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TNIP1 reduction sensitizes keratinocytes to post-receptor signalling following exposure to TLR agonists

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ABSTRACT

Cell level inflammatory signalling is a combination of initiation at cell membrane receptors and modulation by cytoplasmic regulatory proteins. For keratinocytes, the predominant cell type in the epidermis, this would include toll-like receptors (TLR) and cytoplasmic proteins that propagate or dampen post-receptor signalling. We previously reported that increased levels of tumor necrosis factor α induced protein 3-interacting protein 1 (TNIP1) in HaCaT keratinocytes leads to decreased expression of stress response and inflammation-associated genes. This finding suggested decreased TNIP1 levels, as seen in some cutaneous disease states, may produce the opposite effect, sensitizing cells to triggers of inflammatory signalling including those sensed by TLR. In this study of TNIP1-deficient HaCaT keratinocytes we examined intracellular signalling consequences especially those expected to produce gene expression changes downstream of TLR3 or TLR2/6 activation by Poly (I:C) or FSL-1, agonists modeling skin relevant pathogens. We found TNIP1-deficient keratinocytes are hyper-sensitive to TLR activation compared to control cells with a normal complement of TNIP1 and receiving the same agonist stimulation. TNIP1-deficient keratinocytes have increased levels of activated (phosphorylated) cytoplasmic mediators such as JNK and p38 and greater nuclear translocation of NF-κB and phospho-p38 when exposed to TLR ligands. This is consistent with significantly increased expression of several inflammatory cytokines and chemokines, such as IL-6 and IL-8. These results describe how decreased TNIP1 levels promote a hyper-sensitive state in HaCaT keratinocytes evidenced by increased activation of signalling molecules downstream of TLR agonists and increased expression of pro-inflammatory mediators. TNIP1 keratinocyte deficiency as reported for some skin diseases may predispose these cells to excessive inflammatory signalling upon exposure to viral or bacterial ligands for TLR.

1. Introduction

The skin's upper layer, the epidermis, provides multiple important barrier roles protecting underlying tissue from desiccation and exposure to damaging UV rays, chemicals, and biological compounds. This latter protection comes in part from surveillance via a family of toll-like receptors (TLR) that act as pathogen sensors and initiate cytoplasmic signalling culminating in significant gene expression changes. TLR are expressed on both skin immune and non-immune cells [1]. The latter includes keratinocytes at 90–95% of the cells in the epidermis, which represent significant sensing and responding capacity to surface-encountered TLR ligands such as bacterial and viral products. Thus, important for any localized reaction will be the keratinocyte's range of TLR and repertoire of cytoplasmic signal regulatory proteins. These include tumor necrosis factor (TNF) α-induced protein 3-interacting protein 1 (TNIP1). Through physical interaction with its cytoplasmic interaction partner A20, TNIP1 dampens NF-κB signalling subsequent to TNFα receptor (TNF-R) stimulation earning it the alias A20-binding inhibitor of NF-κB activation (ABIN-1) [2–4]. TNIP1 repression of NF-κB-mediated signalling suggests other post-receptor signalling pathways, such as those downstream of TLR, that rely on this mediator may also be subject to modulation by TNIP1.

TLR can be separated into two categories based subcellular localization: spanning the cell plasma membrane (TLR1, 2, 4, 5, 6) or vesicle

Abbreviations: TLR, toll-like receptor; TNIP1, tumor necrosis factor (TNF) α-induced protein 3-interacting protein 1; JNK, Jun-amino-terminal kinase; ABIN-1, A20-binding inhibitor of NF-κB activation; NF-κB, nuclear factor of enhancer of immunoglobulin kappa light-chain of activated B cells

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membranes (TLR3, 7, 8, 9) [5]. Different TLR form either homo- (TLR3, 4, 5, 7, 8, 9, 10) or hetero-dimers (TLR1/2, 2/6). Epidermis-encountered ligands such as bacterial lipoprotein and viral dsRNA are recognized by TLR2/6 and TLR3/3 dimers, respectively [6–9]. Ligand binding leads to a cascade of protein recruitment to the cytoplasmic tail of the TLR and subsequent cytoplasmic signalling. This signal relay ultimately results in expression of inflammatory cytokines and chemokines [10] mediated by transcription factors including NF-κB and activator protein 1 (AP-1) [11]. Regulation of this membrane to cytoplasm to nucleus pathway is key to maintaining proper tissue homeostasis; its loss is associated with development of multiple autoimmune and/or hyper-inflammatory conditions. The preponderance of keratinocytes in the epidermis and their TLR expression makes these cells prime suspect responders and contributors in several cutaneous inflammatory disease states such as psoriasis [12–15]. Thus, there is increasing interest in signal modulators post-TLR, such as TNIP1 protein, that might dampen activation otherwise leading to immune and/or inflammatory hyper-responsiveness [13–16].

TNIP1 represses signalling initiated by several cell membrane and nuclear receptors ([2,17] for reviews). It is expressed across several different organs and tissues [18,19], including as we and others reported the stratifying keratinocytes of the epidermis [20–22]. Experimental increases of TNIP1 repress gene expression downstream of TNFα receptor (TNF-R), epidermal growth factor receptor (EGF-R), and TLR; its cell-specific or germline knockout lead to immune cell activation and widespread tissue inflammation [4,16,23–26]. However, somewhat unexpectedly, cultured TNIP1 −/− mouse fibroblasts have only very limited to no increased sensitivity to TNFα challenge as marked by no or very low fold induction of target genes such as cFLIP and iNOS [26]. In contrast, mouse germline replacement of endogenous TNIP1 with a mutant sequence altered to lose interaction with NEMO does lead to increased secretion of IL-6 and IL-12p40 from splenic B cells challenged in culture with several different TLR agonists [16]. Thus TNIP1 repressive control over cytoplasmic signalling following membrane receptor activation is likely to be dependent on cell type.

TNIP1 is repeatedly cited among very highly scoring genetic susceptibility loci [27–31] for psoriasis vulgaris as well as other autoimmune diseases [32–34]. As yet unresolved for psoriatic samples is TNIP1 protein at lower levels, but mRNA at higher levels, compared to normal epidermis [21,35]. We found that when TNIP1 protein levels are increased in keratinocytes there is a resultant decreased expression of several early response genes including IL-6, Jun, and Fos. Functional grouping of genes revealed those most-changed in expression were antiviral defense and stress response clusters [36]. We hypothesized that reduced TNIP1 protein levels, as seen in the psoriatic samples, might predispose keratinocytes to increased cytoplasmic signalling, especially downstream of challenge by TLR agonists. TLR ligands such as bacterial and viral products were chosen to model those likely encountered by epidermal keratinocytes. Investigation of gene expression changes was guided in part by the clustering of responsive genes seen upon increased TNIP1 expression [36]. The results presented here demonstrate a sensitization to TLR agonists following reduction of TNIP1 protein levels, indicating the importance of TNIP1 as a negative regulator of signalling post-TLR activation. This response appears to be occurring through activation of transcription factors, leading to a pro-inflammatory state evidenced by increased expression and secretion of cytokines and chemokines and related receptors. Keratinocyte TNIP1 deficiency leading to hyper-sensitivity to TLR bacterial- and viral-derived agonists may position these cells to be major contributors to the development of cutaneous hyper-inflammatory responses. Continued TLR agonism in TNIP1 deficient keratinocytes would establish these cells as important propagators of microbe-induced signalling possibly exacerbating cutaneous inflammatory conditions like psoriasis [37].

2. Materials and methods

2.1. Cell culture and treatments

HaCaT cells are a spontaneously immortalized, non-tumorigenic human keratinocyte cell line that retains gene induction and differentiation responses as found in primary keratinocytes [38–40]. HaCaT keratinocytes were cultured in a 3:1 Dulbecco's modified Eagle medium (DMEM)/F12 media supplemented with 10% fetal bovine serum (FBS) (ThermoScientific HyClone, Logan, UT) and antibiotics (100 units/mL penicillin G sodium, 100 μg/mL streptomycin sulfate) in a 5% CO_2, humidified 37 °C incubator.

For TLR ligand treatment, HaCaT keratinocytes were plated in triplicates at about 80% confluence. Forty-eight hours after plating, cells were treated with TLR ligands (InvivoGen, San Diego, CA) Pam3CysSerLys4 (Pam3CSK4), TLR1/2 agonist at 10, 100 and 1000 ng/mL; heat-killed *Listeria monocytogenes* (HKLM), TLR2/2 agonist at 10^6, 10^7 and 10^8 bacteria/mL; FSL-1, TLR2/6 agonist at 10, 100 and 1000 ng/mL; Poly (I:C), TLR3/3 agonist at 10, 100 and 1000 ng/mL for 6 h. Cells were collected in RNA lysis buffer (RNeasy kit, Qiagen, Hilden, Germany).

HaCaT keratinocytes were plated at 50–60% confluence in triplicate for each subsequent condition 24 h prior to transfection in a 6-well plate for protein- and a 12-well plate for RNA extraction. Keratinocytes were transfected overnight with 25 nM of individual SMARTpool TNIP1 or non-targeting siRNA (GE Dharmacon; Lafayette, CO) using DharmaFECT2 as per manufacturer's instructions. The next day a second transfection was performed and 48 h later keratinocytes were exposed to TLR ligands Poly (I:C) (1 μg/mL) or FSL-1 (100 ng/mL) for 0, 6, 12 or 24 h followed by extraction with 2× Laemmli sample buffer for protein or RNA lysis buffer for RNA.

2.2. RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)

Cells from triplicate wells were collected in RNA lysis buffer and RNA was extracted using RNeasy kit (Qiagen) according to the manufacturer's protocol. Reverse transcription was performed using a high capacity cDNA kit (Applied Biosystems, Foster City, CA). Primer pairs were obtained from Invitrogen (Waltham, MA) (Table 1) or designed in-house. The latter passed BLAST analysis for specificity and generated a single T_m value in melt-curve analysis indicative of producing a single product. Amplification was performed using an Applied Biosystems 7500 Fast Real-Time PCR System in a 10 μL reaction volume containing 2 μL diluted cDNA, Fast SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and 0.5 μM of each forward and reverse primer. Gene expression changes were quantified by the ΔΔCT method by normalizing to housekeeping gene ribosomal protein L13a (RPL13a) [36,41]. The cycling conditions were: an initial hold at 95 °C for 20 s followed by denaturing at 95 °C for 3 s, annealing and extension at 60 °C for 30 s. The denaturing, annealing, and extension stages were performed for 40 cycles.

2.3. Western blotting

For Western blot analysis, cell lysates were prepared in RIPA lysis buffer (10 mM Tris, 150 mM NaCl, 1% deoxycholic acid, 1% Triton and 0.1% sodium dodecyl sulfate) containing protease inhibitor cocktail (Roche Life Science, Indianapolis, IN) and protein phosphatase inhibitors (2 mM sodium orthovanadate and 50 mM sodium fluoride). Lysates, 45 μg, were electrophoretically resolved using 10% polyacrylamide gels and transferred onto a Whatman Protran® nitrocellulose membrane (GE Healthcare Life Sciences, Marlborough, MA). The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline with 0.05% Tween 20 (TBST) for 1 h and then incubated overnight at 4 °C with specific primary antibody as follows: JNK and