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The Cutaneous Microenvironement Influences Wound Healing Through Toll-Like Receptor Modulation of Inflammation

Abstract:
Chronic wounds are a major source of morbidity in the United States, affecting an estimated 5.7 million people per year at a cost of 20 billion dollars annually. Underlying factors, such as diabetes, poor perfusion due to vascular disease, or infection, complicates many of these wounds. The normal wound healing process proceeds through three stages, which are precisely choreographed. Appropriate early inflammation is required to sterilize and debride the wound, followed by an orderly transition to repair processes. Inappropriate inflammation can result in pathologic wound healing. It is well recognized that the commensal organisms present in the gut play an important role in mediating gastrointestinal immunity and inflammatory responses through their interaction with innate pathogen recognition receptors, such as TLRs. The skin is also continuously exposed to potential pathogens and possesses unique microflora. Recently, the common cutaneous commensal, *Staphylococcus epidermidis*, was shown to help establish an inflammatory homeostasis by reducing inflammation in the skin under normal conditions. This is accomplished through activation of TLR-2 by the bacteria's unique lipotechoic acid, which leads to suppression of a NF-kB mediated inflammatory pathway activated by TLR3. Recent research has also shown that TLR3 recognizes cutaneous injury in addition to its traditional dsRNA ligand, and is important for inducing inflammation and in wound healing. Inflammation is essential early in the wound healing process, but detrimental later on. Therefore, the timing and degree of inflammation must be tightly regulated. In light of these interactions, and the presence of much greater amounts of commensal bacteria in chronic wounds, I evaluated the role of TLRs and *S. epidermidis* LTA in modulating the wound healing process. TLR3 KO mice demonstrated altered gene expression following wounding, but unlike other reports, we observed delayed healing only on days 2-4. Exposure to LTA significantly delayed the wound healing response in WT mice. TLR3 KO mice exposed to LTA had delayed wound closure at later time points as well, similar to WT mice exposed to LTA. These results indicate that LTA exposure leads to delayed wound healing that is TLR3 independent. While TLR3 is involved in early wound healing, LTA primarily affects later stages of the wound healing process.

Background:

Chronic wounds are a significant health problem in both the United States, and throughout the world. In the United States, there are an estimated 5.7 million people affected by chronic wounds every year. They create a significant burden for individual patients and for the health care system, costing an estimated 20 billion dollars annually[1]. Many chronic wounds are complicated by underlying factors, such as diabetes and vascular disease, which lead to poor perfusion and increased infection. Chronic wounds

have also been associated with an increased burden of colonizing bacteria, or frank infection, and the presence of increased inflammatory mediators[2-4]. In wound healing, inflammation is essential to initiating the repair process, but excessive inflammation can lead to improper healing and disease. Therefore, the inflammatory response must be tightly controlled.

The process of wound healing proceeds through three major stages, the inflammatory phase, the proliferative phase and the remodeling phase. The inflammatory phase begins immediately after injury with initial hemostasis and platelet plug formation. Platelets release a number of factors, including PDGF and TGF-b, which attract neutrophils and macrophages. These cells control bacteria introduced into the wound and clear debris. TGF-b, IL-1 b and TNF-a all drive leukocyte recruitment and activity, fueling a positive feedback loop where these cells produce more cytokines, including TNF-a, IL-6 and IL-1, to enhance recruitment and activation of more inflammatory cells. These leukocytes also release factors that attract fibroblasts and initiate the proliferative phase. The inflammatory phase lasts about 2-4 days. The proliferative phase begins about day 3 and includes angiogenesis, granulation tissue formation, collagen deposition, re-epithelialization and wound contracture. This phase is largely driven by growth factor elaboration and fibroblast activity. Collagen, mostly type III, is laid down and re-epithelialization occurs from the wound edges, or basement membrane if it has remained intact. This may last 2-4 weeks after wounding. Finally, the wound progresses to the remodeling phase, where collagen synthesis and degradation occur at about equal rates and maturation of the wound ensues. The originally deposited type III collagen is replaced with stronger type I collagen and arranged along tension lines. The process of wound maturation may take a year or more[5]. This is a finely orchestrated process, where disruption of any phase, or transition between phases, can result in dysfunctional healing. For example, TGF-β is crucial to the inflammatory phase, however it also inhibits keratinocyte migration, which is essential in the proliferative phase. Therefore, failure to down-regulate this molecule at the appropriate time may result in formation of chronic wounds. Similarly, other causes of continued inflammation, such as infection, may also delay wound closure.

Mammalian immune defense relies on integrated responses from the innate and adaptive elements of the immune system. The innate response is comprised of both physical barriers and genetically encoded defense molecules, such as antimicrobial peptides and pathogen recognition receptors. The adaptive response utilizes somatic recombination in leukocytes to develop highly antigen specific responses to potential pathogens. These systems work together in an integrated fashion, mediated by cytokines and other signaling molecules, to produce an appropriate response to environmental stimuli. The Toll-Like Receptor (TLR) family of pathogen recognition receptors is critical to initiating innate responses, as well as enhancing adaptive immune responses to pathogens [6,7]. They can trigger the release of a broad spectrum of cytokines and chemokines, including interferons and pro-inflammatory NF-κB chemokines. These, in turn, can modulate cellular responses and even initiate adaptive immunity, if required.

Despite the abundance of innate defense mechanisms and microbial recognition receptors, such as the TLRs, most epithelial surfaces are heavily colonized with symbiotic microbes. These organisms do not trigger an immune response and appear to be beneficial in many ways[8]. In fact, commensal organisms have been recognized as important for modulating immune responses, with the gut being the most studied microbiome. For example, normal gut flora has been shown to influence the magnitude and duration of TLR mediated responses and play an important role in modulating normal mucosal immunity[9-11] via the regulation of proinflammatory and anti-inflammatory cytokine synthesis [12,13]. The commensal bacteria *Lactococcus lactis* and *Bacteroides vulgatus* can prime the host to produce IL-10, trefoil factors and TGF-b, which have been suggested to modulate inflammation in the intestine[14-16]. In light of this evidence, it is surprising that there has not been more investigation of the role that the cutaneous microbiome may play in skin immunity and wound healing. Recent work by Lai et al, however, has demonstrated a novel interaction between the common cutaneous commensal microbe, *Staphylococcus epidermidis* (*S. epidermidis*), and TLR3 signaling[17]. Normally, TLR3 is thought to recognize dsRNA in endosomes, triggering both IFNa/b responses and NF-κB pro-inflammatory responses to ward off potential viral pathogens. However, Lai has also demonstrated that TLR3 on keratinocytes is an important sensor of cutaneous injury and dying cells. Despite the sensitivity of keratinocytes to these signals, inappropriate inflammation is normally held in check. This is accomplished through the interaction of lipotechoic acid (LTA) from *S. epidermidis* on TLR2 homodimers, which leads to suppression of the intracellular NF-κB inducing pathway triggered by TLR3[17]. This allows normal anti-viral surveillance without excessive inflammation. This demonstrates the critical role these innate immune receptors play in balancing appropriate inflammation and responses to environmental stimuli. Understanding the regulation of TLR function is important due to its central role in protecting the skin against infection and its influence on many inflammatory skin conditions [18].

Appropriate timing and magnitude of the inflammatory response is essential to effective wound healing. The emerging role of TLR3 in recognizing injury and the ability of *S. epidermidis* to influence inflammatory responses in intact skin through TLR2 mediated suppression of TLR3 proinflammatory responses begs the question of whether the inflammatory response in wounding is modulated by *S. epidermidis* as well. Alteration of the inflammatory response may effect wound healing and could play a significant role in pathologic wound healing.

Hypothesis:

Based on recent publications and ongoing research, I hypothesize that skin commensal bacteria, like *S. epidermidis*, modulate the inflammatory response during wound healing. TLR3 is an important mediator of proinflammatory responses and is involved in wound healing. I hypothesize that its deficiency will lead to a delay in the initiation of these responses and an overall delay in wound closure. LTA from the commensal organism, *S. epidermidis*, has also been shown to suppress TLR3 activation in normal keratinocytes. I hypothesize that exposure of wounds to LTA will lead to a suppression of TLR3 mediated responses, and thus suppressed early inflammatory responses, which will delay wound healing.

Methods:
Mice: C57Bl/6 wild type mice or TLR3 deficient mice on C57Bl/6 background (Jackson Labs) were housed in the VA San Diego VMU. All experiments were approved by the VA IACUC.

Wound healing: Full thickness wounds were made in dorsal skin using 6mm punch biopsies. Briefly, mice were anesthetized, their backs clipped and hair removed by chemical depilation (Nair). Skin was cleaned with 70% ethanol, picked up and sterilely punched through, generating 2 identical wounds on each side of the dorsal midline. Animals were anesthetized with 2% isoflurane during the procedure and buprenorphine (0.1mg/kg) was administered SQ before the procedure and during the first 24 hours post-op for analgesia. Wounds were digitally photographed daily and the area was quantified using NIH ImageJ software. The statistical significance of differences observed in the rates of wound healing was determined by repeated measures ANOVA and Newman-Keuls post-test in the GraphPad Prism v4 statistical software package.

LTA pretreatment: Dorsal fur was removed, as above, from the mice 48 hours before wounding. Mice received an intradermal injection of 50mg LTA from *S. epidermidis* (Invivogen, San Diego, CA.) in 100uL PBS in their left flank and 100ul of PBS vehicle control in their right flank. Injections were given 24 hours and 2 hours before wounding and the area of the bolus was marked to ensure that both injections and the wound would be in the same area.

Microarray Analysis: Microarray analysis was performed on the skin surrounding wounds of WT and TLR3 deficient mice using Affymetrix Mouse Gene 1.0 ST Arrays. Wound tissues were harvested from 2 WT and 2 TLR3 deficient mice 18 hours after wounding and RNA was extracted using Trizol Reagent (Invitrogen, San Diego, CA.). RNA was further purified using an RNeasy kit from Qiagen (Valencia, CA). RNA was then submitted to the VMRF Genechip Microarray Core Facility for QC, hybridization and scanning. Initial differences in expression were determined using Genespring 12 software, The Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 and VAMPIRE. Genespring 12 was used to determine clustering and initial analysis, as well as to produce a heat map. Hierarchical clustering was performed and transcripts were filtered based on a greater than 1.5 fold difference, either increased or decreased, with p-values <0.05. VAMPIRE software was used to compare all transcripts on the chips and assess differences as fold change with statistical significances in TLR3 deficient wounds compared to WT wounds, utilizing a Bonferoni post-test. DAVID v6.7 was used to compare transcripts that were significantly increased or decreased more than 1.5 fold and generate lists of pathways that were significantly changed between WT and TLR3 deficient wounds.

Results:
TLR3 deficiency delays early wound healing:
TLR3 is important for detection of injury and triggering early inflammatory responses through NF-κB. Its role in normal wound healing was also demonstrated recently, as

TLR3 deficient mice had delayed wound closure[19]. In our study, we sought to determine how TLR3 deficiency effects the rate of wound healing by comparing wild type and TLR3 deficient mice (Fig. 1 A&B). Overall, we found no significant difference between wild type and TLR3 deficient mice (P>0.05), however there was a trend towards smaller wound sizes in wild type mice on days 2-4, with day 2 being significantly different (P<0.05 by student t-test) (Fig. 1B). Thus, TLR3 deficiency may slow early wound healing, although there is no difference in the time to complete healing, with both TLR3 deficient mice and wild type mice achieving complete closure by day 9-10. These results indicate that TLR3 is important for early wound healing and deficiency appears to slow initial wound closure, although there is no difference in the final time to full closure.

TLR3 Deficient Mice Demonstrate Altered Gene Expression After Wounding:
TLR3 is important for detecting cutaneous injury, inducing early inflammatory responses and appears to be involved in wound healing. Our observation that TLR3 deficiency led to an early delay in wound healing begged the question of whether TLR3 deficient mice would respond to injury with differentially expressed early genes due to the loss of TLR3 mediated proinflammatory responses. Microarray analysis of wild type and TLR3 deficient mouse wounds at 18 hours showed a broad range of differences in gene expression. Hierarchical cluster analysis of genes altered more than 1.5 fold using Genespring 12 demonstrated clustering of wild type and TLR3 deficient mouse wound samples. There were significant differences in expression between several groups of genes, as demonstrated by the heat map (Fig. 2). Some of the genes altered in the TLR3 deficient mice included decreased expression of proinflammatory cytokines, like TNF and increased expression of many keratin associated protein family members and some keratins (Fig 2 and Table 1A&B). IL-6 gene expression was significantly depressed more than 2 fold in TLR3 deficient mice and CXCL1 and CXCL13 were lower by more than 3 fold. Similarly, TNF, CCL2, CXCL2 and Traf1 were reduced about 1.5 times the wild type mouse levels (Table 1A). This demonstrates that in the absence of TLR3, there is less early expression of proinflammatory cytokines and increased expression of keratin and keratin associated proteins, which are usually expressed in later stages of the healing process. DAVID was used to determine whether the alteration of these individual genes represented increased, or decreased, activity of particular biologic pathways. All transcripts that were altered more than 1.5 fold were analyzed, demonstrating many significantly affected gene ontology pathways. Pathways important for injury response and early healing were decreased in the TLR3 deficient mice (Table 1C), including inflammatory responses, immune defenses, cell migration and new vessel development. In contrast, many pathways involved in cell cycle and division were increased in the TLR3 deficient mice (Table 1D). This list also included enhanced activity of transcripts involved in epithelium development, which includes keratins and associated proteins.

LTA Delays Wound Repair:
Wound healing is an important and dynamic process, which critically depends on appropriately timed inflammatory events. TLR3 has been show to be a mediator of proinflammatory responses to injury and appears to be important for early wound healing. Additionally, LTA from *S. epidermidis* has been demonstrated to suppress TLR3 mediated inflammatory responses in normal skin through a TLR2 dependant mechanism.

As a result of these observations, we examined whether LTA exposure may alter the rate of wound healing. C57Bl/6 mice were exposed to LTA from *S. epidermidis* or PBS control 24 and 2 hours before wounding. The LTA pretreated group had a significantly slower rate of wound closure ($P<0.05$) as compared to the PBS treated wounds (Fig. 3). This difference became evident after day 2, before which the two groups of mice had identical rates of healing. PBS pretreated control wounds were completely healed by day 9-10, while LTA pretreated wounds failed to close until day 10-11. Interestingly, the largest differences in wound area occurred in the middle of the wound healing process, including days 3 to 7. This stands in contrast to TLR3 deficient mice, whose wound closure lagged behind their wild type controls at days 2-4, but eventually completed healing at the same time.

LTA Delays Wound Closure Via A TLR3 Independent Mechanism:
To determine whether LTA influences wound healing by inhibiting TLR3 mediated NF-κB proinflammatory responses, as it does in intact skin, we examined the effect of LTA on TLR3 deficient mice. TLR3 deficient mice demonstrated a further delay in wound healing after pretreatment with LTA ($P<0.01$) (Fig. 4 A&B). Comparison of the healing curves in these mice mirrored the curves seen in wild type mice pretreated with LTA (Fig 4B vs. Fig 3B). Similar to the wild type mouse results, the curves diverged after day 2 with maximal differences in wound area between days 4-8. This indicates that LTA is influencing wound closure through a TLR3 independent pathway. Furthermore, wound closure in LTA pretreated TLR3 deficient mice significantly lagged wound closure in wild type mice after treatment with LTA ($P<0.05$), however the differences were only seen at early time points, including days 1-4 (Fig. 4C). After day 4, the rates of wound closure in TLR3 deficient and wild type mice treated with LTA were almost identical, with both groups completing wound closure by day 11. The differences seen in these curves mirrors the comparison of non-LTA treated TLR3 deficient and wild type mouse wounds, which only showed a difference on days 2-4. This suggests that TLR3 influences early wound healing, while LTA is effecting later processes through non-overlapping pathways.

Discussion:
Chronic wounds are a major health concern, leading to significant morbidity, patient burden and cost[1]. Understanding the cutaneous microbiome's role in modulating inflammation through TLRs during wound healing is important to understanding the pathophysiology of chronic wounds and developing more effective treatment strategies. The role of the skin's microbiome has not been well studied, yet emerging data suggest that it may be important for a broad array of homeostatic functions and defects may lead to disease[8]. The microbiome in the gut has been much more extensively studied and it is clear that it contributes significantly to shaping and directing appropriate host immune responses, including inflammation. Recent research has shown that the cutaneous commensal, *Staphylococcus epidermidis*, contributes to the regulation of TLR mediated inflammation in the skin. Other work has demonstrated a correlation between overgrowth of *Staphylococcus spp* and chronic wounds[20], as well as a link between TLR2 activation

and impaired keratinocyte migration[21], chronic inflammation[22,23] and inhibition of fibrocyte differentiation[24]. In light of this role, and the importance of an appropriate degree of inflammation in wound healing, I investigated the effect of *S. epidermidis* LTA modulation of inflammation on wound healing. These studies are important due to the emergence of TLR3 as a sensor of cutaneous injury with a role in wound healing and staphylococcal LTA's inflammatory homeostatic function of inhibiting TLR3's proinflammatory activity in intact skin[17, 19]. Insufficient inflammation early in the process may lead to impaired wound healing and infection, while excessive inflammation is also detrimental to the process. Wound healing proceeds through a number of precisely coordinated phases. The initial inflammatory phase, which is driven by factors such as TGF-b, TNF-a, and IL-1b, results in a robust leukocyte response to sterilize the wound and remove debris. Once this is accomplished, this process is down-regulated and inflammatory cells yield to fibroblasts, which drive the formation of granulation tissue and collagen deposition, and keratinocytes, which re-epithelialize the defect[5]. Failure to develop these responses, or appropriately transition from the inflammatory to proliferative phase, results in ineffective wound healing or chronic wounds.

Qing *et. al*. demonstrated that TLR3 deficient mice had delayed wound closure by two days compared with WT mice[19]. This was attributed to defective recruitment of leukocytes and release of cytokines. Our experiments utilizing a larger number of animals failed to reproduce the delay in final closure noted in that study. We did, however, establish a trend towards delayed healing early after wounding, which was significant on day 2. This confirms the importance of TLR3 in the early events of the wound healing process. In our experiments, the TLR3 deficient mice had more rapid wound closure after day 2, until day 5, when they had similar wound areas to the WT controls. After this point, the mice continued to heal at a similar rate, with complete closure at the same time. Of note, our experiments utilized only two wounds per animal, while Qing *et. al*. used four, leaving the possibility that the extent of trauma may effect the ability of the skin to heal or for other mechanisms to compensate for the loss of TLR3 driven responses. This would also explain why our mice healed sooner (WT 9 days vs 12 days). Similarly, our mice were housed under standard conditions after wounding, leaving them exposed to the normal microorganisms in their environment, while Qing *et. al*. housed their mice in pathogen free conditions. This may also suggest the role of normal flora in wound healing.

The early response to wound healing involves the elaboration of chemokines and cytokines following injury in order to initiate local defenses, recruit lymphocytes and begin the inflammatory phase of the repair processes. Since TLR3 appears to be involved in early wound healing, we used microarray analysis to determine how TLR3 deficiency alters the initial transcriptional response to injury. While the significance of enhanced hair-related keratin and keratin associated protein family members remains unclear, and translational significance needs to be correlated, the relative decrease in expression of proinflammatory cytokines clearly correlates with TLR3's involvement in injury detection and early response. Other studies have confirmed the decreased levels of MIP-2/CXCL2, MIP-1a/CCL3 and MCP-1/CCL2 in TLR3 deficient mouse wounds. Our findings that transcription of important initiators of the inflammatory phase of wound

healing are decreased in the absence of TLR3 18 hours after wounding correlates well with the early delay in wound healing seen in these mice. In wounding, the initial source of proinflammatory and chemotactic molecules is the local environment. Once neutrophils and macrophages arrive, about 12-24 hours after wounding, they become the predominant source of these factors to further intensify the inflammatory response. Lack of TLR3 mediated inflammatory signals shortly after wounding may delay the recruitment of these inflammatory cells, thus delaying the entire inflammatory phase, which normally occurs during the first 2-4 days. This is consistent with the findings of Qing et. al.[19], who saw decreased neutrophil infiltration on days 1-3 and macrophage recruitment on days 3-6. This is also consistent with our findings that wound closure was delayed in TLR3 deficient mice during this time. The fact that the TLR3 deficient mice were able to catch up to the wild type controls, and complete healing at the same time, likely represents recovery of the wound healing processes by cells responding to injury through non-TLR3 mechanisms.

These studies, combined with evidence that TLR3 is important for detection of injury and initiation of proinflammatory responses though NF-kB pathways, strongly suggest a significant role for TLR3 in early wound healing. We were able to confirm an early wound healing phenotype in these mice, which correlated with decreased expression of important NF-kB dependent cytokines. However, isolated abrogation of TLR3 in aseptic wound models, where the mice are exposed to normal flora, does not seem to be sufficient to alter the overall time to wound closure.

In light of this role of TLR3, and the evidence that TLR2 activation by LTA from normal skin flora suppresses TLR3 mediated inflammation in keratinocytes, we looked at whether LTA from *S. epidermidis* might recapitulate the effects of TLR3 deficiency, causing a delay in early wound healing through inhibition of TLR3 proinflammatory pathways. Mice pretreated with *S. epidermidis* LTA indeed demonstrated a delay in wound closure, however this effect was not the same as in TLR3 deficient mice. The overall rate of wound closure in LTA exposed mice was delayed beginning on day 2 and persisted throughout the healing process with untreated mice achieving closure one day earlier. The greatest difference in both rate and wound area was seen on days 3 to 7. This stands in contrast to TLR3 deficiency, where the difference was significant at day 2 and disappeared by day 5. These results suggest that TLR2 activation effects wound healing through an alternative mechanism and influences later wound healing responses. This was confirmed by examining the effect of LTA pretreatment of TLR3 deficient mice. Similar to wild types, the TLR3 deficient mice exposed to LTA evidenced a delay in wound healing that began after day 2 and persisted until final closure, which was delayed by one day compared to untreated controls. Furthermore, the early delay in wound healing seen in TLR3 deficient mice not treated with LTA was still noted in the LTA treated TLR3 deficient mice compared to LTA treated wild type controls, but no differences between LTA treated TLR3 deficient and wild type mice were seen in later phases of wound healing. This indicates that LTA effects wound healing identically in wild type and TLR3 deficient mice, but does not influence the role of TLR3 in early wound healing. The inability of LTA to suppress TLR3 mediated inflammation in these experiments, in contrast to its previously demonstrated ability to do so in normal skin,

may be indicative of the specificity of this TLR2 function to keratinocytes but not other cells. Wounding involves multiple cell types in the response. The suppression of inflammatory responses of keratinocytes in intact skin, which are exposed to various environmental microorganisms and conditions, may be a beneficial homeostatic function. However, blunting of proinflammatory responses to injury would be detrimental. Therefore, it is likely that this effect in keratinocytes is either suppressed following injury, or is absent in the cells of deeper epidermal or dermal layers, which may still utilize TLR3 mediated detection of injury and inflammatory responses upon injury.

There are many possible causes of the late effect that LTA pretreatment has on wound healing. The later processes of wound healing rely on suppression of the proinflammatory cascade initiated in the inflammatory phase. Some of the same molecules that drive leukocyte recruitment and activation also suppress fibroblast collagen deposition and keratinocyte migration[5]. Failure to transition correctly from the inflammatory phase to the proliferative phase would clearly delay wound closure. TLR2 activation has been show to effect keratinocyte migration, fibrocyte differentiation, increase macrophage activation and induce inflammation[5,21,22,23,24]. In fact, many chronic wounds are colonized with staph species at levels much greater than normal skin[20,3,4]. As we have demonstrated the ability of TLR2 activation by LTA to delay wound closure, this mechanism may be similar to the pathophysiology of chronic wounds. Whether the TLR2 effect is to increase macrophage activity and prolong the inflammatory phase, delay cell migration during healing or promote chronic inflammation, the overall result is retardation of the healing process. In these experiments, LTA was administered prior to wounding, but wounds were not retreated. This leads to the possibility that the effect of LTA on triggering TLR2 mediated delays in wound healing diminished throughout the process, allowing eventual healing of the wound. In chronic wounds, persistent exposure to TLR2 ligands from colonizing bacteria may prevent their closure all together.

Our data suggest that commensal microorganisms may play an important role in host interaction with the environment. *S. epidermidis* plays a role in the inflammatory homeostasis of normal skin. Our work here indicates that while the TLR3 response to injury is important for the initiation of wound healing, the same mechanism for suppression of TLR3 driven inflammation by *S. epidermidis* in normal skin does not effect its ability to help initiate the inflammatory phase of wound healing. Instead, activation of TLR2 by LTA in wounds leads to a more persistent delay in wound healing that is TLR3 independent. Therefore, what is a beneficial homeostatic relationship in intact skin may become a cause of pathologic wound healing if TLR2 ligands are present inappropriately in the wound environment. Understanding the role of commensals in health and disease may open therapeutic avenues for treating pathologic wound healing more effectively.

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Figure Legends:

Figure 1: TLR3 deficiency results in delayed early wound healing.
TLR3 KO mice and C57Bl/6 controls were wounded with full thickness 6mm punch biopsies and the rate of wound healing determined. Photographs of the wounds were taken daily and the area of the wound was measured. Photographs from every other day of 2 representative mice are shown here (A). Wound size was plotted, demonstrating larger wounds in the TLR3 KO mice on days 2-4 (B). This difference was significant ($p<0.05$) on day 2 by student t-test, but overall the difference in wound healing rates was not statistical significant, as determined by ANOVA ($p>0.05$).

Figure 2: Early response to wounding is altered in TLR3 deficient mice at the transcriptional level.
Changes in gene expression 18 hours after wounding were determined by microarray analysis. Analysis of data with Genespring 12 software demonstrated clustering of WT and TLR3 KO wound samples and showed significant differences in expression, as demonstrated by the heat map, of transcripts that were altered more than 1.5 fold (A). Areas of the heat map which contain clusters of genes with large degrees of alteration between the WT and TLR3 deficient mice are expanded in B, C and D, which correspond to the lettered area in A. Large numbers of keratins and keratin-associated proteins were increased in the TLR3 deficient mice (B&D), while some proinflammatory mediators, such as TNF, IL-6 and CXCL1 were decreased (C).

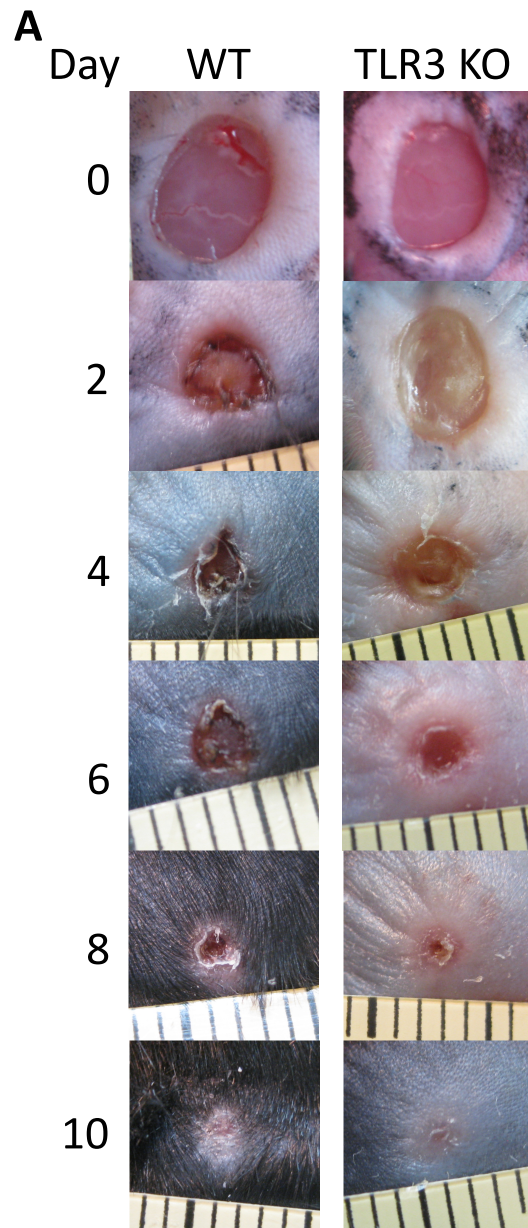
Table 1: Transcription of genes from pathways involved in wound response and inflammation are decreased in TLR3 deficient mice.
Analysis of the differences in transcriptional activity between wild type and TLR-3 deficient wounds was performed utilizing microarrays (Figure 2). Genespring 12 and VAMPIRE software was used to analyze differences between individual transcripts. A number of genes involved in the inflammatory phase of wound healing were significantly decreased in TLR3 KO mice compared to controls, including CXCL cytokines, IL-6 and TNF (A). Most of the genes increased in TLR3 KO mice over wild type controls were keratin associated proteins and keratins, with the top 10 induced genes listed (B). All transcripts that were altered more than 1.5 fold were analyzed with the DAVID software to identify pathways that were different between WT and TLR3 KO mice. The top gene ontology pathways that were significantly increased or decreased are listed. Many pathways involved in the wound response were decreased in the TLR3 KO mice (C), whereas many cell cycle pathways were increased (D).

Figure 3: Exposure to LTA delays wound healing.
C57Bl/6 wild type mice were pretreated with either 50mg *S. epidermidis* LTA or PBS 24 and 2 hours before wounding. Wounds were photographed daily (photographs from every other day of 2 representative mice are shown here (A)) and the wound areas plotted (B). Wounds pretreated with LTA healed significantly slower, beginning after day 2 and

completing closure 1 day later than PBS treated control wounds. This was statistically significant by ANOVA analysis ($p<0.05$)

Figure 4: LTA delays wound healing in a TLR3 independent manner.
TLR3 KO mice were wounded after pretreatment with either 50mg *S. epidermidis* LTA or PBS as in Figure 3. The wounds were photographed daily and images from every other day of 2 representative mice are shown here comparing LTA and PBS treated TLR3 deficient mice (A). The wound areas were calculated and plotted (B). Similar to wild type mice, TLR3 KO mice pretreated with LTA had significantly delayed wound closure compared with PBS pretreated controls. This effect was also seen after day 2 and resulted in a one day delay in final wound closure. Statistical significance by ANOVA $p<0.01$. This independence of the LTA effect from TLR3 was confirmed by comparing TLR3 KO mice and wild type mice that were both pretreated with LTA (C). Both groups of mice demonstrated overall delayed wound healing, with final closure on day 10, instead of day 9 in PBS treated controls (not plotted in C). The early delay in wound closure at days2-4 that was seen when non-LTA pretreated TLR3 KO mice were compared with wild types was also seen in these groups. This indicates that LTA treatment does not suppress TLR3's role in wound healing. ANOVA $p<0.05$.

Figure 1



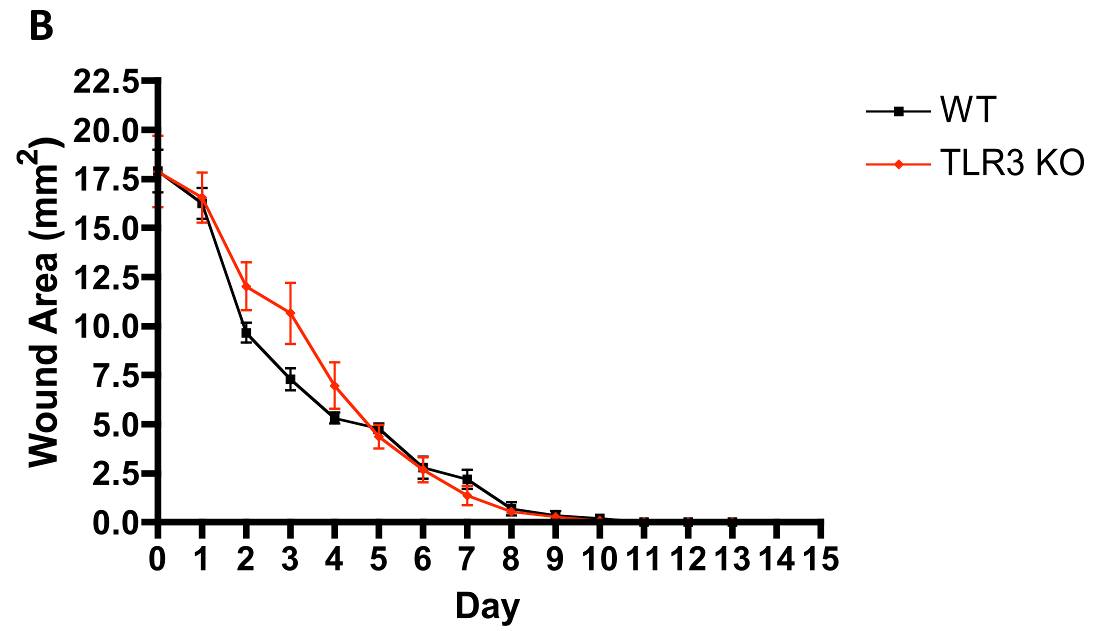


Figure 2

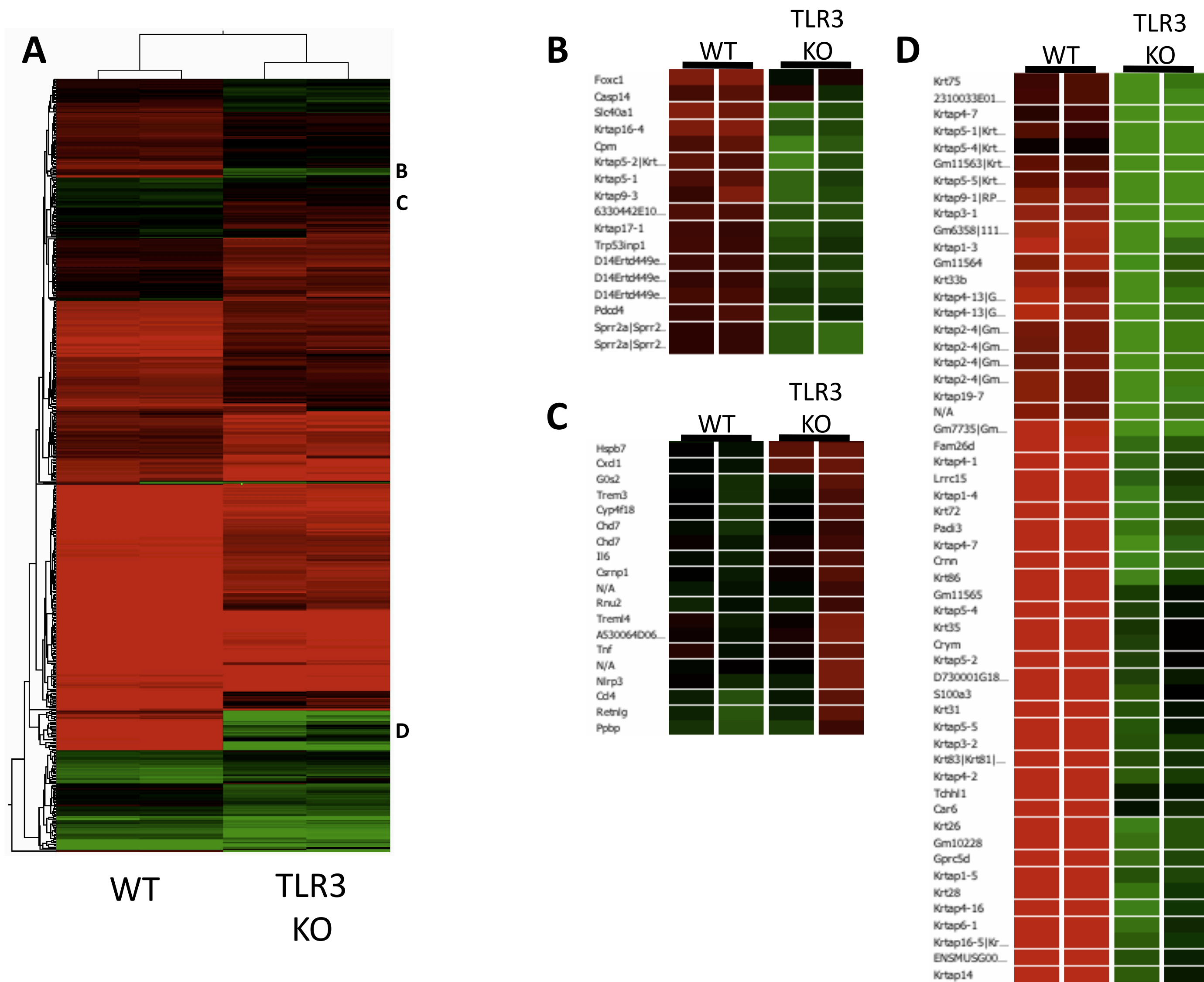


Table 1

A

Transcript Decreased	Fold Change	p
Chemokine (C-X-C motif) ligand 1	3.29	6.14E-42
Chemokine (C-X-C motif) ligand 3	3.21	8.28E-41
Chemokine (C-C motif) ligand 4	2.32	1.79E-23
Interleukin 6	2.05	3.38E-17
Colony stimulating factor 3 (granulocyte)	1.95	9.42E-14
Heparin-binding EGF-like growth factor	1.90	1.84E-14
Chemokine (C-C motif) ligand 2	1.76	1.81E-11
Chemokine (C-X-C motif) ligand 2	1.57	6.48E-08
Tumor Necrosis Factor	1.56	1.84E-07
TNF receptor-associated factor 1	1.56	4.35E-07

B

Transcript Increased	Fold Change	p
Keratin associated protein 15	1027.45	4.06E-33
Keratin associated protein 16-8	525.56	4.60E-33
Keratin 25	480.42	4.71E-33
Keratin associated protein 3-2	468.73	5.09E-33
Keratin associated protein 4-7	425.00	5.02E-33
Keratin 71	396.26	4.99E-33
Keratin associated protein 8-2	375.90	5.48E-33
Keratin 26	344.58	5.78E-33
Keratin associated protein 6-1	311.32	5.48E-33
Keratin 27	281.17	5.82E-33

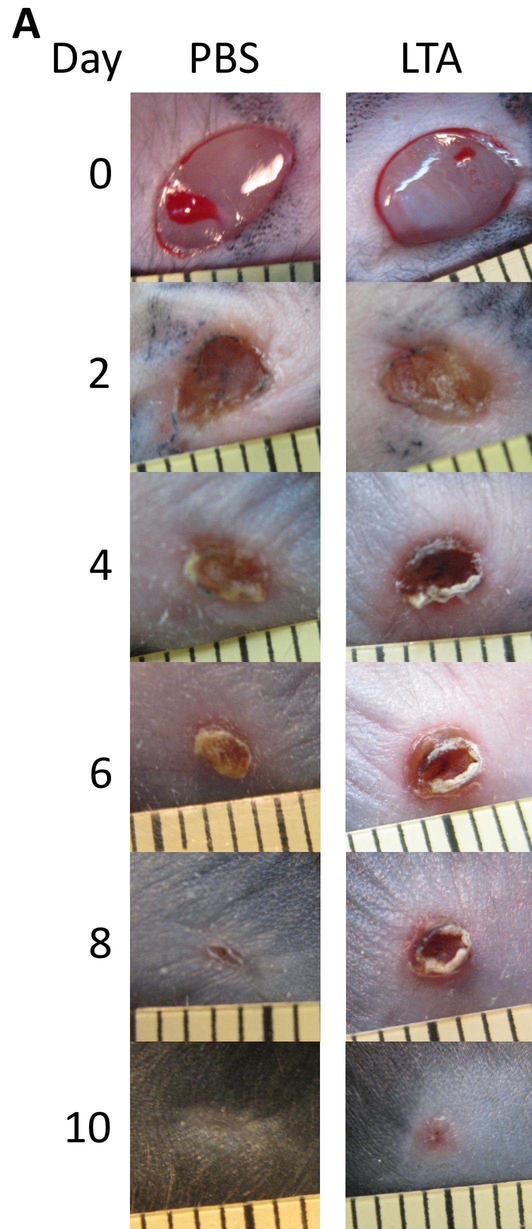
C

Pathway Decreased	% Transcripts Involved
inflammatory response	3.4
blood vessel development	3.6
vasculature development	3.6
response to wounding	4.6
regulation of phosphorylation	3.6
embryonic morphogenesis	4
positive regulation of RNA metabolic process	4.6
positive regulation of transcription	4.9
positive regulation of gene expression	4.9
defense response	4.6
regulation of transcription from RNA polymerase II promoter	6.1
positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	4.9
immune response	4.6
cell motion	3.6

D

Pathway Increased	% Transcripts Involved
M phase of mitotic cell cycle	3.8
nuclear division	3.7
mitosis	3.7
organelle fission	3.7
mitotic cell cycle	4.2
M phase	4.6
cell cycle phase	4.9
cell division	4.2
cell cycle process	5.5
cell cycle	7
epithelium development	2.8
enzyme linked receptor protein signaling pathway	2.7
lipid biosynthetic process	2.7
skeletal system development	2.7

Figure 3



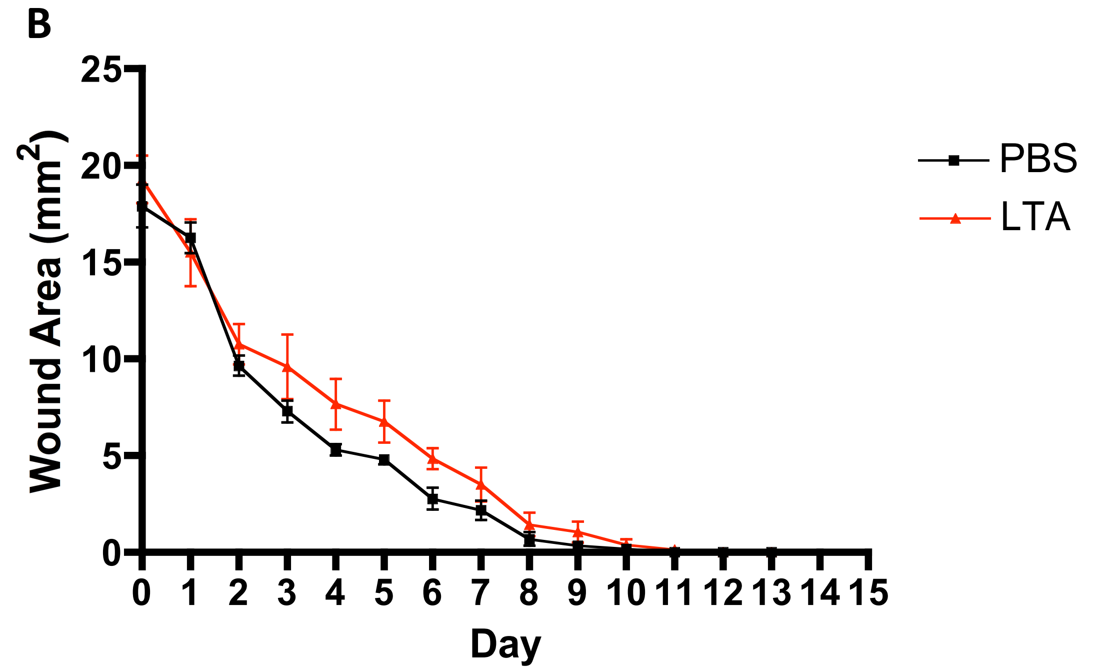


Figure 4