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Interleukin (IL)-6 modulates transforming growth factor- β receptor I and II (TGF- β RI and II) function in epidermal keratinocytes

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Abstract

It been shown that IL-6 modulates TGF- β 1 expression in fibroblasts, however, what role IL-6 plays concerning TGF- β R expression and function in skin is unknown. Therefore, the aim of this study was to investigate the mechanism by which IL-6 might modulates TGF- β receptors in skin. Skin from WT, IL-6 over-expressing mice and IL-6 treated keratinocyte cultures was analysed for TGF- β RI and TGF- β RII expression via histology, PCR and flow cytometry. Receptor function was assessed by cell migration, bromodeoxyuridine (BrdU) proliferation assays, and Smad7 expression and Smad2/3 phosphorylation. Receptor localization within the membrane was determined by co-immunoprecipitation. IL-6 overexpression and treatment increased TGF- β RII expression in the epidermis. IL-6 treatment of keratinocytes induced TGF- β RI and II expression and augmented TGF- β 1-induced function as demonstrated through increased migration and decreased proliferation. Additionally, IL-6 treatment of keratinocytes altered receptor activity as indicated by altered Smad2/3 phosphorylation and increased Smad7 and membrane localization. These results suggest that IL-6 regulates keratinocyte function by modulating TGF- β RI and II expression and signal transduction via trafficking of the receptor to lipid raft pools.

KEYWORDS

skin, receptor endocytosis, smad, migration, caveolin

1 | INTRODUCTION

Cytokines and growth factors are known to play critical roles in cellular responses and can ultimately control the function of the cell. These mediators can serve as molecular signals between cells to communicate changes in the environment or disruptions to homeostasis, which can occur following an injury. The wound environment is characterized by multiple cellular events within interdependent stages including inflammation, tissue formation and tissue remodeling. Cutaneous homeostasis is maintained by permanent crosstalk between dermal fibroblasts, epidermal keratinocytes and through the

production of cytokines. A paradoxical partnership between cytokines such as interleukin (IL)-6 and transforming growth factor (TGF)- β has long been suspected as a mediator of keratinocyte-fibroblast crosstalk in the wound.^[1] Yet, few studies characterizing the mechanism of this partnership exist.

IL-6 is a pleiotropic cytokine that plays a critical role in the inflammatory response as well as in growth and differentiation for numerous cell types.^[2] In the skin, IL-6 is produced by dermal and epidermal cells, while other cells such as macrophages, B and T cells and endothelial cells represent secondary sources.^[3] Dysregulation of IL-6 has been implicated in multiple disease states including rheumatoid

arthritis, inflammatory bowel disease and psoriasis.^[3] Wounds from IL-6-deficient mice demonstrate multiple healing defects, including delayed re-epithelialization, decreased granulation tissue, inhibited neovascularization and greatly increased time to closure, when compared to their wild-type counterparts.^[4,5] While the involvement of IL-6 in the inflammatory process has been well established, much is still unknown about its role in skin.

The TGF- β family consists of TGF- β 1-3, bone morphogenic proteins (BMP) and activins. Of the three TGF- β isoforms, TGF- β 1 predominates in cutaneous repair exerting pro- and antiproliferative effects in numerous cell types, influencing migration and stimulating the production of extracellular matrix proteins.^[6,7] All isoforms are produced in wounds by macrophages, fibroblast, keratinocytes and platelets. Mouse models deficient for TGF- β 1 and its downstream signalling proteins, Smads, demonstrate multiple wound healing deficiencies.^[8] The consequences of TGF- β deficiency on re-epithelialization are of particular interest because of the molecule's ability to induce keratinocyte differentiation and subsequent migration, as these are essential processes in re-epithelialization.^[9] Indeed, late-stage wounds from TGF- β 1 knockout animals demonstrated severely impaired granulation tissue formation, re-epithelialization and collagen deposition; however, these impairments are accompanied with an unchecked inflammatory response which leads to severe wasting.^[10] Thus, it is difficult to discern whether wound healing impairments are a direct consequence of TGF- β 1 deficiency or overall physiological effects associated with this animal model.

TGF- β 1 exerts its biological activity by binding to a heterotetrameric receptor complex composed of TGF- β type I receptor (TGF- β RI) and TGF- β type II receptor (TGF- β RII) dimers.^[7] Ligand binding leads to a conformational change in which TGF- β RII phosphorylates and activates TGF- β RI. The activated TGF- β RI recruits R-Smads (Smad2/3 and Smad4) to the receptor where they are phosphorylated and then translocated to the nucleus to modulate transcription of target genes.^[11] In addition to inhibitory Smads, such as Smad7, TGF- β 1 signalling can be governed by receptor localization within the membrane, specifically within the lipid raft domain vs non-lipid raft. Di Guglielmo et al.^[12] previously reported that clathrin internalization into the early endosome antigen-1 (EEA-1)-positive endosome promotes TGF- β signalling, while lipid raft-caveolin internalization pathway (with the help of Smad7) promotes receptor degradation.

Previous studies concerning the relationship between IL-6 and TGF- β have not provided a definitive mechanistic link between the mediators. Concerning its role in dermal-epidermal crosstalk, IL-6 has been shown to induce keratinocyte migration only when cultured in the presence of fibroblasts.^[13] This finding seems to indicate that IL-6 facilitates re-epithelialization through its partnership with a soluble effector produced by fibroblasts, such as TGF- β 1. This study aims to investigate the relationship of keratinocyte function induced by IL-6 and its association with TGF- β receptor expression and membrane localization. Herein, it was found that IL-6 appears to indirectly modulate keratinocyte activity through functional augmentation of both TGF- β receptors.

2 | MATERIALS AND METHODS

2.1 | Mice

Experimental animals were treated in accordance with the criteria outlined in the *PHS Policy on Humane Care and Use of Laboratory Animals* and *The Guide for the Care and Use of Laboratory Animals* (NIH publication 86-23), in facilities accredited by AAALAC. IL-6-deficient mice were acquired from Jackson Laboratories (Bar Harbor, ME, USA). Frozen embryos of mice that overexpressed IL-6 in epidermis via a K14-IL-6 promoter construct^[16] were graciously provided by Dr. Elaine Fuchs (Rockefeller Institute). Embryos were revived by Jackson Laboratories and maintained at the OUHSC rodent barrier facility.

2.2 | Epidermal keratinocyte isolation and culture

IL-6 knockout (KO) epidermis was collected from neonatal mice and isolated as previously described.^[13] Neonatal human epidermal keratinocytes (Ker-CT, ATCC CRL-4048 and nHEKs, ThermoFisher, Waltham, MA, USA) were cultured according to manufacturer's protocol. Cells were treated with IL-6 (overnight for protein expression studies or 1 hour for signalling assays) or in combination with TGF- β 1 (30 minutes) or specified otherwise. IL-6 neutralizing antibody (Biolegend, San Diego, CA, USA) was used at 500 ng/mL and incubated overnight followed by treatment. LLL-12 was used at .5 μ mol/L for 1 hour.

2.3 | Transwell migration assay

Migration of IL-6 KO keratinocytes was assessed via Transwell assay as described^[14] and quantified using Image J (NIH).

2.4 | Proliferation assays

IL-6 KO keratinocytes were pretreated with IL-6 for 6 hours followed by \pm TGF- β 1. Bromodeoxyuridine (BrdU) (EMD, Gibbstown, NJ, USA) was applied immediately following TGF- β 1 treatment according to manufacturer's instructions and incubated overnight. Results were obtained via a 96-well plate reader (Molecular Devices, Sunnyvale, CA, USA).

2.5 | Semi-quantitative RT-PCR

Total RNA from Ker-CT cultures was prepared, and cDNA was synthesized as described previously.^[13] Primers for TGF- β RI and II, Smad7, and 28S rRNA were constructed from GenBank sequences utilizing Amplify for Mac 3.1.4 (Bill Engles, University of Wisconsin) and synthesized by Life Technologies (Carlsbad, CA, USA). Quantitative real-time (RT)-PCR utilizing SYBR green was performed on an ABI PRISM 7000 SDS. Expression differences were determined relative to 28S rRNA utilizing the $\Delta\Delta$ Ct method.

2.6 | Multiplex assay

nHEKs were cultured as indicated and treated with IL-6 for 16–18 hours followed by TGF- β 1 for 30 minutes. Expression of TGF- β 1, p-Smad2 and p-Smad3 was measured using Millipore Milliplex Map Kit (Cat# TGFBMAG-64K-03 and 48-614MAG) according to manufacturer's protocol and analysed on a Bioplex 200 (Bio-Rad, Hercules, CA, USA).

2.7 | Immunoprecipitation assay

Ker-CT cultures were cultured and treated as described. Protein G Dynabeads Immunoprecipitation Kit was used according to manufacturer's protocol (ThermoFisher). Western blot was performed as described.^[17] Anticaveolin (Cell Signaling, Danvers, MA, USA), anti-TGF- β RII (Cell Signaling) and anti-TGF- β RI (Abcam, Cambridge, MA, USA) were used at the recommended dilution, and IR-labelled secondary antibodies (Licor, Lincoln, NE, USA) were used at 1:10000–1:20 000. Blots were imaged via the Licor Odyssey CLx imaging system (Licor).

2.8 | Flow cytometric analysis

Ker-CT cells were cultured and treated with IL-6 and/or TGF- β 1. Single cell suspensions were obtained, and TGF- β RI (Abcam), p-Smad-PE (Cell Signaling) and p-Stat3-488 (Cell Signaling) antibodies were used according to recommended dilutions and analysed via the Accuri C6 system.

2.9 | Statistical analysis

All experiments were replicated, and representative findings are shown. Statistical significance was determined by two-way analysis of the variance. In all statistical comparisons, a *P*-value of <.05 was used to indicate a significant difference.

3 | RESULTS

3.1 | IL-6 induces TGF- β RI and TGF- β RII expression in keratinocytes

It was previously shown that intra-dermal injection of IL-6 induces TGF- β RII in IL-6 KO mice.^[18] To further confirm these findings, epidermal TGF- β RII expression was determined in mice that specifically over express IL-6 in skin.^[16] Immunohistochemistry revealed that epidermal TGF- β RII expression was increased as compared to control (Figure 1A-I and II). To examine the effect of IL-6 treatment on isolated mouse epidermal keratinocytes, cells from IL-6KO mice (KO) were cultured as described in Methods and treated with IL-6. TGF- β RII was robustly increased in IL-6 treated KO keratinocytes when compared to control (Figure 1A-III and IV). To determine whether IL-6 also modulates TGF- β RI expression, and see whether this modulation translates in a human keratinocyte in vitro model, quantitative

RT-PCR and flow cytometric analysis were utilized to examine mRNA and protein levels for both receptors in human epidermal keratinocytes (Ker-CT) after IL-6 \pm TGF- β 1 treatment. PCR analysis revealed IL-6 significantly modulated both the receptors, while TGF- β 1 alone significantly increased TGF- β RI expression. Treatment with IL-6 and TGF- β 1 significantly decreased TGF- β RII expression as compared to control (Figure 1B). To minimize the effects of endogenously produced IL-6, cultures were incubated with IL-6 neutralizing antibody prior to treatment. Similarly, flow cytometry revealed IL-6 robustly increased TGF- β RI (Figure 1C) and TGF-RII (Figure 1D) when compared to control.

3.2 | IL-6 alters the function of TGF- β 1 in IL-6 KO epidermal cells

TGF- β 1 is a known motogen of keratinocytes.^[6,7,9] To investigate the functional consequences of IL-6 modulation of TGF- β R expression, the migration and proliferation of IL-6 \pm TGF- β 1-treated KO keratinocytes were assessed. Treatment with 2 ng/mL TGF- β 1 alone showed a slight increase in migration; however, this increase was not significant. Migration was significantly increased in a dose-response manner with a maximal response following pretreatment with IL-6 followed by TGF- β 1 as determined by Transwell assay (Figure 2A-I vs III and B). Various studies have shown in respect to skin, TGF- β 1 induces proliferation of dermal fibroblasts but suppresses growth of epidermal keratinocytes.^[19] Therefore, to assess whether IL-6 affects the anti-proliferative activity of TGF- β 1, BrdU assays were performed on IL-6 and TGF- β 1-treated KO keratinocytes. A significant reduction in BrdU incorporation was seen with 10 ng/mL TGF- β 1 alone as compared to control, while pretreatment with IL-6 significantly augmented this response (Figure 2C).

3.3 | IL-6 modulates TGF- β 1-induced signal transduction through Smad2/3 phosphorylation and increases Smad7 expression

TGF- β 1-mediated activation of the TGF- β receptor complex initiates signal transduction through the Smad2/3 and 4 pathways.^[7] To determine whether IL-6 modulation of TGF- β RI and II expression alters signal transduction through the receptor, Ker-CT cells were preincubated with IL-6 and/or followed by treatment with TGF- β 1. Flow cytometric analysis revealed two cell populations, p-Smad^{low} (peak A) and p-Smad^{high} (peak B). After IL-6 treatment, overall p-Smad fluorescence intensity slightly decreased, while the percentage of p-Smad^{high} population decreased and the p-Smad^{low} cells increased (Figure 3A,C, Column 3 and Column 6). Following TGF- β 1 treatment, a similar change was seen in both peaks, albeit more pronounced (Figure 3A,C, Column 4). When the treatments were applied in combination, there was a further enhancement of the shift from the high to low population (Figure 3A,C, Column 5) indicating an additive effect. To further confirm this effect was modulated by IL-6, the Stat3 inhibitor, LLL-12,^[20] was used. Upon inhibition of Stat3, a small increase in p-Smad^{low} was apparent after IL-6 treatment (Figure 3B,D,

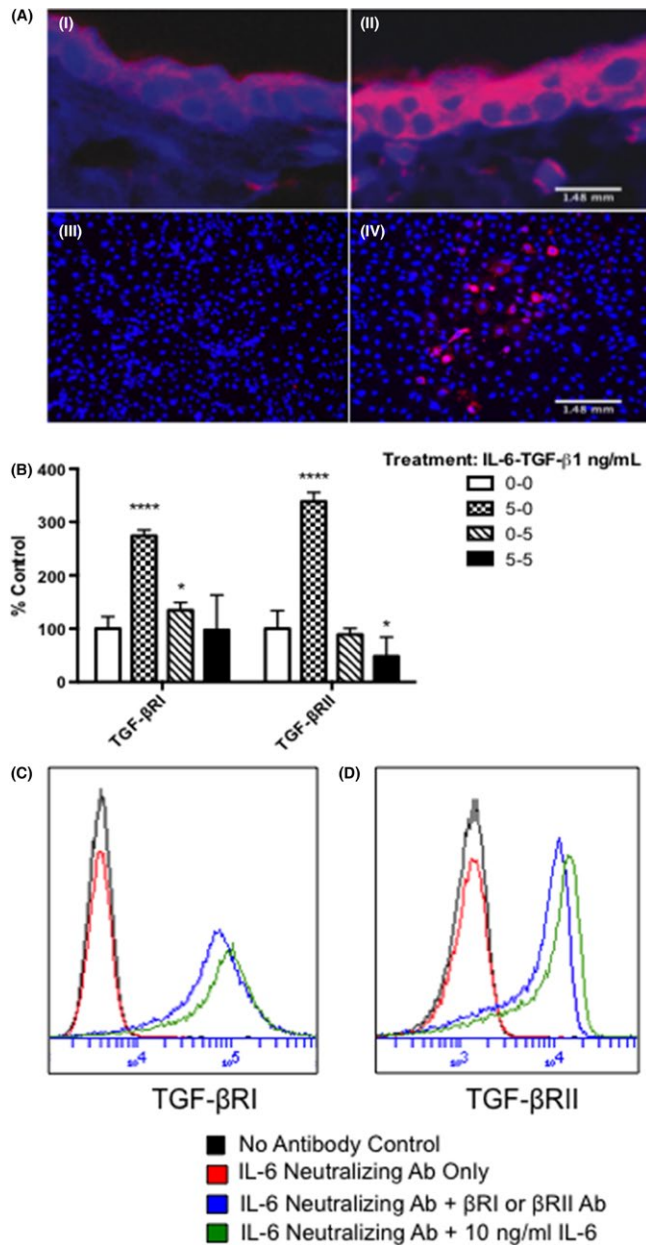


FIGURE 1 Interleukin (IL)-6 augments epidermal expression of transforming growth factor- β type I and II receptors (TGF- β RI and II). Punch biopsies (4 mm) obtained from wild-type (A-I) or transgenic IL-6 over-expressing mice (A-II) were formalin-fixed and paraffin-embedded for histology. IL-6 KO keratinocytes were treated with 0 ng/mL (A-III) or 10 ng/mL (A-IV) IL-6 for 16 hours and then fixed with paraformaldehyde. Slides were stained with monoclonal anti-TGF- β RII (red) followed by Alexa-fluor 546-conjugated secondary antibodies and DAPI (blue) counterstain. Slides were visualized at 20 \times objective. (B) Ker-CTs were cultured and treated as described. Treatments indicated as ng/ml of IL-6-TGF- β 1. Expression of TGF- β RI and II mRNA was determined by SYBR Green real-time PCR. Data are expressed as per cent negative control of average CT fold change \pm per cent SEM (* P <.05, n =3 cultures per data point). (C,D) Ker-CTs were cultured as described, treated with IL-6 neutralizing antibody and \pm IL-6. Flow cytometry for TGF- β RI and II was performed, and histograms for each are presented. (C) TGF- β RI (D) TGF- β RII

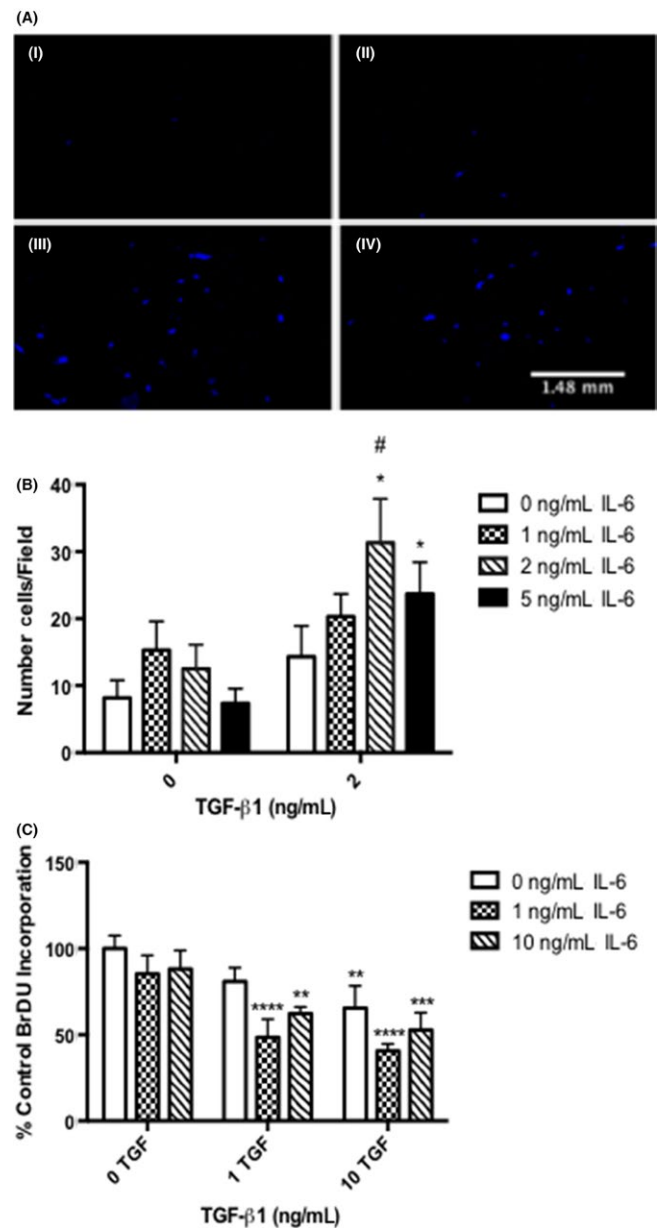


FIGURE 2 Interleukin (IL)-6 modulates transforming growth factor (TGF)- β 1-induced migration and antiproliferative effects. (A) IL-6 knockout (KO) keratinocytes were seeded on a porous (8 μ m) membrane Transwell culture insert and incubated for 2 hours before 16-hour TGF- β 1 and IL-6 treatment. Non-migratory cells were removed and remaining cells stained with DAPI. Migration was visualized using representative photographs under a fluorescent microscope. Treatments indicated as ng/ml of IL-6-TGF- β 1: (I) 0-0 ng/mL, (II) 0-2 ng/mL, (III) 2-2 ng/mL, (IV) 5-2 ng/mL. Migration was quantified using ImageJ particle analysis. (B) Data expressed as mean number of migratory cells \pm SEM/field (* P <.05, n =6). (C) Isolated IL-6 knockout (KO) keratinocytes were cultured and treated with various concentrations of IL-6 and TGF- β 1. Bromodeoxyuridine (BrdU) incorporation was quantified via commercial ELISA. Data expressed as per cent of the negative control optical density (OD) value \pm SEM (* P <.05 different from 0 ng/mL IL-6, # P <.001 different from 0 ng/mL TGF- β 1, n =6)

Column 3), but decreased upon TGF- β 1 only and IL-6 pretreatment (Figure 3B,D, Column 3). However, following TGF- β 1 treatment, a profound increase in the p-Smad^{high} cell population (peak B) was observed accompanied by increased intensity of staining of this population (Figure 3B,D). Interestingly, the decrease in peak B cells that was seen in Figure 3A with the combination treatment of IL-6 and TGF- β 1 was no longer present when treated with LLL-12, indicating Stat3 involvement (Figure 3 C vs D, Row 6). Multiplex analysis also confirmed that upon IL-6 treatment alone, Smad2/3 phosphorylation was minimal, while TGF- β 1 alone showed a marked increase in Smad2/3 phosphorylation (Figure 3E). Similar to Figure 3A, pretreatment with IL-6 followed by TGF- β 1 treatment, exhibited a significant decrease in Smad2/3 phosphorylation as compared to TGF- β 1 treatment alone. IL-6 KO keratinocytes were also treated in the same manner and multiplex data showed very similar phosphorylation trends (data not shown). Furthermore, research has indicated that expression of the inhibitory Smad7 can also be directly correlated with the transcription from TGF- β R target genes in human keratinocytes and is associated

with inhibition of Smad2/3 signalling.^[21] Ker-CT cultures were treated with IL-6 and/or TGF- β 1, and Smad7 expression was measured via quantitative PCR. Pretreatment with IL-6 and TGF- β 1 treatment showed a much more pronounced increase in Smad7 mRNA expression as compared to IL-6 or TGF- β 1 treatment alone further corroborating the link between IL-6 and TGF- β 1 signalling (Figure 3F).

3.4 | IL-6 alters localization of TGF- β RI and II within the cell membrane following IL-6 treatment in human epidermal cells

As the multifunctional role of TGF- β 1 has been well documented, the spatial organization of the receptor and its signalling components is less clear. Receptor activation not only initiates signal transduction, but also triggers clathrin- or non-clathrin-mediated receptor endocytosis, which serves to further regulate TGF- β 1 activity. Therefore, to determine whether IL-6 plays a role in TGF- β R-mediated endocytosis, Ker-CT cells were treated with IL-6 and/or TGF- β 1 and immunoprecipitation and immunohistochemistry assays were performed. As shown in Figure 4A,B, treatment with 5 ng/ml IL-6 induced TGF- β RII immunoprecipitation with caveolin as compared to no IL-6 treatment (Lane 5 vs Lane 6) but decreased the association with TGF- β RI (Lane 1 vs Lane 2). Activation of the receptor with 2 ng/mL TGF- β 1 significantly decreased this association of TGF- β RII with caveolin but had no effect on the TGF- β RI interaction. Pretreatment with IL-6 followed by TGF- β 1 significantly decreased both receptors interaction with caveolin compared to control (Lane 1 vs Lane 4 and Lane 5 vs Lane 8). Similarly, immunohistochemistry revealed diffuse, punctate staining of TGF- β RII (green)^[22] and little to no co-localization with caveolin (red) in the absence of IL-6 treatment (Figure 4C-I). However, upon IL-6 treatment, TGF- β RII and caveolin expression increased and co-localized within the same area (Figure 4I-C-I, orange). Co-localization with clathrin heavy chain and the early endosomal antigen (EEA-1) were also assessed and found that IL-6 treatment did not increase co-localization (results not shown).

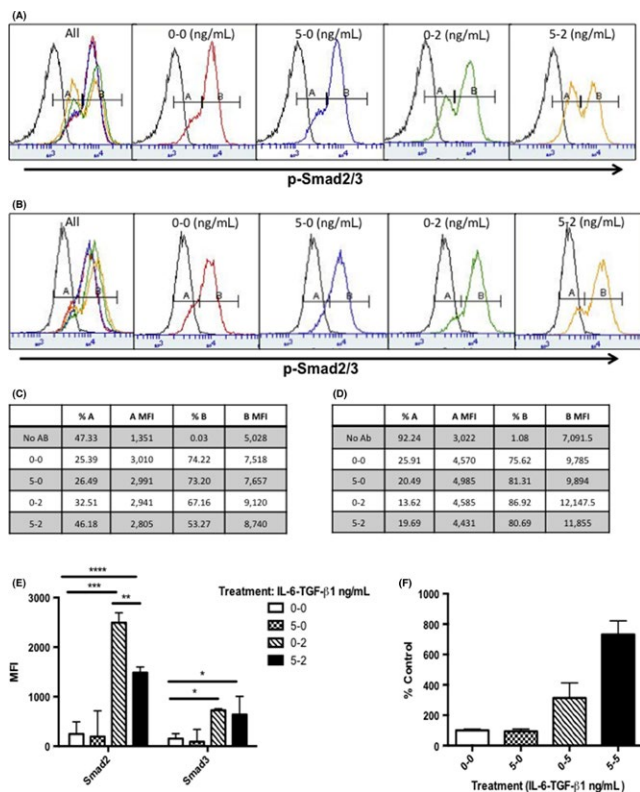


FIGURE 3 Interleukin (IL-6) modulates Smad2/3 phosphorylation and enhances transcription of Smad7 in epidermal keratinocytes. (A) Ker-CTs were cultured and treated with IL-6 for 1 hour followed by TGF- β 1 for 30 minutes. Treatments indicated as ng/ml of IL-6-TGF- β 1 and p-Smad and p-Stat3 histograms are presented. (B) Ker-CT cells treated as in (A) with addition of LLL-12. (C,D) Mean fluorescent data of histograms in A and B. (E) nHEK protein lysates were isolated, and multiplex analysis was performed using the Millipore TGF- β Signaling 6-plex kit. (F) Ker-CTs were cultured and treated, and total RNA was isolated. Expression of Smad7 mRNA was determined by real-time PCR. Data are expressed as per cent negative control average CT fold change \pm per cent SEM (* P < .05, n = 3)

4 | DISCUSSION

Regeneration of dermal and epidermal tissue following cutaneous wounding is a complex process involving the intricate coordination of many cell types in a sequential and predictable pattern. IL-6 and TGF- β are key factors in the normal procession through these events, and both knockout mice display multiple defects in repair.^[4,5] Similar to IL-6, both TGF- β RI and II are minimally distributed in areas such as the epidermis, hair follicles and sebaceous glands, all of which express TGF- β ligands.^[6,23] Following injury, receptor expression increases in the epidermis adjacent to the wound, as does IL-6 expression and tends to persist in older wounds.^[24] Herein, it was demonstrated that IL-6 treatment of keratinocytes enhances expression of TGF- β RI and II as compared to controls (Figure 1A-D). These results are similar to those found by Zhou, D. et al., which showed IL-6 increased the number of cells expressing TGF- β receptors in the spleen.^[15]

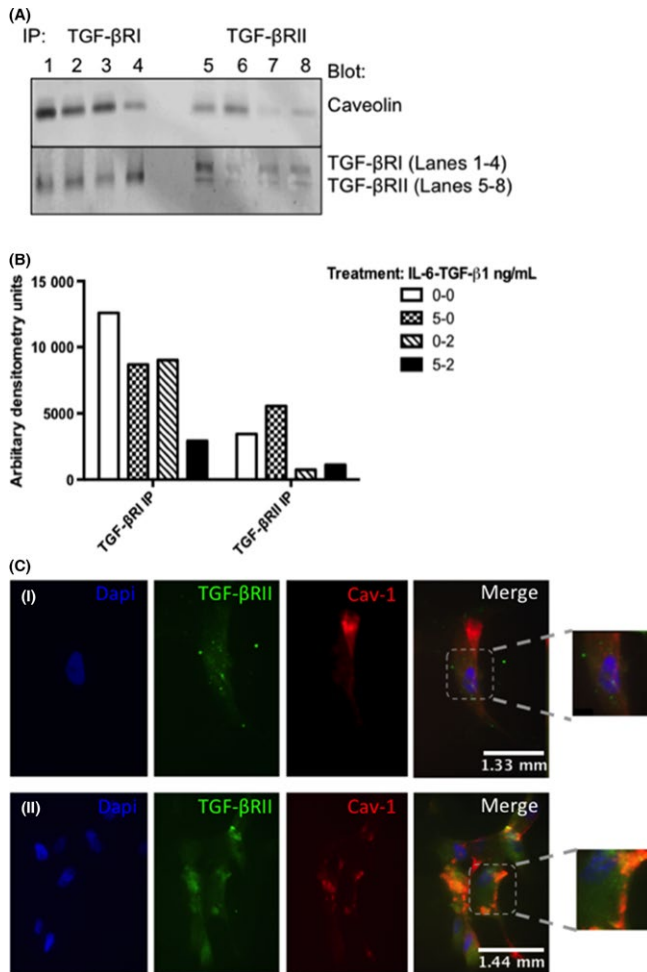


FIGURE 4 Interleukin (IL-6) alters localization of transforming growth factor (TGF)- β R within the cell membrane following IL-6 treatment in human epidermal cells. (A) Ker-CTs were cultured as described and treated with IL-6 for 16 hours followed by TGF- β 1. Cell lysates were immunoprecipitated with anti-TGF- β RI or II (bottom panel), and blots were incubated with anticaveolin (top panel). Treatments indicated as ng/mL of IL-6-TGF- β 1: Lane 1 and Lane 5=0-0 ng/mL, Lane 2 and Lane 6=5-0 ng/mL, Lane 3 and Lane 7=0-2 ng/mL, Lane 4 and Lane 8=5-2 ng/mL. (B) nHEKs were cultured in eight-chamber slides and treated with (I) 0 ng/mL and (II) 10 ng/mL IL-6. Slides were stained with anti-TGF- β RII (green) and anticaveolin (red) and detected using Alexa-Fluor 488 or 546-conjugated secondary antibodies, respectively. Stained slides were visualized at 40 \times objective; photographs are representative of three replicate experiments

The low levels of TGF- β produced by monocultures of keratinocytes and fibroblasts can be increased substantially through co-culturing these two cell types.^[1] These findings and the motogenic properties of TGF- β are particularly interesting when considering that IL-6 has been shown to indirectly induce keratinocyte migration in conjunction with a soluble factor secreted by the dermal fibroblasts.^[13] Furthermore, IL-6 treatment has been shown to increase TGF- β 1 mRNA expression in a dose-response manner in isolated fibroblasts and dermis.^[18] This evidence demonstrates a partnership between the epidermis and the dermis with regard to TGF- β expression and function seemingly driven

by IL-6. While IL-6/TGF- β cooperation is well documented in the realm of collagen deposition and myofibroblast contraction,^[17,18,25,26] few studies have characterized this partnership with respect to re-epithelialization. The present data demonstrate IL-6 pretreatment elicits keratinocyte migration in response to TGF- β 1 to a greater extent than TGF- β 1 alone (Figure 2A-B). These results support the notion of IL-6 mediating the keratinocyte-fibroblast interaction exhibited during wound healing through its regulation of the TGF- β ligand and receptor. While often conflicting, TGF- β has been demonstrated to modulate cell proliferation.^[6] Several studies have demonstrated an inhibitory effect on keratinocyte proliferation^[19,27,28] whereas other have shown that overexpression of TGF- β 1 increases the proliferative phenotype of keratinocytes^[29,30]. In our cell system, it was anticipated that following IL-6 treatment, the antiproliferative activity of TGF- β would be augmented if in fact it facilitates TGF- β -mediated migration of these cells and that was what was demonstrated (Figure 2C). Although still significantly different, the decrease in proliferation in response to TGF- β 1 may seem lower than expected. However, with the exact modulatory nature of TGF- β 1 conflicting as mentioned above, as well as variations in cell types and culture environment, these results are not necessarily surprising. This further illustrates the complexity of TGF- β signalling in wound healing and emphasizes the importance in which small changes in growth factor expression and timing may play a role in the cellular activity or function of a cell.

It is well established that TGF- β signal transduction is carried out through the intricate and highly regulated Smad2/3 signalling pathway, which is involved in repair by regulating migration, proliferation and differentiation^[7,31-33]. In Smad3 null mutant mice, inflammatory infiltration into the dermis as well as keratinocyte proliferation is altered during repair.^[22] Unfortunately, little is known regarding the role of Smad2 in repair as the null genotype is embryonic lethal.^[34] Herein, it is shown that pretreatment with IL-6 and addition of TGF- β 1 resulted in a marked increase in function of TGF- β R as evidenced by increased migration (Figure 2B). Further, it was found that following treatment, keratinocyte cultures form two distinct populations, an apparent p-Smad^{low} and p-Smad^{high} (Figure 3A,B, peaks A and B, respectively). While not previously reported, this may indicate cells at different stages during the signalling process, where peak A is perhaps recycling (or postsignalling) and peak B is actively signalling as indicated by increased fluorescence intensity (Figure 3C,D, Column 3 vs Column 5). Inhibition of Stat3 seems to alter this population of cells indicating a potential mechanism for the shift from Smad^{high} to the Smad^{low} population. Thus, this may be indicative of very active TGF- β R signalling in these cells. Interestingly, increased Smad2/3 expression as well as increased Smad2/3 phosphorylation has been shown to exhibit an inhibitory effect on keratinocyte migration during re-epithelialization^[35] while increased Stat3 can induce migration and inhibit differentiation^[36,37]. This may indicate that peaks A and B could be separate populations of differentiated and undifferentiated cells. However, this was not assessed directly herein and further study will be needed to determine this definitively.

Inhibitory (I)-Smads can terminate TGF- β signal transduction through a variety of complex mechanisms. Most notably, I-Smads aid in the degradation of Smad2/3 and Smad4 and exert feedback inhibitory

effects on transcription of TGF- β -induced genes.^[38-40] Herein, it was demonstrated that Smad7 mRNA expression appears to be directly correlated with TGF- β 1 treatment in keratinocytes as indicated by a significant increase in mRNA expression (Figure 3F). Furthermore, pretreatment with IL-6 enhances TGF- β 1-induced Smad7 mRNA expression supporting the observed decrease in TGF- β R signalling (Figure 3). These results were similar to studies that identified a molecular link by which Smad7 mediates crosstalk with IL-6-mediated Stat3 hyperactivation and attenuation of TGF- β R signalling.^[41] It was shown that increased Stat3 activation in mouse embryonic fibroblasts treated with an IL-6/sIL-6r fusion protein resulted in impaired phosphorylation and nuclear translocation of Smad2.^[41] Additionally, Stat3 directly interacts with Smad3 and interferes with the Smad3-Smad4 complex^[42] and activated Stat3 also induces Smad7 expression to desensitize TGF- β R signalling.^[41] Indeed, IL-6 induced Stat3 expression and activation is increased in injured epidermis as compared to uninjured epidermis;^[43] therefore, Stat3 activation may be a modulatory link between IL-6R and TGF- β RII function during repair. Inhibition of Stat3 signalling, through the use of the novel small molecule inhibitor LLL-12, further supports this association in Figure 3B, where TGF- β 1-induced signalling significantly increased upon inhibition of Stat3.

TGF- β receptors are constitutively internalized via clathrin-dependent or lipid raft (caveolin-rich)-dependent endocytic pathways^[40,44,45] upon ligand binding to regulate receptor activity. The balance between the two internalization pathways is critical in modulating receptor activity. Data presented herein show an increased association between caveolin and TGF- β RII following IL-6 treatment but decreased TGF- β RI association with caveolin, although the interaction is still considerably higher than TGF- β RII association. Interestingly, the reciprocal immunoprecipitation found that only TGF- β RI showed association with caveolin and IL-6 and TGF- β 1 increased this association (data not shown). Irrelevant IgG antibody was also used as a control, and no bands were indicated confirming the specificity of our pull down (data not shown). This suggests IL-6 may modulate TGF- β activity by trafficking the receptor to the lipid raft portion of the membrane (Figure 4). These data support previous findings that a direct interaction occurs between both TGF- β RI and II, and caveolin^[46,47]. These interactions have functional consequences as evidenced by a significant decrease in Smad2 phosphorylation upon receptor activation (Figures 3 and 4). Interestingly, a previous study in human renal cells found that IL-6 increased compartmentalization of TGF- β RI and II into the non-lipid raft portion of the cell membrane, which further enhances TGF- β signalling.^[48] Thus, it might be concluded that in the current model, IL-6-induced compartmentalization of TGF- β R into lipid rafts would decrease signalling, and indeed, this is the case (Figure 3). This would seem paradoxical when considering the apparent increase in TGF- β RI and II expression and function in keratinocytes when pretreated with IL-6 (Figure 1 and 2). However, it may be that this apparent IL-6-mediated shift into the lipid raft is a regulatory mechanism initiated to keep the resulting increase in TGF- β R expression and further or persistent signalling in check through increased receptor turnover. It was recently shown that TGF- β RII was detected in EEA-1-positive compartments and as inflammation progressed (by day 5), the receptor

appeared in caveolin-1-positive intracellular structures as well, indicating a potential mechanism to control TGF- β R activity in inflammatory conditions.^[47] Perhaps once receptor expression attains a certain threshold or a particular Smad-regulated function is required (such as migration), TGF- β RII is directed into the lipid raft domain ultimately resulting in degradation of the receptor in an attempt to terminate any potential (over) signalling, and this may be represented by peak A in Figure 3. Interestingly, kinase activation by bFGF and PDGF signalling was suppressed by manipulation of caveolin-1 expression itself, which is likely to influence trafficking of the receptor.^[49] Similarly, our studies found that in nHEKs treated with IL-6, total caveolin protein expression increased in a dose-response manner (data not shown). It is also shown that the ratio of TGF- β binding to either TGF- β R can also determine the internalization pathway^[11] and IL-6 may modulate the receptor expression and ultimately alter this ratio. It has also been shown in that 10% of IL-6R subunits associate with lipid rafts and treatment with a lipid raft inhibitor disrupted IL-6 signalling further supporting this link.

Herein, it was demonstrated that IL-6 plays a role in keratinocyte migration and proliferation through modulation of TGF- β R expression and function. The current findings, when viewed with previous results, illustrate a model of IL-6 involvement in skin TGF- β function where enhanced TGF- β 1 secretion in the dermis^[18] and upregulation of TGF- β R in the epidermis may facilitate the differentiation of epidermal keratinocytes to the pro-migratory phenotype observed during re-epithelialization. However, further research into the mechanisms by which IL-6 mediates receptor upregulation and membrane localization is necessary for a more complete understanding of the IL-6/TGF- β interaction in skin and in the dynamic process of wound healing.

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CONFLICT OF INTEREST

The authors have declared no conflicting interests.

AUTHOR CONTRIBUTIONS

Lerin R. Lockett-Chastain and Mackenzie L. Cottrell designed and performed the research, analysed the data and wrote the article. Bethany Kavar assisted with performing the research. Michael A. Ihnat contributed essential reagents and tools. Randle M. Gallucci contributed to critically revising and providing final approval of the manuscript.

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