) with NovaRed substrate and counterstaining with haematoxylin. Images were captured using an Eclipse E600 microscope (Nikon, Surrey, UK) and a SPOT camera (Image solutions Inc, Preston, UK). Total cell numbers, bacterial counts, granulation tissue wound area and re-epithelialisation were quantified using Image Pro Plus software (Media Cybernetics, Buckinghamshire, UK).

Fluorescence *in situ* hybridisation (FISH)

The deparaffinised tissue sections were systematically analysed by FISH using peptide nucleic acid probes. A mixture of a CY3-labelled universal bacterium peptide nucleic acid probe in hydridisation solution (AdvanDx Inc, *Massachusetts*, USA) was added to each section and hybridised in a peptide nucleic acid FISH workstation at 55°C for 90 min. Slides were washed for 30 min at 55°C in wash solution (AdvanDx Inc) and mounted in DAPI containing mountant and stored in the dark at -20°C. Slides were visualised using a DMLB 100s Leica Microsystems microscope attached to a Leica Microsystems fluorescence system (Lecia, Milton Keynes, UK). Images were captured using a RS Phototmetrics Coolsnap camera (Photometrics®, Tucson, USA) and overlaid using Adobe Photoshop Elements version 6.5 (Adobe, San Jose, USA).

Electron microscopy

Samples were processed as previously described (Kimura et al., 2007), with the exception that 4% paraformaldehyde and 2 mM $CaCl_2$ were used in the primary fixative and 2% OsO_4

in the secondary fixative. Images were acquired using the Orius® CDD SC1000 camera (Gatan, Oxon, UK).

Statistical analysis

All data are presented as means + s.e.m. Normal distribution and statistical comparisons between groups were determined using Shapiro-Wilk test, Student's *t*-test (two tailed) or two-way ANOVA with Bonferroni post-test where appropriate using GraphPad Prism 7 Version 7.01 (GraphPad Software, Inc. La Jolla, CA) as indicated in the figure legends. For all statistical tests, the variance between each group was determined and probability values of less than $P<0.05$ were considered statistically significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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FIGURE LEGENDS

Figure 1. Skin microbiome dysbiosis in *Nod2*-deficient mice. (a) Representative H+E stained sections of normal skin from WT and *Nod2*$^{-/-}$ mice demonstrate histological equivalence. (b) UPGMA dendrogram profiling of WT (green) and *Nod2*$^{-/-}$ (purple) skin postnatal development (P0-P14). (c-d) DGGE profiles and sequencing of differentially expressed bands showed ≤40% inter-strain similarity bacteria between WT and *Nod2*$^{-/-}$ mice and the corresponding bacterial species are illustrated. (e) Real-time PCR (16S region) confirmed these species-specific differences between WT and *Nod2*$^{-/-}$ normal skin. (f) Gram-stain of representative histological sections showed there was a trend towards altered eubacterial abundance in the *Nod2*$^{-/-}$ skin. All data are representative of two independent experiments, with n = 2 mice/time point (b), and n = 5 mice/group (c-f). * P<0.05. Mean + s.e.m. Scale bar = 200 μM (a), 20 μM (f).

Figure 2. Wound microbiome dysbiosis in *Nod2*-deficient mice. (a) Representative wounds (day 3) histology showed significantly delayed healing in *Nod2*$^{-/-}$ versus WT (arrows denote wound margins). (b) *Nod2*$^{-/-}$ mice had significantly increased total eubacterial abundance (16S qPCR) in their wounds at day 3 post-wounding compared with WT, which was visualised with FISH using a total eubacterial FISH probe (red) as illustrated in (c). (d) UPGMA dendrogram of wound tissue DGGE fingerprints showed ≤60% inter-strain similarity in the *Nod2*$^{-/-}$ wound microbiome profile versus ≤40% in WT controls. (e) Real-time PCR (16S region) confirmed bacterial species-specific differences between WT and *Nod2*$^{-/-}$ wounds. All data are representative of two independent experiments with n = 5 mice/group (a-e). * P<0.05. Mean + s.e.m. Scale bar = 200 μM (a), 100 μM (c).

Figure 3. Injury-induced antimicrobial peptide production is altered in *Nod2*-deficient mice. (a) Cutaneous β-defensins-1 (mBD-1) and -14 were significantly increased from birth (P0) in *Nod2*$^{-/-}$ mouse skin versus WT. (b-e) In adult *Nod2*$^{-/-}$ mice wounding specifically induced both mBD-3 and mBD-14 and IL-22. (f-h) Immunohistochemical analysis revealed increased epidermal and dermal BD-14 at 3 days post-wounding in *Nod2*$^{-/-}$ mice compared to WT. All data are representative of two to three independent experiments with n = 8 mice/group (a), n = 5 mice/group (b-h). *** P<0.001, ** P<0.01, * P<0.05. Mean + s.e.m. Scale bar = 100 μM (h).

Figure 4. Co-housing from birth shows the skin microbiome directly influences healing outcome. Newborn WT and *Nod2*$^{-/-}$ litters were mixed from birth with a *Nod2*$^{-/-}$ mother, and then wounded in adulthood. (a) Representative H+E stained sections of excisional wounds (day 5) from WT and *Nod2*$^{-/-}$ control (non-mix), and co-housed (mix) cages (arrows denote wound margins). (b) Quantification showed significantly delayed healing in WT mice co-housed with *Nod2*$^{-/-}$ mice, with (c) increased local neutrophil influx. All data are representative of two independent experiments with n = 6 mice/non-mix groups and n = 5 mice/mix group. * P<0.05. Mean + s.e.m. Scale bar = 200 μM (a).

Figure 5. Wild-type mice co-housed with *Nod2*$^{-/-}$ mice from birth acquire pathogenic bacteria. WT and *Nod2*$^{-/-}$ litters were mixed from birth, wounded in adulthood and their wound microbial communities sequenced. (a) NMDS plot showing differences in clustering of microbial communities. Alpha diversity of wound tissue across (b) all microbial communities and (c) the Bacteriodetes phylum was compared using the Shannon-Wiener index. (d) Taxonomic classification of the skin microbiome showing proportions of bacteria in each treatment group at the phylum level, genus level and genera that were significantly

altered between treatment groups. Individual taxa with abundances too low to visualise clearly and unassigned reads are grouped into the 'other' category, which comprises 8 additional phyla plus unassigned reads at the phylum level, and 219 additional genera plus unassigned reads at the genus level. (e) UPGMA dendrogram of WT and *Nod2*$^{-/-}$ wound tissue DGGE fingerprints. (f) Total wound eubacterial abundance (16S real-time PCR) was significantly increased in WT mice co-housed with *Nod2*$^{-/-}$ mice. Mean + s.e.m. All data are representative of two independent experiments with n = 3 mice/non-mix groups and n = 5 mice/mix group. ** P<0.001, * P<0.05.

Figure 6. *Pseudomonas aeruginosa* biofilm delays healing in wild-type mice. WT mice were inoculated at wounding with a *Pseudomonas aeruginosa* biofilm. (a) Representative images of 3 days post-wounding macroscopic and SEM images of excisional wounds from normal and *Pseudomonas aeruginosa* infected mice. Note the characteristic rod-shaped *P. aeruginosa* bacterial cells within the host wound tissue (arrows). (b) Representative histological sections illustrate delayed healing in *P. aeruginosa* biofilm wounds (arrows denote wound margins). (c-e) Quantification revealed significantly delayed healing in the *P. aeruginosa* group with increased wound area, decreased re-epithelialisation and increased local neutrophil influx at 3 days post-wounding. All data are representative of two independent experiments with n = 5 mice/group (a-e). ** P<0.001, * P<0.05. Mean + s.e.m. Scale bar = 5 mm (a, left), 150 nm (a, right), 200 μM (b).

SUPPLEMENTARY MATERIAL

Supplementary Figure S1. Gram-stain quantification. Quantification of Gram-staining showed there was a no significant difference in total eubacterial burden in both WT vs. *Nod2*$^{-/-}$ normal skin (a) epidermis, (b) dermis and (c) combined epidermal and dermal scores. There

was, however, a trend towards altered eubacterial abundance in the dermis (b) of *Nod2*$^{-/-}$ normal skin. All data are representative of two independent experiments, with n = 5 mice/group. Mean + s.e.m.

Supplementary Figure S2. Wound microbiome dysbiosis in *Nod2*-deficient mice. (a-c) Quantification of 16S FISH confirmed a significant increase in total eubacterial abundance in *Nod2*$^{-/-}$ day 3 wounds. (d-e) 16S rDNA sequencing of differentially expressed bands from the DGGE fingerprints revealed the bacterial species in wounded WT and *Nod2*$^{-/-}$ mice. All data are representative of two independent experiments, with n = 5 mice/group. * P<0.05. Mean + s.e.m.

Supplementary Figure S3. Absence of altered antimicrobial peptide production in *Nod2*-deficient mice obtained via caesarean birth. (a) There was no significant difference in cutaneous mBD-1, -3 or -14 in *Nod2*$^{-/-}$ mouse skin versus WT from mice born via caesarean section. All data are representative of two independent experiments with n = 8-10 mice/group. Mean + s.e.m.

Supplementary Figure S4. Co-housing from birth with a WT mother shows the skin microbiome directly influences healing outcome. Newborn WT and *Nod2*$^{-/-}$ litters were mixed from birth with a WT mother, and then wounded in adulthood. (a) Representative H+E stained sections of excisional wounds (day 5) from WT and *Nod2*$^{-/-}$ control (non-mix), and co-housed (mix) cages (arrows denote wound margins). (b) Quantification showed significantly delayed healing in *Nod2*$^{-/-}$ mice co-housed with WT mice, with (c) increased local neutrophil influx. All data are representative of two independent experiments with n = 6

652 mice/non-mix groups and n = 5 mice/mix group. * P<0.05. Mean + s.e.m. Scale bar = 250
653 μM (a).

654 **Supplementary Table 1. Primer sequences for DGGE, 16S Illumina high-throughput**
655 **sequencing and qPCR.**

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657 **Supplementary Table 2. Average proportions of genera for all mapped reads from the**
658 **16S Illumina high-throughput sequencing data, including probability values as**
659 **determined by one-way ANOVA.**