IL-22 Promotes Fibroblast-Mediated Wound Repair in the Skin

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Skin wound repair requires complex and highly coordinated interactions between keratinocytes, fibroblasts, and immune cells to restore the epidermal barrier and tissue architecture after acute injury. The cytokine IL-22 mediates unidirectional signaling from immune cells to epithelial cells during injury of peripheral tissues such as the liver and colon, where IL-22 causes epithelial cells to produce antibacterial proteins, express mucins, and enhance epithelial regeneration. In this study, we used *IL-22*$^{-/-}$ mice to investigate the *in vivo* role for IL-22 in acute skin wounding. We found that *IL-22*$^{-/-}$ mice displayed major defects in the skin's dermal compartment after full-thickness wounding. We also found that IL-22 signaling is active in fibroblasts, using *in vitro* assays with primary fibroblasts, and that IL-22 directs extracellular matrix (ECM) gene expression and myofibroblast differentiation both *in vitro* and *in vivo*. These data define roles of IL-22 beyond epithelial cross talk, and suggest that IL-22 has a previously unidentified role in skin repair by mediating interactions between immune cells and fibroblasts.

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INTRODUCTION

Breaches to epithelial barriers such as the skin result in an inflammatory response that prevents infection and augments tissue repair via the release of cytokines that signal between the immune system and cells in the damaged tissue (Eming *et al.*, 2007; Barrientos *et al.*, 2008). In the skin, wound repair requires interactions between keratinocytes, fibroblasts, immune cells, and endothelial cells. Communication between these cells restores the epithelial barrier via keratinocyte proliferation and migration while reestablishing the dermal architecture through extracellular matrix (ECM) production and myofibroblast-mediated wound contraction.

Members of the IL-10 cytokine family are soluble factors that orchestrate interactions between the immune system and peripheral tissues (Dumoutier *et al.*, 2001; Kotenko, 2002). One member of the IL-10 cytokine family, IL-22, is produced by both adaptive and innate lymphoid cells (Liang *et al.*, 2006; Cella *et al.*, 2009; Cupedo *et al.*, 2009; Eyerich *et al.*, 2009; Goto *et al.*, 2009; Martin *et al.*, 2009; Takatori *et al.*, 2009). IL-22 mediates its functions in tissues by binding to its heterodimeric receptor consisting of IL-10 receptor β (IL-10Rβ) and IL-22 receptor α (IL-22Rα) and activating signaling pathways that stimulate STAT3 phosphorylation and nuclear translocation (Xie *et al.*, 2000; Dumoutier *et al.*, 2001, 2003; Kotenko *et al.*, 2001; Lejeune *et al.*, 2002). Whereas IL-10Rβ is expressed ubiquitously on both immune cells and tissue-specific cell types, IL-22Rα is thought to be exclusively expressed on epithelial cells, such as keratinocytes in the skin and hepatocytes in the liver (Dumoutier *et al.*, 2003; Radaeva *et al.*, 2004; Wolk *et al.*, 2004). The restriction of IL-22Rα expression allows IL-22 to mediate unidirectional signaling from the immune system to tissue-specific cells.

Many similarities can be found between the repair processes that occur in the skin and those that occur in other organs containing epithelium, such as the thymus, liver, and intestine, where IL-22 mediates signaling to epithelial cells (Radaeva *et al.*, 2004; Zenewicz *et al.*, 2007, 2008; Sasaki *et al.*, 2011; Dudakov *et al.*, 2012). In the skin, IL-22 is expressed after burn wounding (Sasaki *et al.*, 2011), elevated in human psoriasis patients (Lowes *et al.*, 2008), and promotes dermal inflammation (Zheng *et al.*, 2007; Ma *et al.*, 2008; Zhang *et al.*, 2012). *In vitro* studies have suggested that IL-22 signals to epithelial keratinocytes of the skin to promote proliferation and migration while repressing keratinocyte

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Abbreviations: ECM, extracellular matrix; mRNA, messenger RNA; PBS, phosphate-buffered saline; α-SMA, α-smooth muscle actin; STAT3-P, phosphorylated STAT3; WT, wild type

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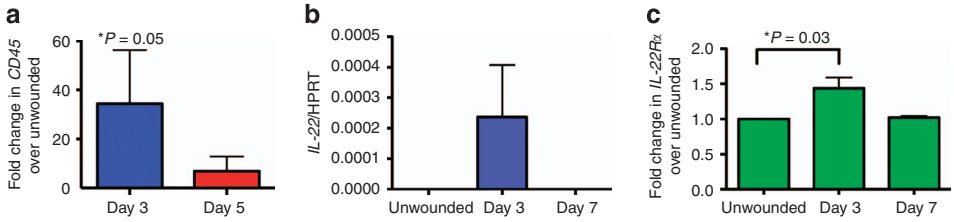

Figure 1. *IL-22* messenger RNA (mRNA) is expressed during acute skin wounding. (**a**) *CD45*, (**b**) *IL-22*, and (**c**) *IL-22Rα* mRNA expression via real-time PCR in unwounded skin or wounds of mice after the indicated time points. $n = 3$ wounds from three mice for each time point. All data are ± SEM. Asterisks indicate significance.

differentiation (Boniface *et al.*, 2005; Wolk *et al.*, 2006); however, the function *of* IL-22 *in vivo* during acute skin wound healing has not been explored.

In this study, we investigated the role of IL-22 in a murine model of acute skin wounding. We find that *IL-22* messenger RNA (mRNA) is upregulated during the inflammatory response following tissue injury. Surprisingly, keratinocytes in the skin of *IL-22*$^{-/-}$ mice display only minor defects in their ability to repair the epithelial barrier during wound healing. However, *IL-22*$^{-/-}$ wounds exhibit severe defects in the dermal compartment because of deficiencies in fibroblast function. We show that IL-22-induced signaling in fibroblasts regulates ECM production and myofibroblast differentiation during proper wound healing. Therefore, our results demonstrate that IL-22 mediates signaling beyond epithelial cells and is required for normal fibroblast function during skin wound repair.

RESULTS

IL-22 mRNA expression during acute skin wounding

To determine whether IL-22 might have a role in acute skin wounding, we analyzed the expression of *IL-22* and *IL-22Rα* mRNA in a murine model of full-thickness skin wounds. The dorsal skin of wild-type (WT) mice was wounded using 2- or 8-mm skin punch biopsies as indicated. For each wound size, wounds were harvested during and after the inflammatory phase of wound healing, as indicated by *CD45* mRNA and protein expression in 3-day skin wounds of 2-mm biopsies (referred to as small wounds) and in 5-day skin wounds of 8-mm biopsies (referred to as large wounds; Figure 1a and data not shown; Eming *et al.*, 2007). *IL-22* mRNA was not detected in unwounded skin (Figure 1b). However, *IL-22* mRNA was upregulated during the inflammatory phase of wound healing, and its expression returned to a steady state after inflammation (Figure 1b). In contrast, *IL-22Rα* mRNA expression increased only slightly during the inflammatory phase compared with unwounded skin, but its expression returned to baseline levels after inflammation (Figure 1c). These data are consistent with the induction of *IL-22* mRNA expression after infectious or injurious stimuli in the liver and colon (Radaeva *et al.*, 2004; Zenewicz *et al.*, 2007, 2008). Taken together, these data demonstrate that IL-22 signaling components are expressed in the skin during acute wound healing.

Delayed wound healing in *IL-22*$^{-/-}$ mice

As *IL-22* mRNA expression is upregulated in the skin after wounding, we investigated whether acute wound healing was defective in *IL-22*$^{-/-}$ mice. No overt skin defects were observed in *IL-22*$^{-/-}$ mice during development (data not shown) or in adult mice (Supplementary Figure S1a online). To investigate whether IL-22 has a role in wound healing *in vivo*, we generated small and large full-thickness wounds in the dorsal skin of sex-matched WT and *IL-22*$^{-/-}$ mice during the telogen phase of the hair cycle (Supplementary Figure S1a online). Immune cell recruitment and blood vessel formation were similar between wounds of WT and *IL-22*-null mice, as indicated by the expression of CD45 and isolectin GS-IB4 (Tammela *et al.*, 2008), respectively (Supplementary Figure S2a and b online).

Surprisingly, keratinocyte function during wound healing was not significantly impaired in *IL-22*-null mice in this wound model, as indicated by the lack of defects in epidermal area (Supplementary Figure S1b online), proliferation of keratinocytes in the leading edge (Supplementary Figure S1c and d online), and rates of reepithelialization (Supplementary Figure S1e online). In addition, induction of the antimicrobial protein, cathelicidin (Dorschner *et al.*, 2001), occurred normally in *IL-22*-null wounds despite the ability of IL-22 to regulate antimicrobial gene expression (Liang *et al.*, 2006; Supplementary Figure S2c online).

In contrast, a major difference in external wound area and the appearance of the dermal compartment was noted in both small and large wounds of *IL-22*$^{-/-}$ mice. In WT mice, the external area of small wounds was $\sim 1.2 \times 10^3\,\mu m^2$ at day 3, and it decreased to $\sim 500\,\mu m^2$ by day 5. In contrast, the average external area of small wounds in *IL-22*$^{-/-}$ mice was $\sim 2 \times 10^3\,\mu m^2$ at day 3, and it decreased slightly to $\sim 1.5 \times 10^3\,\mu m^2$ at day 5 (Figure 2a). Morphometric analysis of hematoxylin and eosin–stained sections from 2- or 8-mm wounds of WT and *IL-22*$^{-/-}$ mice post injury revealed a striking defect in the dermis of *IL-22*$^{-/-}$ mice. The area underneath the wound, referred to as wound bed area, was decreased in both small and large wounds (Figure 2b). Furthermore, hematoxylin and eosin–stained sections revealed alterations in the organization of the dermal compartment of both small and large wounds in *IL-22*$^{-/-}$ mice (Figure 2b and data not shown).

Defects in extracellular matrix production in *IL-22*$^{-/-}$ mice

To determine whether the reduced wound bed area in *IL-22*$^{-/-}$ mice was due to defects in ECM production, we analyzed skin sections of WT and *IL-22*$^{-/-}$ mice using histological stains: Movat pentachrome to reveal total collagen

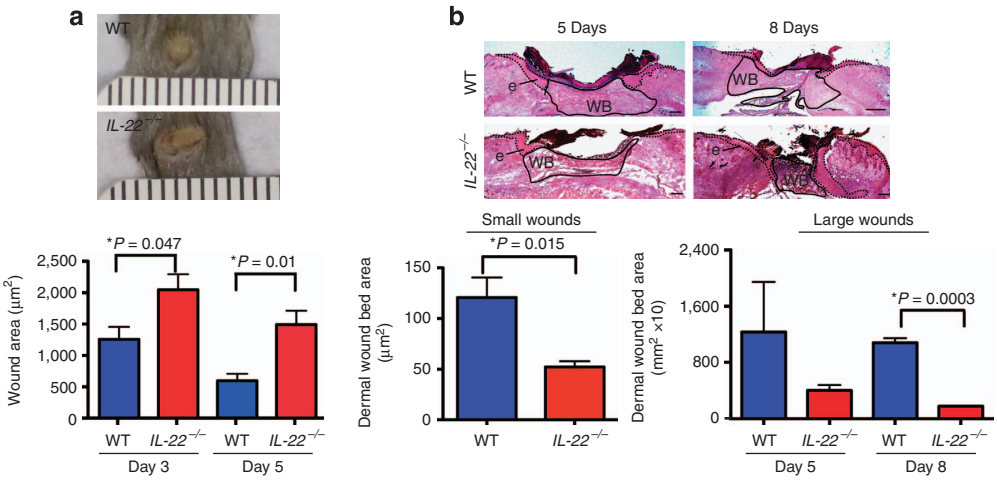

Figure 2. *IL-22*$^{-/-}$ mice exhibit defects in the dermis during wound healing. (**a**) Images of wild-type (WT) and *IL-22*$^{-/-}$ back skin 3 days after wounding with 2-mm punch biopsies. Quantification of the external area of WT and *IL-22*$^{-/-}$ small wounds at 3 and 5 days post wounding. $n > 18$ wounds for each genotype (six wounds from six mice for each of three independent experiments). Ruler notches = 1 mm. (**b**) Hematoxylin and eosin–stained sections of WT and *IL-22*$^{-/-}$ back skins wounded with 8-mm punch biopsies (large wounds). The epidermis (e) is indicated by a dotted line and the wound bed (WB) is outlined by a solid line. Quantification of wound bed area (i.e., dermal area) of small wounds (2 mm) 3 days post wounding and large wounds (8 mm) 5 and 8 days post wounding in WT and *IL-22*$^{-/-}$ mice are shown. For small wounds, $n = 18$ (six wounds from six mice for each of three independent experiments). For large wounds, $n = 12$ wounds from six mice. Bars = 400 μm. All data are ± SEM. Asterisks indicate significance.

and Sirius Red to stain collagen type III. During the inflammatory phase of wound healing, collagen was localized adjacent to the wound bed and was decreased in *IL-22*$^{-/-}$ mice (Figure 3a). After inflammation, collagen localization in *IL-22*$^{-/-}$ wounds resembled that of WT wounds at day 3, suggesting a delay in collagen production in the absence of IL-22 (Figure 3a). Moreover, Sirius Red staining revealed that Collagen Type III fibers were disorganized in *IL-22*$^{-/-}$ mice at day 28 in the remodeling phase of wound healing.

To determine whether the reduction in collagen protein content in *IL-22*$^{-/-}$ skin results from defects in the transcription of ECM genes, we analyzed *Fibronectin*, *Collagen Type IαI*, and *Collagen Type IIIαI* mRNA expression from WT and *IL-22*$^{-/-}$ skin post wounding (Figure 3b). Wound healing in WT mice was characterized by an initial upregulation of *Fibronectin* and *Collagen Type IIIαI* mRNAs during inflammation, but these gene products decreased after the inflammatory phase. In contrast, *Collagen Type IαI* mRNA levels increased and remained elevated during these time points in WT mice. Our data show that the levels of *Fibronectin*, *Collagen Type IαI*, and *Collagen Type IIIαI* were significantly reduced in *IL-22*$^{-/-}$ skin compared with WT skin (Figure 3b). Therefore, delayed wound healing in *IL-22*$^{-/-}$ mice is consistent with a delay in the expression of ECM genes during wound healing.

Considering that ECM components are produced by fibroblasts within the dermis, we hypothesized that fibroblast number or ECM production was decreased in *IL-22*$^{-/-}$ mice. To determine whether IL-22 affects the number of fibroblasts, we isolated WT and *IL-22*$^{-/-}$ dermal cells from unwounded skins and from skins post wounding. Using flow cytometry, we analyzed the percentage of fibroblasts by staining dermal cells with an antibody against the fibroblast-specific protein ER-TR7 (Van Vliet *et al.*, 1984). We found no significant difference in the percentage of ER-TR7^{+} fibroblasts isolated from *IL-22*$^{-/-}$ wounds compared with WT wounds (Figure 3c).

To confirm that the dermal defects are due to an absence of IL-22 during dermal remodeling, we injected recombinant IL-22 intradermally into *IL-22*$^{-/-}$ wounds 3 days after wounding when the initial defects in the dermis are detected in *IL-22*$^{-/-}$ mice. The severe defect in collagen production in *IL-22*$^{-/-}$ mice was restored by intradermal IL-22 (Figure 3d). This result confirms that IL-22 is essential for proper dermal organization after wounding.

IL-22 activates extracellular matrix gene expression in fibroblasts

To determine whether IL-22 acts directly on fibroblasts during wound healing, TaqMan real-time PCR was used to measure *IL-22Rα* expression in FACS-purified murine keratinocytes, naive CD4^{+}CD25^{-}CD44^{-}CD62L^{+} T cells, and purified dermal fibroblasts (Kunz *et al.*, 2006; Festa *et al.*, 2011). We used real-time PCR to determine the purity of the isolated keratinocyte and fibroblast populations on the basis of *K14* and *vimentin* mRNA expression (Supplementary Figure S3b online). As expected, *IL-22Rα* mRNA was not detected in naive T cells, but was expressed in keratinocytes (Kunz *et al.*, 2006; Figure 4a). Surprisingly, *IL-22Rα* mRNA was also expressed in fibroblasts. This expression was confirmed by immunostaining with antibodies against ER-TR7 and IL-22Rα in skin sections (Figure 4b). Specificity of the IL-22Rα staining was confirmed by immunostaining with rabbit isotype control antibodies (Supplementary Figure S3c online).

To determine whether IL-22 can activate signaling downstream from IL-22Rα in fibroblasts, we analyzed STAT3 phosphorylation, as IL-22 induces STAT3 phosphorylation and nuclear translocation in other cell types (Lejeune *et al.*, 2002). Primary fibroblasts were serum-starved and then stimulated for 20 minutes with vehicle, Oncostatin M, which

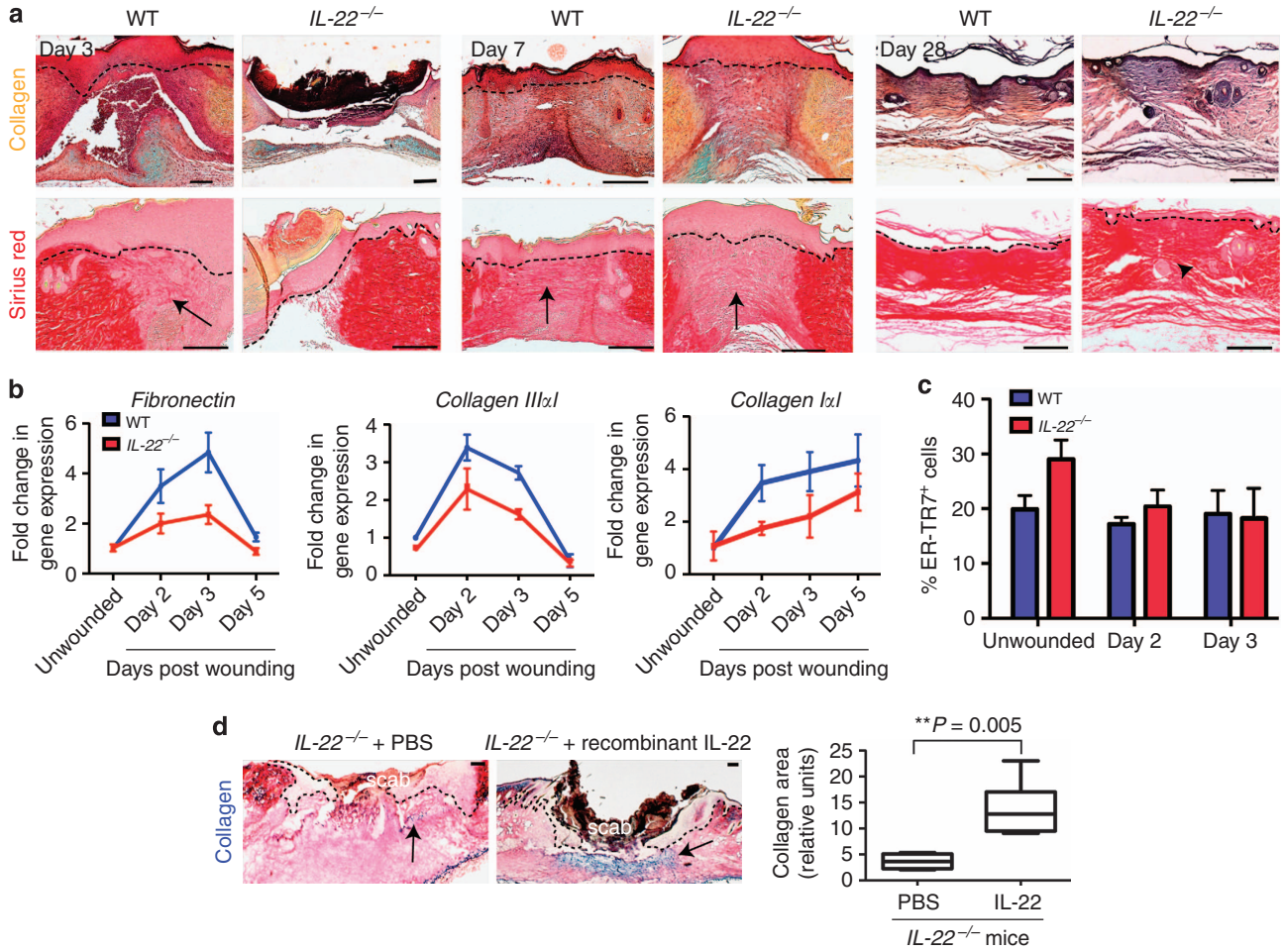

Figure 3. *IL-22*$^{-/-}$ mice exhibit defects in extracellular matrix production. (**a**) Skin sections of wild-type (WT) and *IL-22*$^{-/-}$ mice 3, 7, and 28 days post wounding with 2-mm punch biopsies were stained with Movat pentachrome (collagen, yellow; mucin, blue) and Sirius Red (Collagen Type III, red). Bars = 200 μm. Arrows indicate collagen, and arrowhead indicates disorganized collagen. (**b**) Real-time PCR analysis reveals decreased *Fibronectin*, *Collagen Iα1*, and *Collagen IIIα1* messenger RNA levels in 2-mm *IL-22*$^{-/-}$ skin wounds. $n = 4$ mice for each genotype and time point. (**c**) Quantification of % ER-TR7+ cells in the total dermal cell population derived from the dermis of WT and *IL-22*$^{-/-}$ mice wounded with 2-mm punch biopsies. $n = 4$ mice for each genotype. (**d**) Skin sections of *IL-22*$^{-/-}$ skin 5 days post wounding that were injected with phosphate-buffered saline (PBS) or recombinant IL-22 on day 3 and stained with trichrome histological stain (collagen, blue). Bars = 200 μm. Quantification of the area of collagen in the wound beds of *IL-22*$^{-/-}$ mice injected with PBS or 25 ng recombinant IL-22. $n = 6$ mice. Asterisks indicate significance. All data are mean ± SEM.

activates STAT3 in fibroblasts (Snyder *et al.*, 2008), or recombinant IL-22. Immunostaining of fibroblasts with antibodies against phosphorylated STAT3 (STAT3-P) revealed activation of STAT3 after stimulation with either OSM or IL-22 (Figure 4d). Western blot analysis of fibroblast cell lysates after OSM or IL-22 stimulation confirmed that IL-22 stimulates phosphorylation of STAT3 in fibroblasts (Figure 4c). Taken together, these data demonstrate that fibroblasts express functional IL-22Rα and that IL-22/IL-22Rα signaling is sufficient to activate STAT3-P in fibroblasts.

To determine whether IL-22 can directly stimulate the production of the ECM components in fibroblasts, primary cultured dermal fibroblasts isolated from WT or *IL-22*-null mice were stimulated with recombinant IL-22 for 48 hours. We analyzed mRNA expression by real-time PCR. IL-22 induced a dose-dependent increase in mRNA expression of *Fibronectin*, *Collagen Type Iα1*, and *Collagen Type IIIα1*, but not *Integrin α5* or *Metalloproteinase 9*, which mediate adhesion to or degradation of ECM molecules, respectively (Figure 4e; Sawicki *et al.*, 2005; Liu *et al.*, 2010). As *IL-22*$^{-/-}$ fibroblasts also induce ECM genes in response to IL-22, no intrinsic defects exist in the fibroblasts from *IL-22*$^{-/-}$ mice. These changes in gene expression were confirmed by western blot analysis of fibronectin protein expression after IL-22 stimulation (Figure 4f). Taken together, these data suggest that IL-22 signaling in fibroblasts can directly induce the expression of ECM components.

IL-22 activates myofibroblast differentiation

As *IL-22*$^{-/-}$ mice display defects in wound contraction and ECM production after wounding, we hypothesized that IL-22 may influence the differentiation of dermal myofibroblasts, which contract wounds and produce ECM during wound healing (Montesano and Orci, 1988). Myofibroblasts are identified by their characteristic expression of α-smooth muscle actin (α-SMA; Darby *et al.*, 1990). To determine

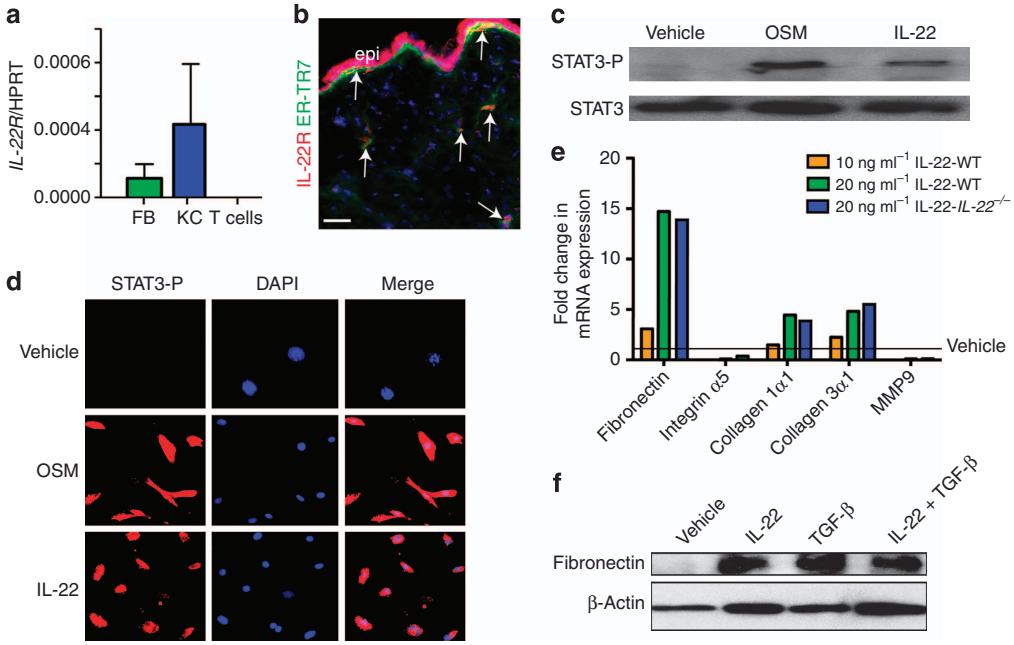

Figure 4. IL-22 stimulates STAT3 activation and extracellular matrix (ECM) production in dermal fibroblasts. (**a**) Real-time PCR reveals that isolated dermal fibroblasts (FB) and keratinocytes (KC) express *IL-22Rα* messenger RNA (mRNA). $n = 3$ mice. Data are mean ± SEM. (**b**) Skin sections were immunostained with antibodies against IL-22Rα and ER-TR7. Arrows indicate ER-TR7+ and IL-22Rα+ cells. Bar = 100 μm. epi, epidermis. (**c**) Representative images of primary mouse fibroblasts immunostained with antibodies against phospho-STAT3 (STAT3-P) after treatment with IL-22 or oncostatin M (OSM) for 20 minutes. (**d**) Western blot analysis confirms the increased activation of STAT3-P in primary dermal fibroblasts treated with IL-22 or Oncostatin M. (**e**) Real-time PCR analysis of mRNA in wild-type (WT) or *IL-22*$^{-/-}$ primary mouse fibroblasts reveals enhanced expression of ECM genes after IL-22 stimulation for 48 hours. (**f**) Western blot analysis shows that IL-22 increases the expression of fibronectin protein in WT fibroblasts. β-Actin is included as a loading control.

whether the skin of *IL-22*$^{-/-}$ mice displays a reduction in the number of α-SMA^{+} myofibroblasts, we investigated whether the number of α-SMA^{+} fibroblasts was altered during wound healing in the absence of IL-22. Immunostaining of WT and *IL-22*$^{-/-}$ wounds after wounding revealed a reduction in the number of α-SMA^{+} cells in the dermis of small and large *IL-22*$^{-/-}$ wounds compared with WT wounds (Figure 5a and data not shown). In addition, we isolated unwounded skin and day 3 wounded skin from WT and *IL-22*$^{-/-}$ mice and measured α-*SMA* mRNA expression by real-time PCR. Whereas α-*SMA* mRNA expression increased in response to wounding in both WT and *IL-22*$^{-/-}$ mice, α-*SMA* mRNA expression was significantly reduced in wounds from *IL-22*-null mice compared with WT wounds (Figure 5b). These data suggest that there was a reduction in the number of myofibroblasts in the absence of IL-22.

As the reduced number of α-SMA^{+} fibroblasts could be due to alterations in the differentiation of fibroblasts into myofibroblasts (Barisic-Dujmovic *et al.*, 2010), we investigated whether IL-22 was sufficient to induce myofibroblast differentiation. Primary dermal fibroblasts from WT or *IL-22*-null mice were treated *in vitro* with recombinant IL-22 for 48 hours and α-*SMA* mRNA and protein expression was quantified. Real-time PCR analysis revealed a nine-fold increase in α-*SMA* mRNA expression after the addition of IL-22 to fibroblasts from both WT or *IL-22*$^{-/-}$ mice (Figure 5c). In addition, WT cultures displayed an increase in α-SMA protein as shown via western blotting (Figure 5d). Taken together, these data suggest that IL-22 promotes myofibroblast differentiation and function in the skin after wounding. These data suggest that the lack of myofibroblasts is most likely responsible for the dermal remodeling defects in *IL-22*-null mice.

DISCUSSION

Given the essential functions of the epithelia to protect mammalian tissues from the external environment, highly coordinated mechanisms between multiple cell types have evolved to repair epithelial tissue damage. The recruitment of immune cells following acute skin injury is essential for multiple stages of wound healing including keratinocyte reepithelialization, extracellular matrix deposition, granulation tissue remodeling, and wound contraction by fibroblasts (Eming *et al.*, 2007). Although extensive research has focused on cytokines and growth factors that mediate keratinocyte reepithelialization, very little is known about the signals that mediate the cross talk between immune cells and fibroblasts. Here we identify IL-22 as an essential signal that mediates communication between immune cells and fibroblasts in the skin.

Several studies have linked IL-22 to inflammatory conditions in tissues containing epithelial cells such as the thymus, the colon, and the skin (Zenewicz *et al.*, 2008; Kagami *et al.*, 2010; Sekikawa *et al.*, 2010; Yamamoto-Furusho *et al.*, 2010). *In vitro* studies have suggested that IL-22 induces keratinocyte proliferation and migration while inhibiting keratinocyte differentiation (Boniface *et al.*, 2005; Wolk *et al.*, 2006; Zheng *et al.*, 2007). Our data suggest that the

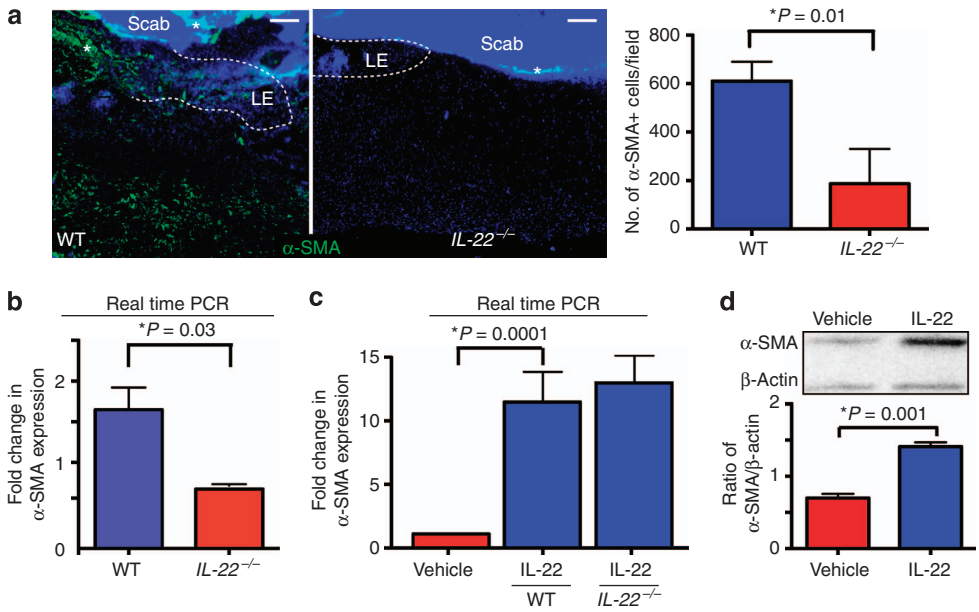

Figure 5. IL-22 induces myofibroblast differentiation. (**a**) Skin sections of wild-type (WT) and *IL-22*$^{-/-}$ mice 3 days after wounding with 2-mm punch biopsies were immunostained with antibodies against α-smooth muscle actin (α-SMA). Quantification of α-SMA staining; $n = 6$ mice for each genotype. LE, leading edge. (**b**) Real-time PCR reveals reduced messenger RNA (mRNA) expression of α-SMA in 2-mm *IL-22*$^{-/-}$ wounds 3 days post wounding. $n = 4$ mice for each genotype and each time point. (**c**) Real-time PCR analysis of WT or *IL-22*$^{-/-}$ primary dermal fibroblasts cultured with vehicle or recombinant IL-22 for 48 hours confirms that α-*SMA* mRNA expression increases with IL-22 stimulation. $n = 3$ independent experiments. (**d**) α-SMA protein expression increases in primary fibroblasts after stimulation with IL-22 for 48 hours as indicated by a representative image of western blot analysis. Quantification of the ratio of α-SMA/β-actin expression is shown. $n = 3$ independent experiments. All data are mean ± SEM.

loss of IL-22 alone does not cause major defects in early events involved in skin wound healing, such as immune cell recruitment or blood vessel formation. Future experiments examining specific immune cell subsets, blood vessel architecture, or keratinocyte function in the absence of IL-22 may reveal functions for this cytokine in additional aspects of skin wound healing. Furthermore, our experimental system did not detect defects in keratinocyte function during wounding *in vivo,* suggesting that additional signaling molecules such as FGF7 and EGF (Werner and Grose, 2003) may compensate for a loss of IL-22 in the skin. Wound models that challenge the wound epidermis, such as with a stinted wound technique, may reveal a role for IL-22 in keratinocyte function.

Interestingly, our study uncovered a role for IL-22 in directing fibroblast function during skin wound repair. Fibroblasts have several functions that are defective in *IL-22*$^{-/-}$ mice, leading to abnormal granulation tissue formation, extracellular matrix protein production, and wound contraction. These defects are probably due to direct action of IL-22 on fibroblasts, as primary dermal fibroblasts express IL-22Rα and IL-22 can induce STAT3 phosphorylation in dermal fibroblasts downstream of the IL-22Rα. Our data show that IL-22 directly stimulates extracellular matrix gene expression in primary dermal fibroblasts *in vitro,* and that *IL-22*$^{-/-}$ mice lack extracellular matrix components. Future studies will focus on whether IL-22 also acts on mesechymal cells in the other organs during acute regeneration.

Furthermore, the defects in ECM production may be linked to our finding that IL-22 promotes myofibroblast differentiation. As the number of myofibroblasts is proportional to the rate of wound contraction and the amount of ECM produced (Gabbiani *et al.*, 1976), the defects in wound contraction and the delay in ECM production in *IL-22*$^{-/-}$ mice is likely due to a reduced number of α-SMA^{+} myofibroblasts in the wound bed. This study illustrates a role for IL-22 on mesenchymal cells during cutaneous wound healing, which to our knowledge is previously unreported. These data change the paradigm of IL-10 family cytokines as mediators of epithelial cell biology, showing that IL-22 also has a key role in mediating immune cell–stromal cell interactions during tissue repair.

IL-22 signaling may directly or indirectly stimulate the expression of ECM genes. Although IL-22 can signal via Jak/STAT, MAPK, and Akt pathways (Lejeune *et al.*, 2002), the majority of the effects of IL-22 on epithelial cells have been linked to STAT3. Our work highlights a role for IL-22-induced STAT3 phosphorylation in dermal fibroblasts. Therefore, it is possible that IL-22 signaling directly activates the promoters of *Fibronectin*, *Collagen IIIαI*, and *Collagen IαI* through STAT3 activation. Alternatively, it is possible that IL-22 indirectly regulates ECM production by regulating myofibroblast differentiation.

Although IL-22 has been highlighted for its ability to protect epithelial cells in the liver and colon during inflammation, it also has been suggested to have a pathogenic role in psoriasis and collagen-induced arthritis (Wolk *et al.*, 2006; Lowes *et al.*, 2008; Geboes *et al.*, 2009; Kagami *et al.*, 2010). The results presented here could help explain some aspects of IL-22's pathogenicity, as IL-22 may act on fibroblast-like

chondrocytes in the joints and fibroblasts in the skin to induce excessive collagen production in patients with rheumatoid arthritis or psoriasis, respectively (Koivukangas *et al.*, 1995; Steenvoorden *et al.*, 2006). In fact, IL-22 was recently shown to worsen synovitis by inducing the production of RANKL from synovial fibroblasts and therefore indirectly promoting osteoclastogenesis (Kim *et al.*, 2012). IL-22 was also shown to have a role in the pathogenesis of systemic sclerosis, which is consistent with our data (Truchetet *et al.*, 2011).

Although normal levels and kinetics of IL-22 can enhance wound healing by inducing myofibroblast differentiation and ECM production, these physiologic processes may become pathogenic if excessive ECM production destroys normal tissue architecture and interferes with organ function (Jun and Lau, 2010a, b). Therefore, further studies on the role of IL-22 in conditions characterized by abnormal wound healing, such hypertrophic scarring and fibrotic diseases, will clarify the role of IL-22 in the pathogenesis of diseases characterized by defects in fibroblast-mediated tissue repair (Kong *et al.*, 2012).

MATERIALS AND METHODS

Mice

IL-22$^{-/-}$ mice were generated using the VelociGene method (VelociGene Allele Identification Number: VG204) in collaboration with Regeneron Pharmaceuticals (Valenzuela *et al.*, 2003). *K14-H2BGFP* mice were described previously (Tumbar *et al.*, 2004). For wounding studies, 7–8-week-old male mice were used during the telogen phase of the hair cycle. Littermates and C57BL/6 (B6) mice were used as WT controls. A maximum of four wounds were made on the dorsal skin of each mouse. All animal care and experimental procedures were approved by the Yale University Animal Resource Center and were conducted in accordance with requirements approved by the Institutional Animal Care and Use Committee of Yale University.

Skin wounding and morphogenetic measurements

WT and *IL-22*$^{-/-}$ mice were anesthetized with isofluorane, and full-thickness wounds were made on shaved dorsal skins using sterile dermal biopsy punches, 2, 4, and 8 mm, as indicated (Accuderm, Fort Lauderdale, FL). Wounds were air-exposed throughout the healing process. Images of the external appearance of the wounds were taken and the area of wound was measured using the ImageJ software (NIH, Bethesda, MD).

To measure histological characteristics of wounds, each wound was embedded and sectioned through its entirety. Sections from the middle of the wound, as indicated by the largest length of hyperproliferative epidermis, were used for all analyses. We determined the percentage of epidermal reepithelialization by measuring the total length of the hyperproliferative keratinocyte region and dividing by the total wound length (the sum of the lengths of the epithelium and unepithelialized dermis; Qiu *et al.*, 2003). The maximal rate of wound closure was calculated using linear regression of the epithelialization data.

For rescue experiments, 20 μl of phosphate-buffered saline (PBS) or recombinant IL-22 (25 ng ml^{-1}, R&D Systems, Minneapolis, MN) was injected intradermally at day 3 post injury. The wound bed area was determined at the indicated time points after wounding, using the ImageJ software.

For FACS, RNA, and protein analysis, a circular area ~1 mm larger than the wound was isolated at the indicated time points in order to ensure that activated dermal fibroblasts in the wound margins were included.

Gene expression analysis

Quantitative real-time PCR was performed as previously described (Horsley *et al.*, 2008; O'Connor *et al.*, 2009). The following accession numbers were used for the Taqman primers: Mm00444241_m1 (*IL-22*) and Mm00663697_m1 (*IL-22Rα*). For quantitative PCR using SYBR green, the following primers were used: β-actin For 5′-ATC AAG ATC ATT GCT CCT CCT GAG-3′, Rev 5′-CTG CTT GCT GAT CCA CAT CTG-3′; Fibronectin For: 5′-CTA CCC TGC AGC CTC TGC GC-3′, Rev: 5′-TCA CCT CCC TGG CTC GGT CG-3′; Collagen IIIαI For: 5′-CTG GTC CTG TTG GTC CAT CT-3′, Rev: 5′-CTT CTC CAG CGG TAC CAG AG-3′; Collagen IαI For: 5′-ACG TCC TGG TGA AGT TGG TC-3′, Rev: 5′-TCC AGC AAT ACC CTG AGG TC-3′; MMP-9 For: 5′-ATC CCC AGA GCG TCA TTC GCG-3′, MMP-9 Rev: 5′-CAC GTA GCC CAC GTC GTC CAC-3′; α-SMA For: 5′-GTC CCA GAC ATC AGG GAG TAA-3′, Rev: 5′-TCG GAT ACT TCA GCG TCA GGA-3′; CD45 For: 5′-TAC GCA AAG CAC GGC CTG GG-3′, Rev: 5′-CTC CGG GGT TCC CAC CCC TC-3′; Vimentin For: 5′-CAG GGC AGC AGT GAG GTC-3′, Rev: 5′-TCC TCG AGC AGC AGA ACA AAA TCC-3′.

Dermal fibroblast isolation and IL-22 stimulation

Primary dermal fibroblasts were isolated from 8- to 12-week-old mice using either Collagenase 1A (2.5 mg ml^{-1}, Sigma, St Louis, MO) and DNaseI (62.5 U ml^{-1}, Sigma) digestion or tail skin explant cultures. After skin digestion, isolated cells were centrifuged after filtering and plated with high-glucose DMEM (Invitrogen, Grand Island, NY) containing 10% fetal bovine serum and 1% Penicillin/Streptomycin/Amphotericin. Fibroblasts were grown to 85% confluence and recombinant IL-22 (R&D Systems) was added at indicated doses (10 or 20 ng ml^{-1}) for 48 hours. Cells were harvested in Trizol for RNA extraction, and cell lysates were harvested in radioimmuno precipitation assay buffer for western blot analysis.

Isolation, culture, and analysis of primary fibroblasts

Primary dermal fibroblasts were isolated from the wounded skin of 12-week-old mice using Collagenase 1A (2.5 mg ml^{-1}, Sigma) and DNaseI (62.5 U ml^{-1}, Sigma) digestion. After filtering the digested tissue with a 70-μm^2 mesh, cells were centrifuged and if cultured, plated in DMEM media containing glucose, 10% fetal bovine serum and 1% Pen/Step/Amphotericin. For FACS analysis, freshly isolated cells were fixed, permeablized, and stained with rat α-ER-TR7 (1:250, Novus Biologicals, Littleton, CO) and Alexa 488–conjugated α-rabbit IgG (1:250, Invitrogen). Samples were analyzed on a FACS Aria and analyzed with the FlowJo software (Tree Star Inc., Ashland, OR).

Western blotting

To collect protein lysates from wounds *in vivo*, wounded skin was isolated and flash-frozen in liquid N_2. Skin was minced and added to radioimmuno precipitation assay buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% Na Deoxycholate, 1% NP-40) containing protease inhibitors (Roche, Indianapolis, IN). The tissue was homogenized, sonicated for 10 minutes, and frozen at –80 °C. To collect protein lysates from cell culture, cells were washed once with PBS (Invitrogen) and then lysed with radioimmuno precipitation assay buffer.

Total protein was measured using the Bradford assay, and 10 μg of protein was run on 4–20% Tris-Glycine gels (Invitrogen) before being transferred to nitrocellulose membranes. Membranes were blocked with blocking buffer (5% nonfat dry milk, 10 mM Tris, 100 mM NaCl, 0.1% Tween-20) and probed with the following primary antibodies: Rabbit polyclonal α-STAT3 (1:1,000, Cell Signaling Technology, Danvers, MA), murine monoclonal α-STAT3-P (1:1,000 Cell Signaling), murine monoclonal α-Fibronectin (Calbiochem, San Diego, CA), goat polyclonal α-alpha-SMA (Sigma), murine monoclonal α-β-actin (1:20,000, Sigma), and secondary antibodies (1:5,000, peroxidase-conjugated α-mouse IgG, α-rabbit IgG, and α-goat IgG). Western blots were visualized via chemiluminescence with the ECL Plus Detection System (Amersham, Pittsburgh, PA).

Cell sorting

To purify keratinocytes from mouse skin, *K14-H2BGFP* mice were shaved and dorsal skins were removed. The dermal side of the skin was scraped to remove fat and floated on 0.25% trypsin overnight at 4 °C. To release epidermal cells, the dorsal side of the skin was scraped, cells were filtered, washed in PBS, and resuspended in PBS + 3% Serum + 1% Penicillin/Streptomycin/Amphotericin containing 50 μg ml^{-1} DNaseI. Cells were stained with propidium iodide α-CD49f-PE (1:75; BD Biosciences, San Jose, CA) antibodies and CD49f$^+$, GFP$^+$, PI$^-$ keratinocytes were purified via FACS.

To purify naive CD4$^+$ T cells from spleens, splenocytes were isolated from 6- to 8-week-old C57BL/6 mice and a single-cell suspension was treated with red blood cell lysis buffer. Cells were enriched for CD4$^+$ cells (L3T4 microbeads and magnetic-activated cell sorting columns, Miltenyi Biotech, Auburn, CA). CD4$^+$CD25$^-$CD44$^-$CD62L$^+$ cells were purified by cell sorting on a FACS Vantage sorter with FITC-conjugated α-CD44, PE-conjugated α-CD25, APC-conjugated α-CD4, and PECy7-conjugated α-CD62L (all from BD Biosciences). Sorted cell purity was >97%.

Histological stains and immunofluorescence

Wounded dorsal skins were mounted in OCT compound (Tissue-Tek) on dry ice and cryosectioned for histological staining or immunostaining with the indicated antibodies. For Movat pentachrome and Sirius Red stains, skin samples were fixed in 10% formalin-buffered saline, embedded in paraffin, sectioned through the entire length of the wound, and stained. Trichrome staining was performed using the Masson's trichrome staining kit according to the manufacturer's protocol (Polysciences, Warrington, PA).

Immunostaining was performed as described previously (Festa *et al.*, 2011). Briefly, tissue sections were fixed with 4% formaldehyde and blocked with PBS containing 0.02% Gelatin, 0.25% Triton-X, 1% BSA, and normal goat and donkey serum. When applicable, the M.O.M. kit (Vector labs, Burlingame, CA) was used to prevent nonspecific binding of mouse antibodies. Slides were stained with rat polyclonal α-ER-TR7 (1:250, Novus Biologicals), rabbit polyclonal N-terminal α-IL-22R (1:250, QED Biosciences, San Diego, CA), rabbit polyclonal α-phospho-STAT3 (1:100, Cell Signaling), murine monoclonal α-SMA (1:250, Thermo Scientific, Asheville, NC), rabbit polyclonal α-Ki67 (1:300, Leica Microsystems, Buffalo Grove, IL), rat monoclonal CD45-PE-Cy7 (1:500, eBioscience, San Diego, CA), rabbit polyclonal cathelicidin (1:300, Novus Biologicals), or Isolectin GS-IB4 Alexa 488–conjugated antibodies (1:100, Invitrogen) overnight at 4 °C, and then with required secondary antibodies (1:250).

Immunofluorescent staining of fibroblasts was performed on microcoverslips in six-well tissue culture plates. Cells were serum-starved for 24 hours and then treated with 25 ng ml^{-1} recombinant Oncostatin M (R&D Systems) or 25 ng ml^{-1} recombinant IL-22 (R&D Systems) for 20 minutes. Cells were washed and fixed in 4% formaldehyde before being stained with murine monoclonal α-STAT3-P (1:100, Cell Signaling). Image acquisition was performed using a Zeiss confocal microscope or an upright Zeiss microscope with Axiovision software (Zeiss, Oberkochen, Germany).

Statistical analysis

To determine significance between groups, comparisons were made using Student's *t*-tests. Analyses of multiple groups were performed using one-way ANOVA with Bonferroni's post-test with GraphPad Prism version for Macintosh (GraphPad Software, La Jolla, CA). For all statistical tests, the 0.05 level of confidence was accepted for statistical significance.

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 http://www.nature.com/jid

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