Consistent with this, thickening of the skin assessed by histology was significantly reduced in the C3−/− mice (Figure 2b and c). The altered skin pathology in the IMQ-treated C3-deficient mice appeared to affect mainly the epidermis. Untreated skin of WT and C3−/− mice was histologically indistinguishable.

In summary, we demonstrate that C3 is involved in the development and resolution of the psoriasiform skin inflammation induced by short-term treatment with IMQ. The proinflammatory effect of C3 is likely to be mediated by several mechanisms. In the absence of C3 the expression of psoriasis-relevant genes in the skin was impaired, neutrophil infiltration into the inflamed site was decreased, and IL-17 production by γδ T cells in the skin and the draining lymph nodes was reduced. Taken together, these data support a proinflammatory role of C3 during psoriasis-like skin inflammation.

CONFLICT OF INTEREST
The authors state no conflict of interest.

ACKNOWLEDGMENTS
We thank the staff of the Biological Services Unit at our institution for the care of the animals involved in this study. This work was supported by the Wellcome Trust (grant reference number 108008/ Z/15/Z). CG was supported by a Wellcome Trust Institutional Strategic Support Fund (ISSF) Inflammation Science PhD studentship and NB by a studentship from Majlis Amanah Rakyat (MARA).

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SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2016.11.011.

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Integrin-β4–TNS4–Focal Adhesion Kinase Signaling Mediates Keratinocyte Proliferation in Human Skin


TO THE EDITOR
TNS4, also known as CTEN, is a cytoplasmic scaffold protein containing Src homology 2 and phosphotyrosine-binding domains (Lo, 2014). TNS4 localizes to focal adhesions involved in integrin-mediated signaling, which plays an important role in cell adhesion, migration, and proliferation in a context-dependent manner (Duperret and Ridky, 2013). Although TNS4 was initially identified as a tumor suppressor, accumulating evidence suggests that TNS4 promotes tumorigenesis in many cancers (Lo, 2014). Although TNS4 is proposed as a prognostic
marker for melanoma (Sjoestroem et al., 2013), the expression and physiologic role of TNS4 in the normal skin remain poorly understood. All materials from humans used in this study were approved by the institutional review board at Seoul National University Hospital, and written informed consent was received from all subjects. The study was conducted according to the Declaration of Helsinki.

We investigated TNS4 expression and physiologic roles using primary human keratinocytes and normal skin tissues in vivo. Immunofluorescence staining of normal human skin showed that TNS4 was localized in the basal membrane side of basal keratinocytes (Figure 1a), implying that TNS4 may be involved in epidermal proliferation and/or differentiation. Consistently, TNS4 protein expression progressively decreased in keratinocytes differentiated by calcium treatment (Figure 1b), although differentiation per se was not affected by TNS4 inhibition (see Supplementary Figure S1 online). To investigate the role of TNS4 in epidermal proliferation, TNS4 was knocked down using small interfering RNA (siRNA) in normal human epidermal keratinocytes (NHEKs). TNS4 down-regulation induced a significant 40% decrease in cell numbers and significantly reduced the number of BrdU-positive proliferative cells (control scrambled siRNA vs. siTNS4 = 78% vs. 56%; Figure 1c and d). However, TNS4-silenced cells showed no significant increase in the levels of cleaved caspase 3 and cleaved PARP proteins compared with scrambled siRNA-treated cells, indicating that the reduced cell number was not associated with increased apoptosis (see Supplementary Figure S2 online). Conversely, TNS4 overexpression by adenovirus in NHEKs significantly increased the cell number compared with that from cells infected with a control vector (see Supplementary Figure S3a—c online).

Next, we explored the mechanism underlying TNS4-mediated keratinocyte proliferation. Activated focal adhesion kinase (FAK) acts in cell cycle progression and proliferation through phosphorylation (Cox et al., 2006). Additionally, tensin proteins, including TNS4, interact with phosphorylated FAK through the Src homology 2 domain (Muharram et al., 2014). Therefore, we examined whether FAK activation was affected by TNS4 silencing or overexpression. Compared with control siRNA, TNS4 silencing strongly reduced FAK Tyr397 phosphorylation (pY397FAK) (Figure 1e). The initial step of FAK phosphorylation is Tyr397 autophosphorylation, which leads to the phosphorylation of other Tyr residues to activate FAK (Cox et al., 2006). The target of activated FAK signaling in cell cycle progression is cyclin D1 through the extracellular signal-regulated kinase (ERK) pathway. (Cox
Consistent with previous literature (et al., 2006), TNS4 silencing decreased FAK activation and down-regulated both ERK phosphorylation and cyclin D1 expression in NHEKs (Figure 1e). Conversely, TNS4 overexpression upregulated pY397FAK, phosphorylated ERK, and cyclin D1 expression (see Supplementary Figure S3d). Next, we examined whether TNS4-mediated cell proliferation was suppressed by FAK or ERK inactivation. NHEKs were infected with an adenovirus expressing TNS4 and treated with FAK inhibitor 14 or ERK inhibitor (PD98059), which prevents FAK Tyr397 autophosphorylation or ERK phosphorylation, respectively. Both inhibitors significantly abrogated keratinocyte proliferation induced by TNS4 overexpression (Figure 1f), suggesting that TNS4-induced keratinocyte proliferation is mediated by FAK and ERK.

To investigate the upstream molecule that regulates TNS4 levels, TNS4 interaction with integrin-β1 (ITGB1) and -β4 (ITGB4), the major β integrins in keratinocytes, was assessed by immunoprecipitation (Grose et al., 2002). ITGB1 interacts with TNS4 to promote mammary epithelial cell migration (Katz et al., 2007). ITGB4, a laminin-332 receptor, is mainly localized in the basal layer and promotes cell cycle progression in keratinocytes through the ERK pathway (Mainiero et al., 1997; Nikolopoulos et al., 2005). TNS4 co-immunoprecipitated with ITGB4, but not with ITGB1, and co-localized with ITGB4 (see Supplementary Figure S4 online). ITGB4 activation by laminin-332 increased TNS4 protein expression levels and subsequent FAK-ERK signaling. Conversely, ITGB4 silencing decreased both TNS4 and FAK-ERK signaling (see Supplementary Figure S5 online). Moreover, activated ITGB4-ERK signaling is inhibited by TNS4 silencing, indicating that the known ITGB4-ERK signaling is mediated through TNS4 and FAK (Figure 2a). Akt pathway linked to ITGB4 for cell survival was also regulated by TNS4 (see Supplementary Figure S6 online). Moreover, endogenous ITGB4, TNS4, and FAK were co-immunoprecipitated after integrin activation in NHEKs (Figure 2b).

Finally, to evaluate the effects of TNS4 on cell proliferation in vivo, we injected green fluorescent protein (GFP) only or GFP-TNS4 overexpressing adenovirus...
TO THE EDITOR

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Common Delayed Senescence of Melanocytes from Multiple Primary Melanoma Patients


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