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The TGFβ1 Pathway is Required for NFκB Dependent Gene Expression in Mouse Keratinocytes

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Abstract

The transforming growth factor beta1 (TGFβ1) and NFκB pathways are important regulators of epidermal homeostasis, inflammatory responses and carcinogenesis. Previous studies have shown extensive crosstalk between these pathways that is cell type and context dependent, but this has not been well-characterized in epidermal keratinocytes. Here we show that in primary mouse keratinocytes, TGFβ1 induces NFκB-luciferase reporter activity that is dependent on both NFκB and Smad3. TGFβ1-induced NFκB-luciferase activity was blocked by the IκB inhibitor parthenolide, the IκB super-repressor, a dominant negative TGFβ1-activated kinase 1 (TAK1) and genetic deletion of NFκB1. Coexpression of NFκB p50 or p65 subunits enhanced NFκB-luciferase activity. Similarly, inhibition of the TGFβ1 type I receptor with SB431542 or genetic deletion of Smad3 blocked TGFβ1 induction of NFκB-luciferase. TGFβ1 rapidly induced IKK phosphorylation but did not cause a detectable decrease in cytoplasmic IκB levels or nuclear translocation of NFκB subunits, although EMSA showed rapid NFκB nuclear binding activity that could be blocked by SB431542 treatment. TNFα, a well characterized NFκB target gene was also induced by TGFβ1 and this was blocked in NFκB+/− and −/− keratinocytes and by the IκB super-repressor. To test the effects of the TGFβ1 pathway on a biologically relevant activator of NFκB, we exposed mice and primary keratinocytes in culture to UVB irradiation. In primary keratinocytes UVB caused a detectable increase in levels of Smad2 phosphorylation that was dependent on ALK5, but no significant increase in SBE-dependent gene expression. Inhibition of TGFβ1 signaling in primary keratinocytes with SB431542 or genetic deletion of *Tgfb1* or *Smad3* suppressed UVB induction of TNFα message. Similarly, UVB induction of TNFα mRNA was blocked in skin of *Tgfb1*+/− mice. These studies demonstrate that intact TGFβ1 signaling is required for NFκB-dependent gene expression in mouse keratinocytes and skin and suggest that a convergence of these pathways in the nucleus rather than the cytoplasm may be critical for regulation of inflammatory pathways in skin by TGFβ1.

Keywords

TGFbeta1; NFkappaB; keratinocyte; UVB; TNFalpha

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1. Introduction

Transforming growth factor-beta 1 (TGFβ1) is a secreted cytokine that plays a critical role in normal epidermal homeostasis, inflammation and carcinogenesis. TGFβ1 acts through a heterodimeric receptor causing phosphorylation of intracellular Smads, Smad2 and Smad3 that each complex with Smad4 to mediate TGFβ1 dependent gene expression. In addition to its own signaling pathway, TGFβ1 is known to modulate the activity of many other signaling such as, JNK, p53 and NFκB [1]. In the epidermis NFκB acts as a negative regulator of keratinocyte proliferation and modulator of epidermal differentiation and inflammation. TGFβ1 can inhibit and activate NFκB dependent gene expression [2,3,4,5,6]. Further, TGFβ1 mediated gene expression can occur through kappaB sites and require interaction between NFκB subunits and Smads [5]. Conversely, cytokines that activate NFκB such as TNF can block TGFβ1 signaling through the NFκB mediated induction of Smad7 [7], while TGFβ1 can inhibit NFκB activity through induction of IκB [2]. In keratinocytes IKKα an upstream activator of NFκB, can interact directly with Smad2/3 independently of Smad4 to regulate keratinocyte differentiation [8].

NFκB is a major signaling pathway activated by UV irradiation in the skin and other cell types [9]. Exposure of cultured human keratinocytes to UVB causes increased NFκB DNA binding [10] and induction of proinflammatory cytokine gene expression [11]. The impact of TGFβ1 signaling on the UVB response is not well understood. UVB irradiation causes increased expression of TGFβ1 mRNA and protein in cultured human keratinocytes [12], and in sun exposed human skin increased expression of both TGFβ1 mRNA and Smad7, an inhibitory Smad, in the epidermis and dermis [13]. In contrast to these studies UVB reduced levels of TGFβ1 protein and mRNA in human keratinocytes and foreskin [14]. Other studies have shown that UVB causes downregulation of the TGF type II receptor and decreased Smad3/4 binding to the consensus SBE target site in keratinocytes and other cell types [15,13]. Here we used a genetic and pharmacological approach to test the hypothesis that TGFβ1 can directly activate NFκB dependent gene expression in keratinocytes and modulate UVB-induced proinflammatory cytokine gene expression.

2. Materials and Methods

2.1 Cell culture

Keratinocytes were isolated from newborn FVB/n mice or from crosses of *Smad3*+/−, *Tgfb1*+/−, *NFκB1*+/− adults and genotyped using specific PCR primers as described [16,17,18]. Primary keratinocytes were cultured as described [19] for 4 days prior to experimental treatment. SP1 cells [20] were obtained from the laboratory of Stuart Yuspa NCI and were cultured similarly to primary keratinocytes. Cells were pretreated with SB431542 (Sigma, St. Louis, MO) at 1.0uM 1h prior to treatment with TGFβ1 or UVB to block Smad2 phosphorylation [19] or 20 nM parthenolide (Biomol, Plymouth Meeting, PA), to inhibit IκB kinase [21,22].

2.2 UVB irradiation

Mice were shaved 24 hrs prior to exposure to 540 mJ/cm^2 [23] from UV bulbs (American Ultraviolet Light Co.) covered with cellulose triacetate (KODAK) to filter out UVC radiation, and produce UV wavelengths between 280–320 nm as described [24]. For *ex-vivo* studies irradiance and exposure time was determined using a UVX radiometer (UVP, Upland Ca). *In vitro* studies in keratinocyte culture utilized a Cl-1000 ultraviolet crosslinker (UVP, Upland CA) fitted with bulbs that delivered predominantly UVB wavelengths. To generate a constant energy output the time of exposure was corrected by the instrument,

which was on average 25–30 seconds. Culture media was removed from cells and after a PBS wash, cells were exposed to UVB in PBS after which original media was replaced.

2.3 Transfection and Luciferase Assay

Primary keratinocytes were cotransfected with the NFκB reporter pBIIX-Luc [25] or a Smad binding element (SBE)-luciferase reporter pGL-SBE4 [26] and a renilla-luciferase control plasmid (pRLTK) using Lipofectamine 2000 (Invitrogen). Where indicated, cotransfections included the IκBsuper-repressor (IκBsr), TAK1K63W (gift of Jun Nionomiya-Tsuji) or expression plasmids for NFκB p50 and p65 [27] and Smad3. After transfection cells were treated with TGFβ1 (1 ng/ml) or TNF (10 ng/ml) and luciferase activity measured after an additional 24 hrs using the Promega Dual Luciferase Assay System (Promega).

2.4 Analysis of Gene Expression

RNA preparation from whole skin and keratinocytes and quantitative RT-PCR (qPCR) for TNF ’ GATTATGGCTCAGGGTCCAA-3’; 5’-GAGACAGAGGCAACCTGACC-3’) and control genes was done as described [19], using the MyIQ system (BioRad Laboratories, Hercules, CA) and PerfeCTa SYBR Green SuperMix for iQ (Quanta Biosciences, Gaithersburg, MD). All qPCR values were normalized to 18s rRNA (5’-TCAACTTTCGATGGTAGTCGCCGT-3’; 5’-TCCTTGGATGTGGTAGCCGTTTCT-3’). Primer sequences were obtained using Primer 3 [28] software with Genebank sequence information.

2.5 Protein Isolation

Whole cell protein lysates were made using RIPA buffer containing protease and phosphates inhibitors. To fractionate cytoplasmic and nuclear protein confluent 10 cm^2 dishes of keratinocytes were incubated first in ice cold Buffer A (0.33M sucrose, 10mM Hepes pH 7.4, 1mM $MgCl_2$, 0.1% Triton X-100 with protease and phosphatase inhibitors) for 15 minutes followed by centrifugation at 1000×g for 10 minutes to collect the cytoplasmic fraction. The pellet was washed twice with Buffer A then incubated with ice cold Buffer B (0.45M NaCl, 10mM Hepes pH 7.4,1mM $MgCl_2$ with protease and phosphatase inhibitors) and centrifuged at 14,000×g to isolate the nuclear fraction. Immunoblotting and ECL were done as described [19] using the following antibodies: phospho-Smad2 (Cell Signaling 3101), Smad2/3 (Cell Signaling 3102), p50 (Santa Cruz C19), p65 (Santa Cruz F6), IKK (Santa Cruz7607) p-IKK (Cell Signaling 2078), IκB (Santa Cruz C21), p-I (Santa Cruz 8404), Actin (Millipore 1501), GAPDH (Cell Signaling 2118).

2.6 EMSA

Six ug of nuclear protein isolated as described above was incubated with a ^{32}P-labeled double stranded oligo containing a NFκB binding site [29] on ice in binding buffer (20% glycerol, 5mM MgCl2, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mL Tris-HCl ph 7.5) for 1 hr followed by electrophoresis through a 5% Criterion TBE gel (obtained from Biorad, Hercules, CA). Competition with unlabelled NFκB oligo and binding reactions with a labeled mutant NFκB oligo were done to demonstrate specificity of shifted signal [30]. Gels were dried and exposed to autoradiography film. Supershifts were done by adding antibodies to either p50 NLS (Santa Cruz, Santa Cruz CA) or p65 F6 (Santa Cruz, Santa Cruz CA) to the binding reaction for 30 min after preincubating labeled oligo with nuclear protein for 20 min, followed by electrophoresis on gels as described above.

2.7 Statistical Analysis

One way analysis of variance (ANOVA) with Tukey's post test was used as a test of significance. P values of <0.05 were considered significant. Dixon's Q-test was used for identification and rejection of single outlier.

3. RESULTS

3.1 TGFβ1 induction of NFκB-luciferase activity is dependent on NFκB and Smad signaling

To test if TGFβ1 could directly activate NFκB signaling, pBIIX-Luc transfected primary mouse were treated with TGFβ1 in the presence or absence of NFκB pathway inhibitors. TGFβ1 caused a 3 fold increase in NFκB dependent luciferase activity (ranging from 3–5 fold in different experiments) after 24 hrs (Fig. 1A), and this was detectable as early as 6 hrs post TGFβ1 (data not shown). Treatment of transfected cells with parthenolide a pharmacologic inhibitor of the NFκB pathway, or cotransfection with an IκB super-repressor expression plasmid significantly reduced TGFβ1-mediated induction of NFκB-luciferase, similar to their effects on TNF induction of NFκB-luciferase (Fig. 1A). TGFβ1 induction of NFκB-luciferase activity was significantly reduced in *Nfκb1* +/− keratinocytes and nearly abolished in *Nfκb1*−/− keratinocytes (Fig. 1B). Treatment of keratinocytes with SB431542, an inhibitor of the TGF type I receptor kinase, blocked TGFβ1 induction of NFκB-luciferase activity (Fig. 1C) as did genetic ablation of *Smad3* in keratinocytes (Fig. 1D). No significant difference in NFκB signaling molecules were detected between *Smad3*+/+ and −/− keratinocytes (Supplemental Figure 1) When keratinocytes were cotransfected with pBIIX-Luc and a Smad3 expression plasmid pCMV-Smad3, TGFβ1 caused a 9-fold induction of luciferase activity compared to 5-fold by TGFβ1 alone (Fig. 1E). There was no effect of NFκB1 deletion on TGFβ1 induction of a SBE-luciferase construct (not shown).

3.2 TGFβ1 increases nuclear NFκB binding to DNA and NFκB transactivation

To test if TGFβ1 increased nuclear NFκB binding activity in mouse keratinocytes, we performed an NFκB EMSA, and observed an increase in NFκB binding activity as early as 15 minutes post-TGFβ1 treatment that continued to increase through 6 hrs (Fig 2A). When keratinocytes were pretreated with the ALK5 inhibitor SB431542, the increase in NFκB binding activity was blocked (Fig. 2B). Supershifts showed that both p50 and p65 NFκB subunits were present in the shifted complex (Figure 2C). Both NFκB p50 and p65 were detectable in cytoplasmic and nuclear extracts of untreated keratinocytes, but there was no effect nuclear translocation of either subunit following TGFβ1 despite the increase in NFκB binding activity. However, as expected TGFβ1 caused a clear increase in the levels of nuclear phospho-Smad2 (Fig. 2D). There was also no TGFβ1-mediated increase in nuclear p50 or p65 levels in the SP1 papilloma cell line, which has significantly higher levels of p65, even though TGFβ1 caused a 3-fold increase in NFκB-luciferase activity (Supplemental Figure 2).

To test if TGFβ1 could cooperate with NFκB to enhance NFκB-luciferase activity, we cotransfected primary mouse keratinocytes with pCMV-p50 or pCMV-p65 [27] and measured NFκB-luciferase activity in the presence or absence of TGFβ1. Figure 2E shows that by itself pCMV-p50 caused a 2-fold increase in NFκB-luciferase activity but pCMV-p65 did not cause a significant increase. TGFβ1 induced a 3–5 fold increase in NFκB-luciferase activity and with cotransfection of pCMV-p50 there was a 6–7 fold increase in activity. TGFβ1 also enhanced pCMV-p65 driven NFκB-luciferase activity but to a lesser extent. Since there was a rapid increase in NFκB binding activity following treatment with TGFβ1 but no significant increase in translocation we determined whether the canonical pathway was activated. Figure 2F shows that TGFβ1 rapidly increased levels of pIKK but

there was no significant decrease in total IκB levels or increase in p-IκB. Since TAK1 can link TGFβ1 to activation of NFκB signaling [31,32,33] we transfected primary keratinocytes with a dominant negative TGFβ1 activated kinase 1 (TAK1K63W) expression construct [33] and measured the effect on TGFβ1 and TNF induction of NFκB-luciferase. For both cytokines, cotransfection with pTAK1K63W significantly suppressed induction of luciferase activity (Fig. 2G).

3.3 TGFβ1 induction of TNF expression is dependent on NFκB and Smad3

Since TNF is a well characterized NFκB dependent gene we tested whether its expression was induced by TGFβ1 in an NFκB dependent manner. We infected primary keratinocytes with adenoviruses expressing either beta-galactosidase or the IκBsr and then treated cells with TGFβ1. Figure 3A shows that in control adenovirus infected keratinocytes, TGFβ1 caused a 2-fold increase in level of TNF mRNA as expected. However, in IκBsr adenovirus infected keratinocytes this induction was significantly reduced. Similarly, we treated *Nfκb1* +/+, +/− and −/− primary mouse keratinocytes with TGFβ1 and measured TNF induction after 24 hrs. In *Nfκb1*+/+ keratinocytes TGFβ1 caused a 5 fold increase in TNF transcript levels and this was significantly reduced in *Nfκb1*+/− keratinocytes (Fig. 3B). Interestingly, basal expression of TNF was much higher in *Nfκb1* −/− keratinocytes, and there was no significant induction by TGFβ1 (Fig 3B). When TNF transcript induction by TGFβ1 was measured in *Smad3*+/+, +/− and −/− keratinocytes we observed a gene dose dependent reduction in TNF induction (Fig. 3C). Together these results suggest that TGFβ1 induction of TNF requires both intact p50 and Smad3.

3.4 UVB induction of TNFα expression in mouse skin is dependent on intact TGFβ1 signaling

Since UVB is a potent inducer of NFκB activity in keratinocytes we tested whether UVB induction of TNFα mRNA expression was dependent on intact TGFβ1 signaling. Figure 4A shows that within 2 hrs post UVB there was a significant induction of TNFα and this was blocked by pretreatment with SB431542. Similarly, TNFα induction by UVB was suppressed although not completely blocked in *Tgfb1* +/− and *Smad3* −/− primary keratinocytes (Fig 4B, C). As with TGFβ1 induction of TNFα, baseline expression of TNFα was higher in *Nfkb1*−/− keratinocytes and there was no further induction by UVB (Fig. 4D). These data suggest that both intact TGFβ1 and p50 are required for induction of TNFα by UVB. In primary keratinocytes irradiated with UVB at 50 and 75mJ/cm^2 there was an increase in Smad2 phosphorylation within 30 minutes post-UVB which was sustained up to 6 hrs. Treatment with SB431542 blocked Smad phosphorylation indicating dependence on ALK5. Because of difficulties associated with UV induction of transfected luciferase plasmids we used keratinocytes from mice containing a SBE-luciferase transgene [34] to determine if pathway activation resulted in increased Smad dependent gene expression. However, UVB did not cause any detectable increase in luciferase activity in keratinocytes (Fig. 4F) (or fibroblasts, data not shown) isolated from SBE-luciferase mice [34], although a significant induction occurred in the skin of the transgenic mice *in vivo* 48 hrs post UVB which could be due to infiltrating immune cells (data not shown). These results show that the UVB-induced increase in Smad phosphorylation was not sufficient to cause Smad dependent gene expression and suggest that TGFβ1 signaling is likely to directly modulate UVB-induced TNFα rather than indirectly through UVB induced Smad transactivation at SBE sites. Interestingly, pretreatment of these transgenic keratinocytes with TGFβ1 prior to UVB significantly enhanced SBE-luciferase activity and this was blocked by the IKK inhibitor parthenolide (Fig. 4F). To determine if UVB induction of TNFα was dependent on TGFβ1 signaling *in vivo* we isolated whole skin RNA from UVB irradiated wildtype *Tgfb1*+/+ and *Tgfb1*+/− mice. Figure 4G shows that 6 hrs after UVB irradiation there was 1.5–2 fold increase in TNF mRNA levels in skin of *Tgfb*1 +/+ mice, consistent with

published studies. In contrast, the basal level of TNF mRNA was elevated in skin of *Tgfb1* +/− mice and UVB reduced expression.

4. Discussion

Maintenance of cellular homeostasis in multiple cell types and tissues is dependent on cross talk between the NFκB and TGFβ1 signaling pathways [35,36,37,38,39,3]. In the skin both TGFβ1 and NFκB signaling play critical roles in regulating epidermal proliferation and differentiation, inflammation, and tumor development [40,41] Recent studies indicate that interaction of these two pathways plays a critical role in regulation of squamous differentiation [8] as well as tumor progression [42].

Our results provide strong evidence of cross-regulation of these pathways in primary mouse keratinocytes. We observed significant induction of NFκB-dependent reporter gene expression when primary mouse keratinocytes were treated with TGFβ1. In the absence of Smad3, or in the presence of the ALK5 inhibitor SB431542, TGFβ1-dependent transactivation of an NFκB-luciferase reporter plasmid, NFκB binding to DNA, and induction of TNFα mRNA were blocked. Furthermore, TGFβ1 dependent transactivation of the NFκB reporter was increased by exogenous p50 and p65 and blocked in *Nfkb1*−/− primary keratinocytes, suggesting a requirement for both Smads and NFκB. An association between Smads and NFκB subunits has been documented in other epithelial cell types, notably in mink lung epithelial cells in which Smad3 binds to p52 [5]. Although the nature of the putative interaction we observe between NFκB and Smad3 in TGFβ1-treated keratinocytes was not elucidated, one possibility is that Smad3 may serve as a coactivator of NFκB such as it does with the vitamin D receptor [43] and other transcription factors [44]. Alternatively, both transcription factors may bind to distinct DNA binding sites with or without physical interaction as observed in the regulation of the type II collagen promoter in fibroblasts containing both NFκB and SBE consensus sites [45]. However, this is unlikely given the absence of TGFβ1-induced NFκB luciferase and TNFα expression in *Nfkb1*−/− primary keratinocytes. Six experimentally validated NFκB binding sites have been identified in the murine TNF promoter within 2Kb from the transcriptional start site [46,47]. In addition there are 6 putative CAGA SBE binding elements, with the NFκB site at −860–869 and SBE site a −797 in somewhat close proximity. Chromatin immunoprecipitation (ChIP) studies will be necessary to clarify the nature of NFκB and Smad interactions at the TNF promoter that drive expression in response to TGFβ1.

TGFβ1 caused an increase in NFκB binding to DNA in the absence of NFκB translocation, in primary mouse keratinocytes as well as a papilloma cell line, even though TGFβ1 by itself caused Smad nuclear translocation TGFβ1 did not cause a decrease in total IκB levels, or increase in p-IκB consistent with the lack of p50 or p65 nuclear translocation but did cause a rapid increase in pIKKα levels that paralleled the increase in nuclear NFκB binding activity. The suppression of NFκB luciferase activity by a dominant negative TAK1 expression plasmid suggests that TGFβ1 activation of IKK is linked to TAK1. Given the lack of IκB degradation or NFκB subunit translocation, these results suggest that activation of the upstream TGFβ1 signaling pathway modulates the function of a nuclear population of NFκB rather than NFκB sequestered in the cytoplasm. It is possible that the IκB super-repressor inhibition of TGFβ1-induced NFκB transactivation and TNFα expression may reflect genetic repression of nuclear IκB rather than IκB residing in the cytoplasm, as nuclear IκBα, IκBζ, and BCL-3 can both repress or activate NFκB [48,49]. Nuclear IKK may also play a role in directly activating NFκB signaling in some human head and neck cancer cell lines [50]. Although the precise role of a resident population of NFκB is unclear, NFκB-dependent gene expression in resting cells is repressed by a pool of post-translationally modified, transcriptionally inactive nuclear NFκB [51,52]. Our results showing increased

basal NFκB-luciferase activity and TNFα expression in *Nfkb1*−/− relative to *Nfkb1*+/+ keratinocytes support this potential function of p50 as a repressor of NFκB transcription under basal conditions in mouse keratinocytes. Given that several studies demonstrate enhanced NFκB transcriptional activity following post-translational modification such as phosphorylation [53,54,55,56,57] it is possible that TGFβ1 alters NFκB activity through postranslational modification of p50 or p65 activity. Phosphorylated p50 was not detected in 50ug nuclear extracts of TGFβ1 treated keratinocytes (data not shown) suggesting that this is not the major mechanism by which TGFβ1 activates NFκB in the absence of nuclear translocation, but the presence of other modifications remains to be determined.

In addition to direct activation of NFκB reporter construct and the proinflammatory gene TNFα, our results indicate that TGFβ1 signaling is important in modulating NFκB signaling mediated by UV irradiation. Solar radiation leads to activation of NFκB in humans [58] and mice [59], although UVB-induced upstream activation of NFκB signaling molecules including IκB and IKK is inconsistent in the literature [60,61,62,63]. UVB also induces expression of TNFα [64,65], although the mechanism of UVB modulation of the TNFα promoter is poorly understood [66,67]. In mink lung epithelial cells and human fibroblasts UVB can suppress the TGFβ1 signaling pathway [13,68,69,15] while in human keratinocytes UV causes an increase in TGFβ1 mRNA and bioactive TGFβ1 [12,15,70]. Our findings in mouse skin and epidermal keratinocytes demonstrate for the first time that intact TGFβ1 signaling is required for maximal induction of TNFα by UVB. A possible mechanism is activation of TGFβ1 signaling directly by UVB which is is required for TNFα induction. While UV caused a slight increase in levels of phosphorylated Smad2 in keratinocytes, we were unable to demonstrate UVB-induced Smad transactivation in keratinocytes or fibroblasts isolated from transgenic mice containing an SBE-luciferase reporter. Thus the role of TGFβ1 signaling in UV mediated induction of TNFα message is not likely to be through parallel activation of SBE elements in the TNFα promoter but through modulation of NFκB transactivation. This will require ChIP analysis for validation. However, the synergistic increase in Smad-dependent transactivation observed when cells were pre-treated with TGFβ1 prior to exposure to UVB suggests that UVB can amplify TGFβ1 signaling under conditions where the pathway is activated.

4.1 Conclusion

Epithelial cells such as keratinocytes have emerged as important mediators of inflammation [71]. Here we have demonstrated that NFκB-dependent gene expression can be induced by TGFβ1 in mouse keratinocytes and that UVB induction of TNFα, a proinflammatory cytokine, requires intact TGFβ1 signaling both in keratinocytes and skin. These studies establish an important and novel intersection between TGFβ1 and NFκB pathways in epidermal keratinocytes, which may play a significant role in modulating inflammatory responses in the skin. Given that expression of TNFα is critical for skin homeostasis, particularly the initiation and resolution of inflammation any disruption of signaling pathways mediating TNFα expression, such as TGFβ1-induced NFκB transactivation, has the potential to alter chronic inflammatory conditions in the skin such as psoriasis. In addition, TGFβ1 can have a tumor promoting role in squamous cell cancer and this can occur in part through its proinflammatory actions [72,73]. Our results provide a potential mechanism through which TGFβ1 can positively regulate NFκB dependent proinflammatory pathways relevant to cancer development. Thus these studies enhance understanding of the interaction of two potent signaling pathways that regulate keratinocyte homeostasis and inflammatory responses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

EMSA	electrophoretic mobility shift assay
TGFβ1	transforming growth factor-beta 1
TNFα	tumor necrosis factor alpha
NFkappaB	Nuclear Factor kappaB
IKβ	I kappaβ
IKβsr	I kappaβ super repressor
TAK1	TGFβ1 activated kinase 1
IKKα	I kappa Kinaseα
JNK	cJun N-terminal Kinases
SBE	Smad binding elements
ALK5	Activin receptor-like kinase 5

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- In mouse primary keratinocytes TGFβ1 induces NFκB-dependent gene expression.
- NFκB reporter and TNFα expression requires TGFβ1 and NFκB pathways.
- UVB-induced TNFα expression requires intact TGFβ1 signaling.

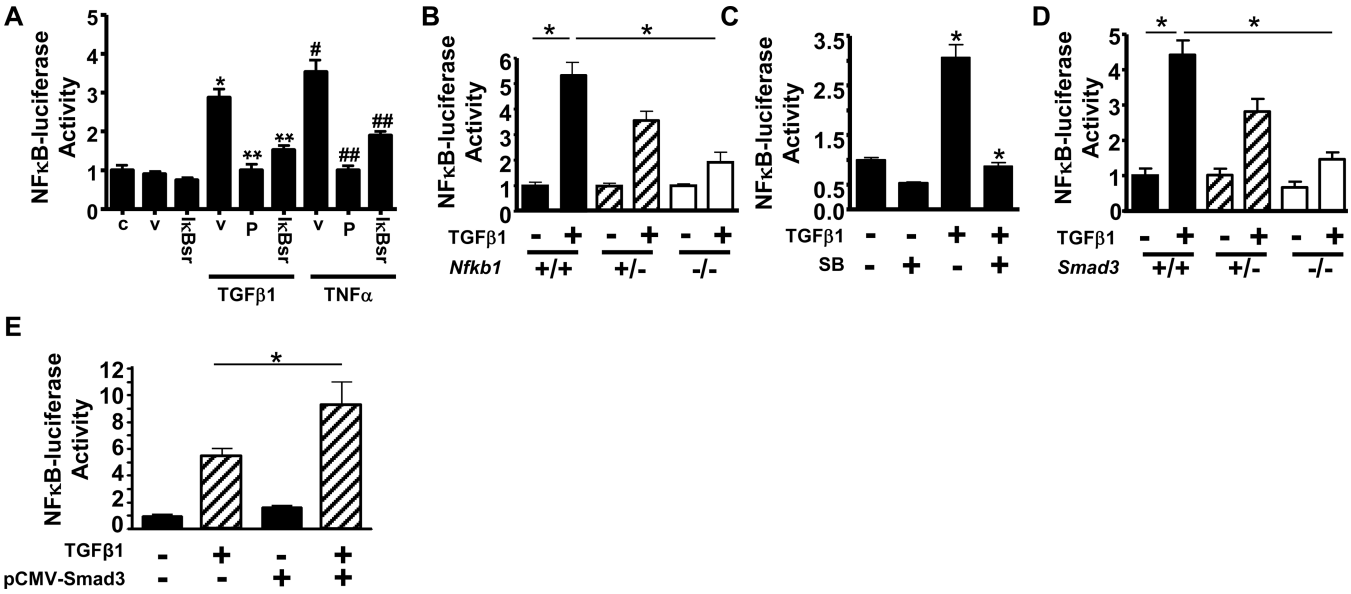

Figure 1. TGFβ1 induces NFκB-luciferase activity in a NFκB and Smad3 dependent manner

(A) pNFκB-luc reporter activity in primary mouse keratinocytes treated with or without TGFβ1 (1 ng/mL), TNFα (10 ng/mL), parthenolide (20 nM) or cotransfection with IκB super-repressor plasmid (IκBsr). (B) TGFβ1 induced pNFκB-luc activity in *Nfκb1* +/+, +/− and−/− primary keratinocytes. (C) pNFκB-luc or pSBE-luc activity in keratinocytes treated with TGFβ1 in presence or absence of ALK5 inhibitor SB431542 (1 uM). (D) pNFκB-luc activity in *Smad3* +/+, +/− and −/− primary keratinocytes. (E) TGFβ1 induced pNFκB-luc in keratinocytes cotransfected with the Smad3 expression vector pCMV-Smad3. All experiments were averaged from N=6 wells repeated 3 times. * indicates significant difference between indicated groups p<.05. For 1A *, # indicate significantly different from control, **, ## significantly different from TGFβ1 or TNFα alone, p<.05. c= no treatment; v=vehicle (DMSO)

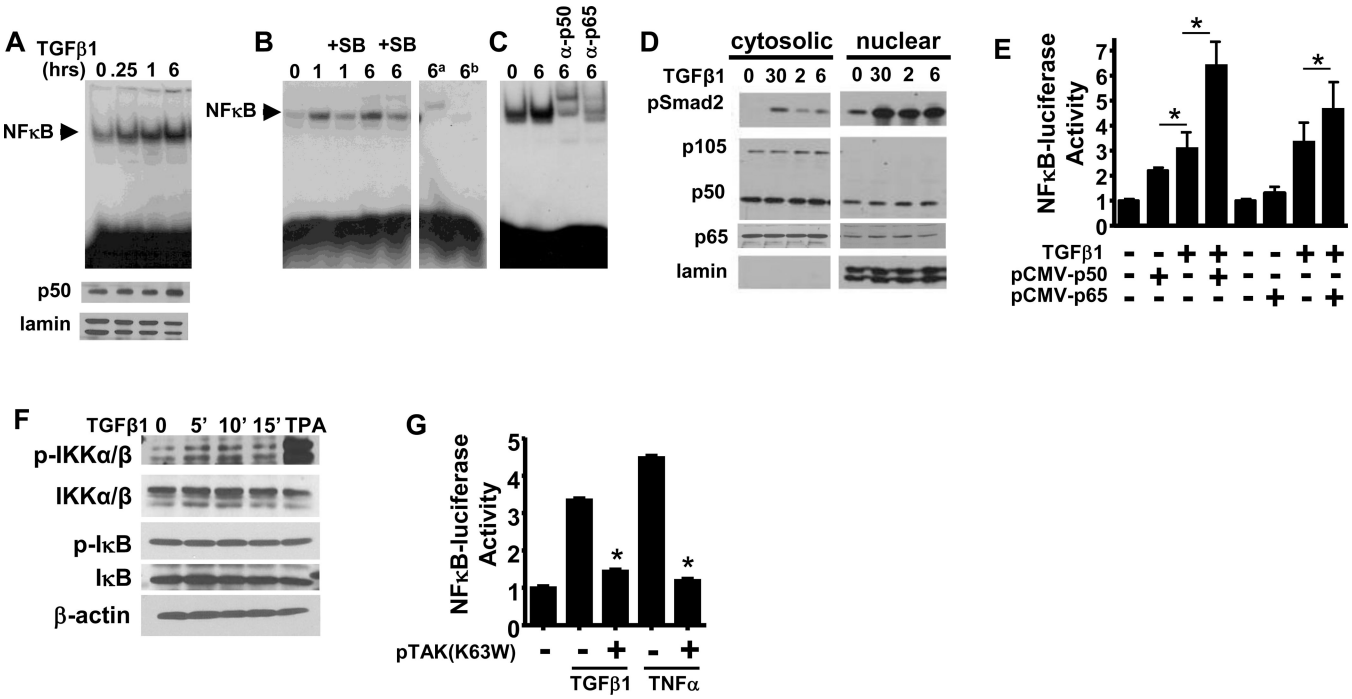

Figure 2. TGFβ1 induces nuclear NFκB binding activity and enhances p50 and p65 driven gene expression

(A) Top: NFκB EMSA of nuclear extracts from primary keratinocytes treated with TGFβ1(1 ng/mL) for the indicated times. Bottom: immunoblot of nuclear extracts showing equal amounts of NFκB p50 and lamin loading control (B) NFκB EMSA of nuclear extracts from primary keratinocytes treated with TGFβ1 for the indicated times +/− the ALK5 inhibitor SB431542 (1 uM). Extracts isolated 6h post TGFβ1 treatment were incubated with labeled mutant NFκB oligo (6a) or unlabelled wildtype NFκB oligo(6b). (C) NFκB EMSA of TGFβ1 treated 6 hr nuclear extracts incubated with anti-p50 and anti-p65 antibodies. (D) Immunoblot of NFκB subunits and phospho-Smad2 levels in nuclear and cytosolic lysates from primary keratinocytes treated with TGFβ1. 50 g of nuclear extract was used to detect p65 in the nucleus. Cytoplasmic and nuclear lysates were run on the same gel, with intervening lanes removed. (E) Effect of pCMV-p50 or pCMV-p65 cotransfection on TGFβ1 induced NFκB-luc activity. All experiments were averaged from N=6 wells repeated 3 times. * indicates significant difference between indicated groups, $p<0.05$ (F) Immunoblots of phospho and total IKKα/β, phospho and total IκB in cytosolic extracts of TGFβ1 treated primary keratinocytes. Repeated 3 times. (G) pNFκB-luc reporter activity in primary mouse keratinocytes treated with or without TGFβ1 (1 ng/mL), TNFα (10 ng/mL) in the presence of TAK1K63W or control vector. Average of 3 wells, repeated twice. * indicates significantly different from TGFβ1 alone, $p<.05$.

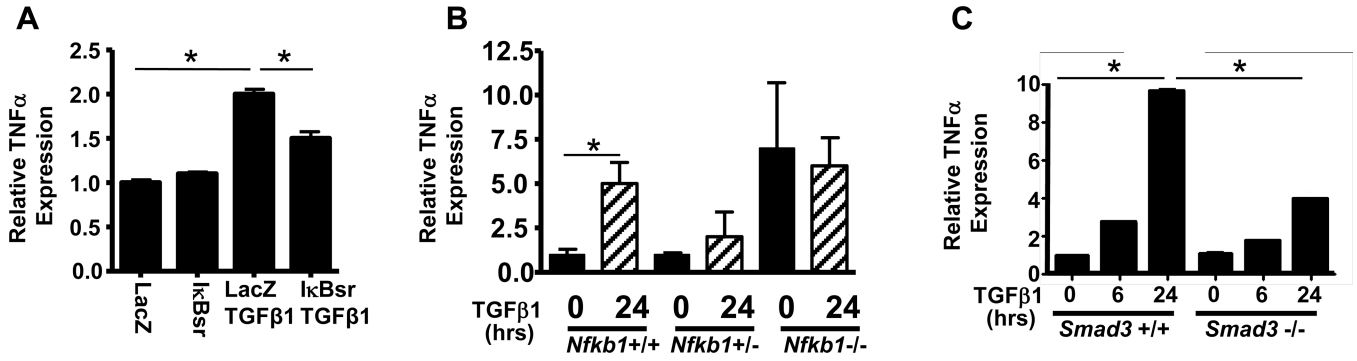

Figure 3. TGFβ1 induces TNF expression in a NFκB dependent manner

(A) qRT-PCR analysis of TNF mRNA expression in primary keratinocytes infected with beta-galactosidase (b-gal) or IκB super-repressor adenoviruses and treated with TGFβ1 (1 ng/mL). (B) qRT-PCR analysis of TGFβ1-induced TNF mRNA expression in *Nfκb1*+/+, +/− and −/− keratinocytes. Samples were normalized to the untreated wildtype. (C) qRT-PCR analysis of TGFβ1-induced TNF mRNA expression in *Smad* +/+, −/− primary keratinocytes. Results were normalized to 18S rRNA. Histograms represent N=3, repeated twice. * indicates significant difference between indicated groups $p<.05$.

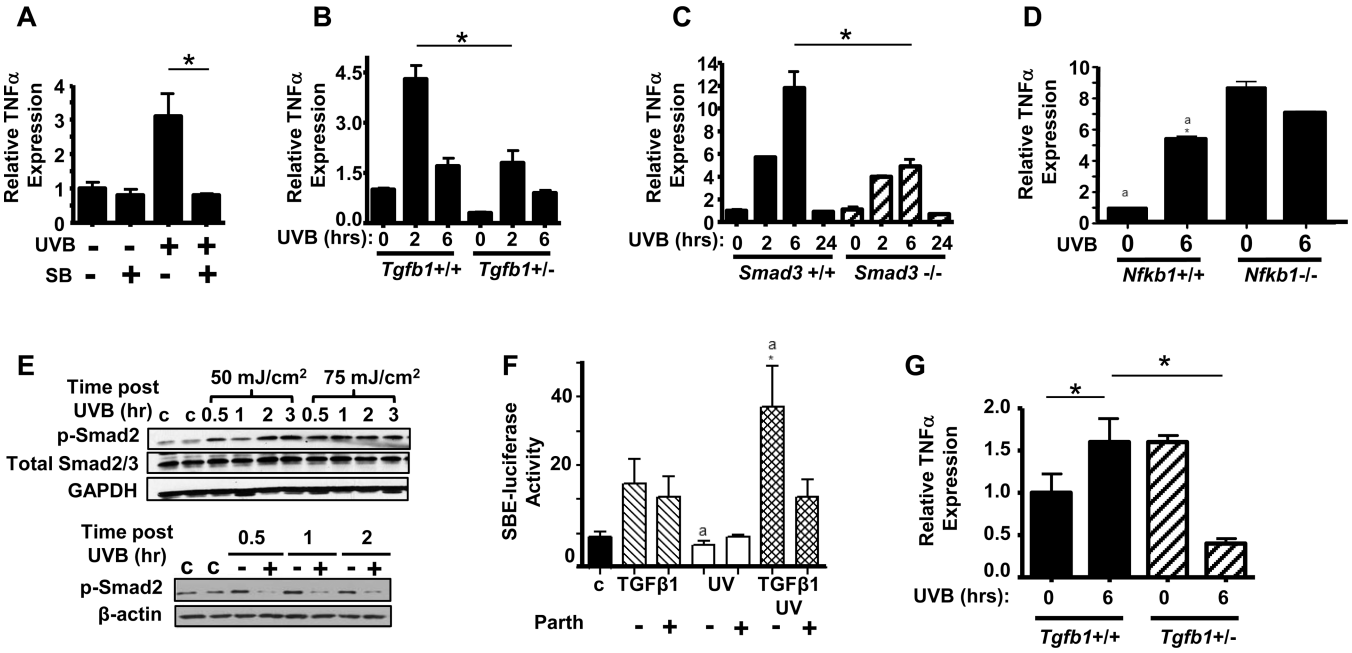

Figure 4. UVB induction of TNF mRNA is dependent on intact TGFβ1 and NFκB pathway

(A) Quantitative rt-PCR analysis of UVB-induced TNF mRNA expression in primary keratinocytes in the presence or absence of ALK5 inhibitor SB431542 (1 M). (B) Quantitative rt-PCR analysis of TNF transcript levels in UVB irradiated *Tgfb1*+/+ and +/− primary keratinocytes. (C) TNF transcript levels in UVB irradiated *Smad3*+/+ and *Smad3*−/− primary keratinocytes. (D) Quantitative rt-PCR analysis of TNF transcript levels in UVB irradiated *Nfkb1*+/+ and *Nfkb1*−/− keratinocytes. Results were normalized to 18S rRNA. (E) Immunoblot of total and phospho-Smad2/3 levels in UVB irradiated primary keratinocytes at either 50 or 75mJ/cm^2 (F) SBE-luc activity in SBE-luc transgenic primary keratinocytes treated with TGFβ1 (1 ng/mL), UVBor TGFβ1+ UVB in the presence or absence of parthenolide (20 nM). (G) Quantitative rt-PCR analysis of TNF transcript levels in RNA isolated from UVB irradiated skin of *Tgfb1* +/+ and −/− mice at the indicated time post UVB (540 mJ/cm^2). N=5–6 mice per group. * significantly different between indicated groups $p<0.05$.