



# TNF- $\alpha$ and IL-1 $\beta$ Do Not Induce Langerhans Cell Migration by Inhibiting TGF $\beta$ Activation

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In the skin, Langerhans cells (LCs) require autocrine latent TGF $\beta$  that is transactivated by the integrins  $\alpha\nu\beta 6$  and  $\alpha\nu\beta 8$  expressed by keratinocytes (KCs) for long-term epidermal retention. Selective expression of a ligand-independent, constitutively active form of TGF $\beta$ R1 inhibits LC migration during homeostasis and in response to UVB exposure. In this study, we found that LC migration in response to inflammatory stimuli was also inhibited by ligand-independent TGF $\beta$ R1 signaling. Contrary to UVB stimulation, which reduced KC expression of  $\alpha\nu\beta 6$ , in vitro and in vivo exposure to TNF- $\alpha$  or IL-1 $\beta$  increased  $\alpha\nu\beta 6$  transcript and protein expression by KCs. This resulted in increased KC-mediated transactivation of latent TGF $\beta$ . Expression of  $\alpha\nu\beta 8$  was largely unchanged. These findings show that ligand-independent TGF $\beta$ R1 signaling in LCs can overcome inflammatory migration stimuli, but reduced KC-mediated transactivation of latent TGF $\beta$  by KCs may only drive LC migration during homeostasis and in response to UV stimulation.

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## INTRODUCTION

The skin serves as a physical barrier against a plethora of biologic and nonbiologic agents alike, including UV, chemical haptens, and commensal as well as pathogenic microbes. Keratinocytes (KCs) in the epidermis create a physical barrier but also form a niche for the cells of the immune system such as Langerhans cells (LCs), CD8<sup>+</sup> tissue-resident memory T cells, and dendritic epidermal gamma delta T cells. These leukocytes, along with KC, work in harmony to maintain barrier integrity and orchestrate effective immune responses (Kobayashi et al., 2019). Within the epidermis, LCs possess the unique ability to migrate from the skin epidermis to regional lymph nodes (LNs). At steady state, LCs migrate to LN transporting self and potentially commensal-derived antigen to promote the development of peripheral tolerance (Hemmi et al., 2001). During infection, LCs transport

potential pathogen-derived antigen and prime naïve adaptive immune responses (Igyártó et al., 2011; Kobayashi et al., 2015).

Inflammatory cytokines, including TNF- $\alpha$  and IL-1 $\beta$ , are well-known to trigger LC migration and are thought to directly interact with LCs (Cumberbatch et al., 1997; 1994). A direct effect on LCs is unlikely, at least for IL-1 $\beta$ , because LCs lacking *Myd88*, which is required for signaling through toll-like receptor and IL-1 receptor family members, migrate normally in response to IL-1 $\beta$  injection (Didovic et al., 2016; Haley et al., 2012). LC migration is also unaffected in response to *Candida albicans* infection and DNFB application and during homeostasis. Another key cytokine involved in LC migration is TGF $\beta$ 1 (TGF $\beta$ ). LC-specific genetic ablation of *Tgfb1*, *Tgfb1* (ALK5), or *Tgfb2* results in fully differentiated LCs that spontaneously migrate from the epidermis into regional LNs (Borkowski et al., 1996; Kaplan, 2017; Kaplan et al., 2007; Kel et al., 2010). On synthesis and secretion, TGF $\beta$  is bound to the latency associated peptide (LAP), resulting in inactive TGF $\beta$  (i.e., LAP–TGF $\beta$ 1) (Travis and Sheppard, 2014). Removal of the LAP protein from TGF $\beta$ 1 can be accomplished by low pH, proteases, mechanical stress, ROS, and integrin-mediated processes (Worthington et al., 2011). In the epidermis, activation of LAP–TGF $\beta$ 1 is accomplished through functionally active integrins  $\alpha\nu\beta 6$  and  $\alpha\nu\beta 8$  that are expressed on KC (Aluwihare et al., 2009; Yang et al., 2007). Integrin activity is positively regulated through the cytoplasmic adaptor proteins Talin1 (*Tln1*) and Kindlin1 (*Fermt1*) (Kerr and Byzova, 2018; Qin et al., 2004; Takada et al., 2007).

Epidermal KCs can be categorized on the basis of their relative spatial relationship to the hair follicles as inter-follicular (IFE), infundibulum/isthmus (IM), and bulge KCs (Nagao et al., 2012). At a steady state, LCs reside intercalated with IFE and IM KCs but are actively excluded from the bulge (Nagao et al., 2012). IFE KCs express integrin  $\alpha\nu\beta 6$  but not  $\alpha\nu\beta 8$ , and IM KCs express  $\alpha\nu\beta 8$  but not  $\alpha\nu\beta 6$  (Mohammed

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Abbreviations: DMBA, 7,12-dimethylbenz[a]anthracene; EpCAM, epithelial cell adhesion molecule; IFE, interfollicular; IM, infundibulum/isthmus; KC, keratinocyte; LAP, latency associated peptide; LC, Langerhans cell; LN, lymph node; MHC, major histocompatibility complex; pKC, primary keratinocyte

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et al., 2016). Integrin  $\beta 6$  (*Itgb6*) and  $\beta 8$  (*Itgb8*) form obligate heterodimers with integrin  $\alpha v$  (*Itgav*) to form functional integrin proteins (Kerr and Byzova, 2018; Takada et al., 2007). Genetic ablation of *Itgb6* results in loss of LCs from the IFE owing to increased spontaneous LC migration into regional LNs. Similarly, ablation of *Itgb8* results in loss of LCs from the IM. Notably, LC-specific expression of a constitutively active, ligand-independent form of the TGF $\beta$ R1 (TGF $\beta$ RCA) prevents spontaneous LC migration in *Itgb6*<sup>-/-</sup> mice. Epidermal exposure to UVB is sufficient to reduce KC expression integrin  $\alpha v\beta 6$  or  $\alpha v\beta 8$  and induce efficient LC migration that can be inhibited by LC-specific expression of TGF $\beta$ RCA (Mohammed et al., 2016). Taken together, these data support a model where autocrine LC-derived LAP–TGF $\beta$  is transactivated either by  $\alpha v\beta 6$  or  $\alpha v\beta 8$  expressed by KCs and then acts directly on LCs to prevent spontaneous migration. Moreover, the data suggest that reduced TGF $\beta$  transactivation after UVB irradiation is a trigger for LC migration. Whether a similar mechanism occurs with inflammatory stimuli remains unexplored.

In this study, we report that enforced TGF $\beta$  receptor signaling in LCs is sufficient to prevent inflammation-induced LC migration. UVB efficiently reduced the expression of  $\alpha v\beta 6$  and  $\alpha v\beta 8$  on primary KCs (pKCs), resulting in reduced TGF $\beta$  transactivation. In contrast, TNF- $\alpha$  and IL-1 $\beta$  increased  $\alpha v\beta 6$  expression in both pKCs and epidermal KCs, resulting in increased TGF $\beta$  transactivation. Thus, loss of TGF $\beta$  transactivation is associated with homeostatic and UVB-induced LC migration but not with inflammation-induced migration.

## RESULTS

### Enforced intrinsic TGF $\beta$ signaling is sufficient to prevent LC migration

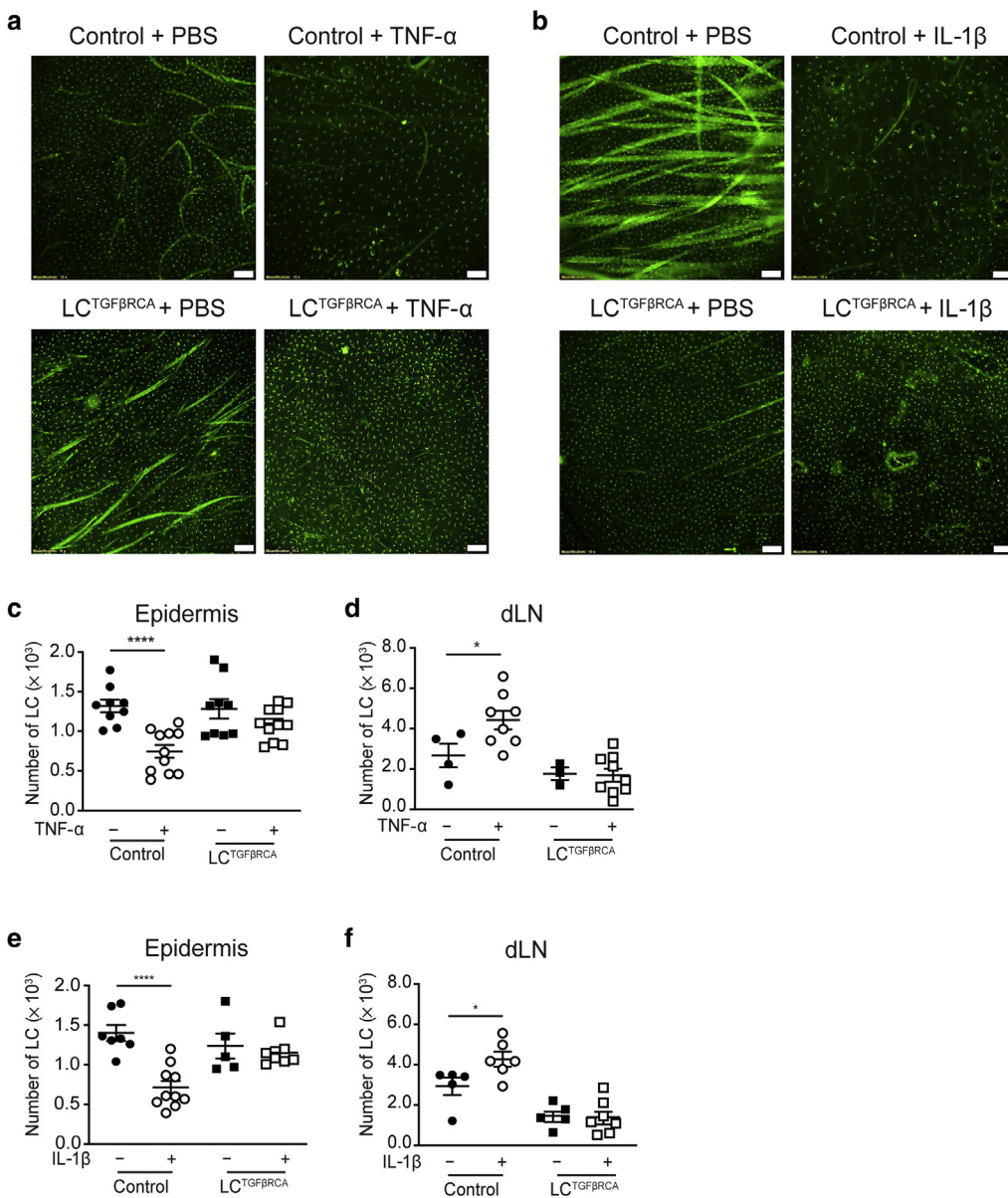
We previously reported that ligand-independent TGF $\beta$ R signaling in LCs was sufficient to prevent LC migration during homeostasis and in response to UVB (Mohammed et al., 2016). To determine whether LC migration in response to other inflammatory stimuli could also be overcome by ligand-independent TGF $\beta$ R signaling, we utilized our hulangerinCreER<sup>T2</sup>  $\times$  lox-stop-lox TGF $\beta$ RCA  $\times$  ROSA26.lox-stop-lox-YFP mice (LC<sup>TGF $\beta$ RCA</sup>). In these mice, tamoxifen treatment results in dual expression of YFP and a ligand-independent, constitutively active form TGF $\beta$ R1 selectively in LCs. To test whether LC migration in response to inflammatory cytokines was affected by enforced TGF $\beta$ R1 signaling, we treated LC<sup>TGF $\beta$ RCA</sup> and control LC<sup>YFP</sup> mice with an intraperitoneal injection of tamoxifen for 5 days, followed by intradermal injection with 100  $\mu$ g of TNF- $\alpha$  or PBS. The number of LCs in the epidermis at 72 hours after injection was determined by immunofluorescent microscopic evaluation of epidermal whole mounts. The number of LCs in the LNs was evaluated by flow cytometry gating LCs as CD11c<sup>+</sup>, major histocompatibility complex (MHC) II<sup>high</sup>, CD11b<sup>+</sup>, CD207<sup>+</sup>, CD103<sup>-</sup>. As expected, TNF- $\alpha$  induced an approximately 50% reduction in the number of epidermal LCs in control mice, but the numbers in LC<sup>TGF $\beta$ RCA</sup> mice were unaffected (Figure 1a and c). Consistent with the known capacity for TNF- $\alpha$  to induce LC migration, we observed increased numbers of LCs in the LNs of TNF- $\alpha$ -treated mice

compared with that in the LNs of PBS-treated control mice (Figure 1d). Notably, LC numbers in the LNs of LC<sup>TGF $\beta$ RCA</sup> mice were unaffected by TNF- $\alpha$  administration. We repeated the experiment with an intradermal injection of IL-1 $\beta$  (100  $\mu$ g) and observed a similar result (Figure 1b, e, and f). Thus, injection of either TNF- $\alpha$  or IL-1 $\beta$  is sufficient to induce LC migration from the epidermis to the draining LNs, and this migration can be inhibited by conditionally expressing a constitutively active form of TGF $\beta$ R in LCs.

Epicutaneous infection with *C. albicans* drives the migration of LCs and dermal LCs from the skin into the draining LNs. To test whether constitutive TGF $\beta$ R1 signaling can inhibit LC migration during *C. albicans* infection, we infected LC<sup>TGF $\beta$ RCA</sup> and control LC<sup>YFP</sup> mice. Although the visualization of epidermal LCs in epidermal whole mounts was technically not feasible during *C. albicans* infection, we did observe increased numbers of LCs in the draining LNs of the control mice 3 days after infection. LC numbers were not increased in LC<sup>TGF $\beta$ RCA</sup> mice, demonstrating an absence of efficient LC migration (Figure 2a). Notably, migration of cDC1 (CD11c<sup>+</sup>, MHC-II<sup>high</sup>, CD207<sup>+</sup>, CD103<sup>+</sup>, CD11b<sup>-</sup>), cDC2 (CD11c<sup>+</sup>, MHC-II<sup>high</sup>, CD207<sup>-</sup>, CD103<sup>-</sup>, CD11b<sup>+</sup>), and double negative DC (CD11c<sup>+</sup>, MHC-II<sup>high</sup>, CD207<sup>-</sup>, CD103<sup>-</sup>, CD11b<sup>-</sup>) were equivalent in both control and LC<sup>TGF $\beta$ RCA</sup> mice, thereby demonstrating that the failure to migrate in LC<sup>TGF $\beta$ RCA</sup> mice is selective to LCs (Figure 2b–d). Similar results also were obtained after a single epicutaneous application of 0.1 mM 7,12-dimethylbenz[*a*]anthracene (DMBA) (Figure 2e). In summary, these findings show that conditional expression of the constitutively active form of TGF $\beta$  receptor in LCs is sufficient to prevent both steady-state and inflammation-induced LC migration.

### Migratory stimuli increase integrin $\alpha v\beta 6$ and active TGF $\beta$ bioavailability in pKCs

We previously reported that exposure of KC to UVB reduced the expression of the TGF $\beta$ -activating integrins *Itgb6* and *Itgb8* in vivo and in vitro (Mohammed et al., 2016). Coupled with the observation that forced TGF $\beta$ R signaling prevented UVB-induced LC migration, we concluded that LC migration in response to UVB resulted from reduced epidermal bioavailability of TGF $\beta$ . To test whether inflammatory stimuli also suppress KC expression of *Itgb6* and *Itgb8*, we treated in vitro pKC cultures with UVB, TNF- $\alpha$ , IL-1 $\beta$ , and DMBA and evaluated mRNA expression by RT-qPCR 24 hours after treatment. As expected, the expression of *Itgb6* and *Itgb8* were reduced by UVB exposure (Figure 3a and b). Similarly, expression of transcripts for the integrin-associated proteins *Itgav* ( $\alpha v$ ), *Tln1* (Talin1), and *Fermt1* (Kindlin1) was reduced, suggesting a broad reduction in the pathway responsible for transactivating latent TGF $\beta$  (Figure 3c–e). Contrary to our expectations, we observed that pKC cultures incubated with TNF- $\alpha$ , IL-1 $\beta$ , or DMBA consistently increased the expression of *Itgb6* (Figure 3a). Expression of *Itgav*, *Tln1*, and *Fermt1* remained relatively unchanged, and expression of *Itgb8* was reduced only with DMBA treatment (Figure 3b–e). Analysis of protein expression by flow cytometry revealed reduced expression of  $\alpha v\beta 6$  after UVB treatment and modest but statistically significant increased expression after TNF- $\alpha$  and DMBA treatment (Figure 3f). Expression of  $\alpha v\beta 8$  was largely



**Figure 1. Enforce intrinsic TGF $\beta$  signaling is sufficient to prevent TNF- $\alpha$ - or IL-1 $\beta$ -induced LC migration.** LC<sup>TGF $\beta$ BRCA</sup> or control LC<sup>YFP</sup> mice received five-daily doses of tamoxifen (0.05 mg/g) to induce constitutive TGF $\beta$  signaling in LCs. The ear pinnae of these mice were subject to intradermal injection of either PBS, TNF- $\alpha$ , or IL-1 $\beta$  (100 ng). (a, b) Representative immunofluorescence and (c, e) summary data of epidermal sheets stained for MHCII (green) 72 hours after treatment with TNF- $\alpha$  or IL-1 $\beta$ ; autofluorescence shows hair shafts. (d, f) The absolute number of LCs in dLNs after TNF- $\alpha$  or IL-1 $\beta$  treatment. The number of LCs in the LN was evaluated by flow cytometry gating LCs as CD11c<sup>+</sup>, MHC-II<sup>high</sup>, CD11b<sup>+</sup>, CD207<sup>+</sup>, CD103<sup>-</sup>. Data are representative of (d, f) two or at least (a–c, e) three separate experiments. Each symbol represents data from an individual mouse. \* $P < 0.05$  and \*\*\*\* $P < 0.0001$ . Bar = 100  $\mu$ m. dLN, draining lymph node; LC, Langerhans cell; LN, lymph node; MHC, major histocompatibility complex.

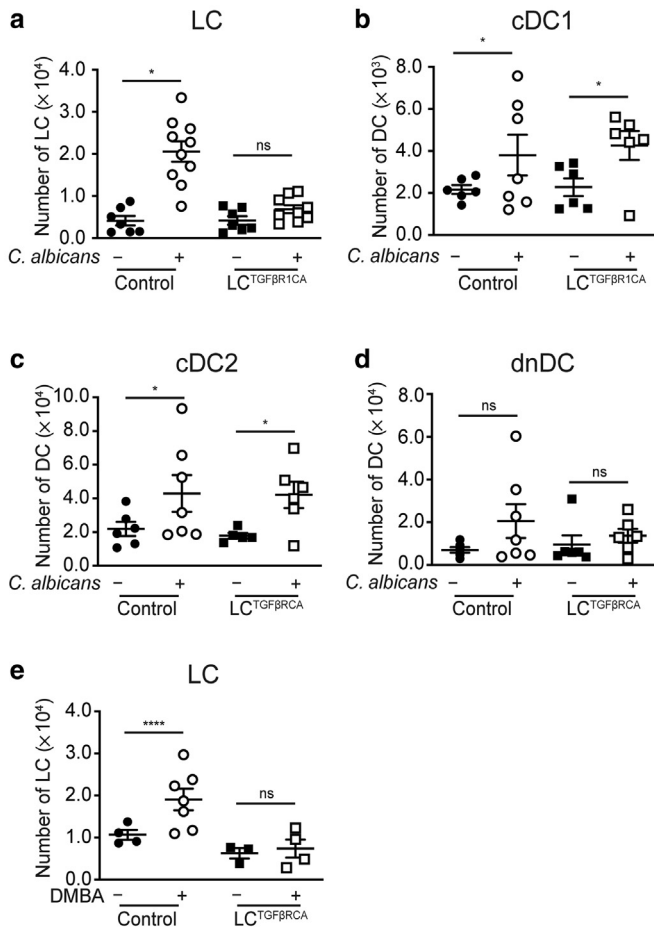
unchanged, with only a minor decrease after DMBA treatment (Figure 3g). Finally, we tested the capacity of pKCs to activate latent TGF $\beta$  by incubating UVB-, TNF- $\alpha$ -, IL-1 $\beta$ -, and DMBA-treated pKCs with a reporter cell line that expresses luciferase when autocrine TGF $\beta$  is transactivated by treated cells. Consistent with the expression data, pKCs treated with UVB showed reduced capacity to transactivate TGF $\beta$ , whereas pKCs treated with TNF- $\alpha$ , IL-1 $\beta$ , or DMBA showed enhanced TGF $\beta$  transactivation (Figure 3h). From these data, we conclude that the three inflammatory stimuli we have examined increase the expression of integrin  $\alpha$ v $\beta$ 6 and the capacity of in vitro pKCs to transactivate TGF $\beta$ .

#### KC expression of *Itgb6* is increased by TNF- $\alpha$ in vivo

We have previously shown that subsets of KCs express different amounts of *Itgb6* and *Itgb8*. In the steady state, IFE KCs primarily express *Itgb6* and not *Itgb8*, IM KCs primarily express *Itgb8* and not *Itgb6*, and bulge KCs express both

(Mohammed et al., 2016). We noted that pKCs expressed high levels of both *Itgb6* and *Itgb8*, suggesting that pKCs may be more representative of bulge KCs rather than IFE or IM KCs. Because LCs are actively excluded from the bulge and reside in the IFE and IM (Nagao et al., 2012), we next examined the expression of *Itgb6* and *Itgb8* in IFE and IM KCs by RT-qPCR. Wild-type mice were injected intradermally with 100  $\mu$ g of TNF- $\alpha$  or PBS on flank skin. After 4 or 24 hours, single-cell epidermal suspensions were FACS sorted as IFE (CD45.2<sup>-</sup>, CD207<sup>-</sup>, MHCII<sup>-</sup>, CD34<sup>-</sup>, epithelial cell adhesion molecule [EpCAM]<sup>-</sup>, Sca1<sup>+</sup>) or IM (CD45.2<sup>-</sup>, CD207<sup>-</sup>, MHCII<sup>-</sup>, CD34<sup>-</sup>, EpCAM<sup>+</sup>, Sca1<sup>-</sup>). As expected, IFE KCs expressed higher levels of *Itgb6* than IM KCs (Figure 4a). Administration of TNF- $\alpha$  transiently increased the expression of *Itgb6* at 4 hours that returned to baseline level by 24 hours in IFE KCs. Somewhat unexpectedly, expression of *Itgb6* also increased in IM KCs with similar kinetics. Expression of *Itgb8* was limited to IM KCs in PBS-injected mice (Figure 4b).





**Figure 2. Migration of dermal DCs is unaffected in TGFβRCA<sup>LC</sup> mice.** LC<sup>TGFβRCA</sup> or control LC<sup>YFP</sup> mice were treated with an intraperitoneal injection of tamoxifen as described earlier. Flank skin of mice was then treated with either epicutaneous *Candida albicans* or DMBA and PBS or vehicle controls, respectively. (a, e) Inguinal LCs were identified by flow cytometry by gating as CD11c<sup>+</sup>, MHC-II<sup>high</sup>, CD11b<sup>+</sup>, CD207<sup>+</sup>, CD103<sup>-</sup>. Different cDC subsets from *C. albicans*-infected mice were detected by gating for cDC1 as CD11c<sup>+</sup>, MHC-II<sup>high</sup>, CD207<sup>+</sup>, CD103<sup>+</sup>, and CD11b<sup>-</sup>; cDC2 as CD11c<sup>+</sup>, MHC-II<sup>high</sup>, CD207<sup>-</sup>, CD103<sup>-</sup>, and CD11b<sup>+</sup>; and dnDC as CD11c<sup>+</sup>, MHC-II<sup>high</sup>, CD207<sup>-</sup>, CD103<sup>-</sup>, and CD11b<sup>-</sup>. (a–e) Data are representative of two separate experiments. Each symbol represents data from an individual mouse. \**P* < 0.05 and \*\*\*\**P* < 0.0001. cDC, cutaneous dendritic cell; DC, dendritic cell; DMBA, 7,12-dimethylbenz[*a*]anthracene; dnDC, double negative dendritic cell; LC, Langerhans cell; MHC, major histocompatibility complex; ns, not significant.

Administration of TNF-α resulted in a transient nonsignificant reduction of *Itgb8* expression in IM KCs that returned to baseline level by 24 hours. Because the expression of *Itgb8* only trended downward in TNF-α-treated IM KCs, we examined whether TNF-α might preferentially drive the migration of LCs out of the follicular ostia. TNF-α was administered intradermally, and flank skin was harvested 48 hours later to visualize LCs by immunofluorescence. Follicular isthmus and LCs were identified by staining with α-EpCAM and α-Langerin, respectively. We observed equivalent LC migration from both the IFE and the IM (Figure 4c). Taken together, these data show that TNF-α transiently increased the expression of *Itgb6* mRNA in IFE and IM KCs in vivo, whereas the expression of *Itgb8* remained largely unchanged.

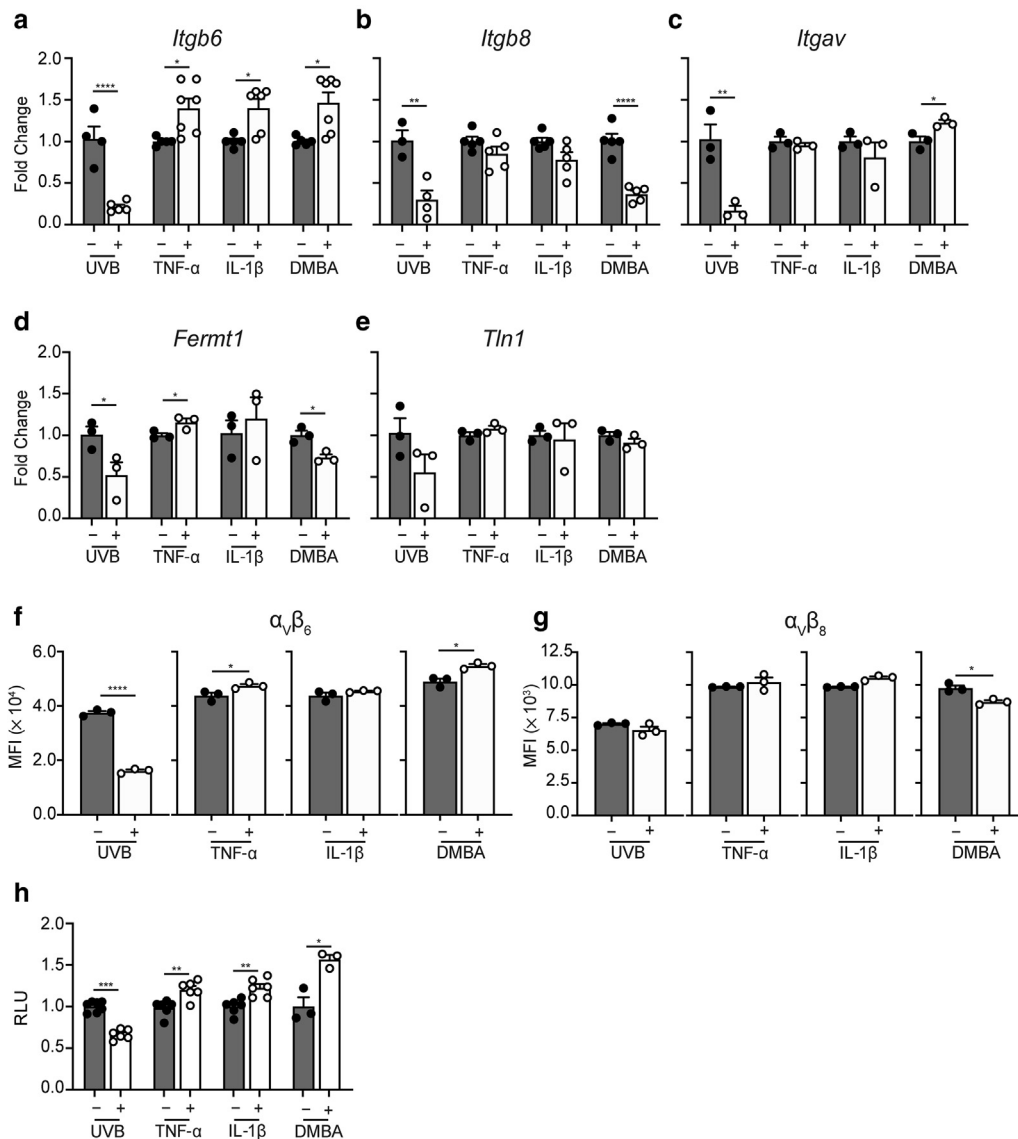
**TNF-α increases epidermal TGFβ bioavailability through integrin αvβ6**

To confirm our finding that *Itgb6* mRNA is increased in TNF-α treated IFE and IM KCs, we examined the surface expression of integrins αvβ6 and αvβ8 proteins by flow cytometry using recently developed mAbs (Takasaka et al., 2018; Weinreb et al., 2004). In PBS-injected mice, we observed modest expression of αvβ6 by IFE KCs that was less evident in IM KCs (Figure 5a and b). Staining of KCs isolated from *Itgb6*<sup>-/-</sup> *Itgb8*<sup>ΔKC</sup> mice was included as a specificity control. Treatment with TNF-α increased the expression of αvβ6 in IFE KCs at 12 hours, which largely persisted at 24 hours. IM KCs also increased the expression of αvβ6, although less robustly than IFE KCs. Expression of αvβ8 was evident in PBS-treated IM KCs and modestly increased after TNF-α administration. We then tested whether the increased expression of αvβ6 on KCs resulted in increased transactivation of TGFβ. TNF-α or PBS was administered intradermally to wild-type mice, and bulk epidermal cells were isolated 12 hours later and incubated in vitro with a reporter cell line for active TGFβ, as described earlier. Epidermal cells from TNF-α-treated skin showed an enhanced capacity to transactivate latent TGFβ compared with epidermal cells from PBS-treated skin (Figure 5c). Finally, to determine whether increased expression of integrin β6 is sufficient to increase the surface expression of αvβ6 and increase TGFβ activation, we overexpressed integrin β6 in a cell line generated from immortalized primary murine KCs. Cells were electroporated with either an empty vector or a vector containing *Itgb6* under the control of the cytomegalovirus promoter (β6), followed by antibiotic selection and cloning by limited dilution. The surface expression of integrin αvβ6 was clearly increased in KCs transfected with *Itgb6* compared with that transfected with the empty vector (Figure 5d). As expected, KCs transfected with *Itgb6* showed increased activation of latent TGFβ (Figure 5d). Thus, increased integrin β6 expression is sufficient to increase TGFβ activation. These data show that overexpression of integrin β6 in KCs or administration of TNF-α in the skin drives increased expression of αvβ6 and αvβ8 and that this is sufficient to increase the epidermal bioavailability of active TGFβ.

**DISCUSSION**

In this study, we have shown that enforced TGFβR signaling in LCs is sufficient to prevent inflammation-induced LC migration. This extends our previous findings that homeostatic and UVB-induced LC migration can be inhibited by TGFβRCA expression. We also confirmed our previous observation that UVB reduced the expression of αvβ6 and αvβ8 in pKCs, resulting in reduced TGFβ transactivation. However, contrary to our expectations, TNF-α and IL-1β did not decrease but rather increased *Itgb6* and αvβ6 expression in both pKCs and epidermal KCs. This was associated with an increased KC-mediated TGFβ transactivation. Thus, loss of TGFβ transactivation by KCs is associated with homeostatic and UVB-induced LC migration but not with inflammation-induced migration.

We had expected that loss of TGFβ signaling through the suppression of KC-mediated transactivation would be a



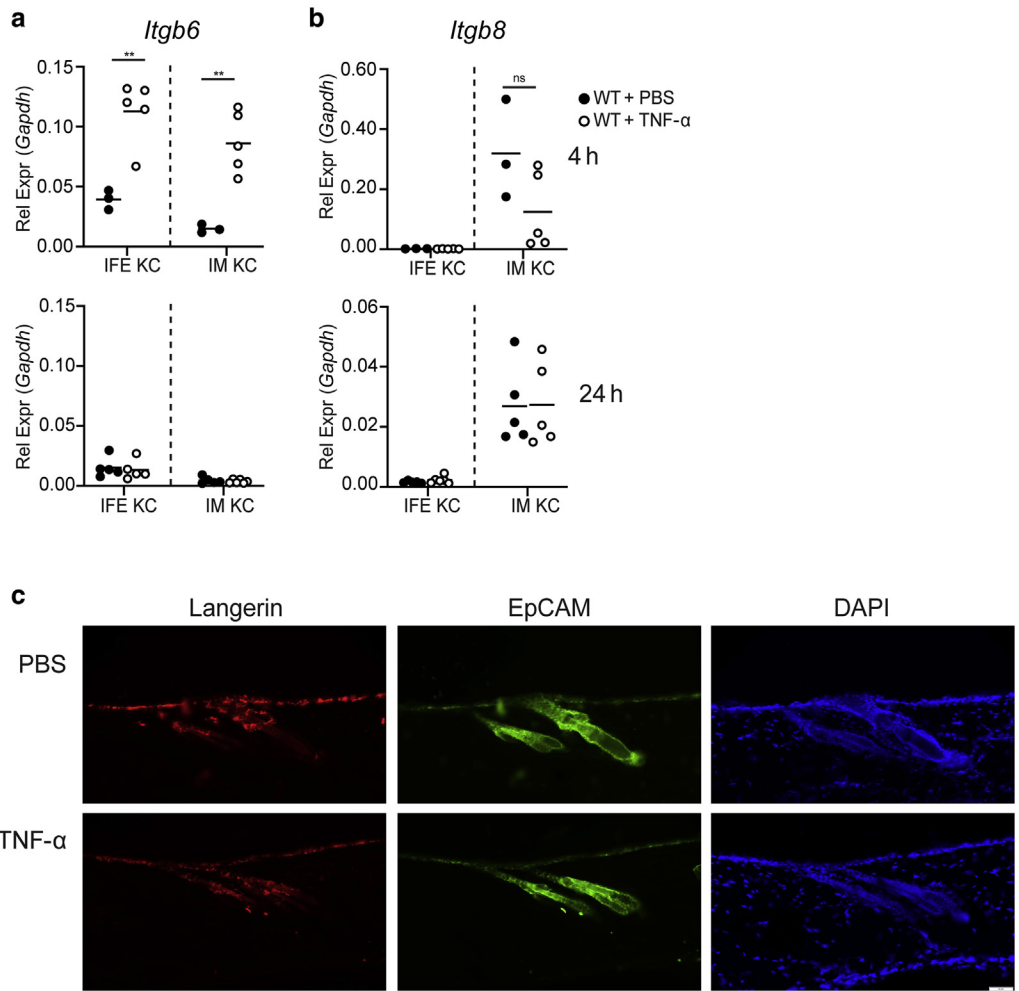
**Figure 3. Migratory stimuli increase the ability of pKCs to convert latent TGF $\beta$ .** pKCs were treated with 100 ngs of TNF- $\alpha$  and IL-1 $\beta$  or 0.1 mM DMBA for 24 hours or 20 mj UVB. (a–e) RT-qPCR analysis of *Itgb6*, *Itgb8*, *Itgav*, *Fermt1*, and *Tln1* is shown. (f, g) Flow cytometry analysis of the expression of integrins  $\alpha_v\beta_6$  and  $\alpha_v\beta_8$  on treated pKCs. Luciferase activity in tMLEC TGF $\beta$  reporter cells cocultured with pKCs pretreated with the indicated stimulus is shown. (h) Data are presented as RLU, normalized to control cells; PBS or vehicle-treated pKCs. Data are representative of (a–e) two independent experiments or (h) three independent experiments. (a–h) Each symbol represents data from an individual mouse. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , and \*\*\*\* $P < 0.0001$ . MFI, mean fluorescence intensity. DMBA, 7,12-dimethylbenz [a]anthracene; pKC, primary keratinocyte; RLU, relative light unit; tMLEC, thymic mink lung epithelial reporter cell.

fundamental component in LC migration. The observation that forced expression of TGF $\beta$ RCA can prevent LC migration indicates that high levels of TGF $\beta$ R signaling can overcome a wide variety of migratory signals. The fact that we did not observe any reduction in KC-mediated TGF $\beta$  transactivation in response to TNF- $\alpha$  or IL-1 $\beta$  indicates that these signals do not induce LC migration through an extrinsic loss of TGF $\beta$  availability. TNF- $\alpha$ -induced LC migration is inhibited by global ablation of TNF receptor 2 (p50) but not of TNF receptor 1 (p75) (Wang et al., 1997; 1996). In the epidermis, TNF receptor 2 is believed to be preferentially expressed by LCs (Eaton et al., 2015; Luo et al., 2006; Yang et al., 2018). Thus, TNF- $\alpha$  could likely induce migration by acting directly on LCs. In contrast, LC migration in response to IL-1 $\beta$  is not affected by the loss of *Myd88*, which is a required component of IL-1 $\beta$  receptor signaling (Didovic et al., 2016; Haley et al., 2012). Thus, IL-1 $\beta$  likely triggers LC migration through an LC-extrinsic mechanism that is independent of reduced TGF $\beta$  transactivation. The functional importance of increased  $\alpha_v\beta_6$

expression in response to TNF- $\alpha$  and IL-1 $\beta$  remains unclear. TGF $\beta$  is required for differentiation of resident memory T cells and could participate in the development of monocyte-derived LCs that are recruited into the skin by inflammation (Ferrer et al., 2019; Mackay et al., 2013). We speculate that increased  $\alpha_v\beta_6$  expression may be required for optimal recruitment and differentiation of these cell types in an inflammatory context.

It is interesting to note that the two conditions where the loss of KC-mediated TGF $\beta$  transactivation triggers LC migration (i.e., homeostasis and UVB) are both associated with the induction of peripheral tolerance (Mutymbizi et al., 2009; Shklovskaya et al., 2011; Yoshiki et al., 2010). This is consistent with the observation that those LCs that have migrated into regional LNs in response to an artificial loss of autocrine TGF $\beta$  maintain an immature activation state (Bobr et al., 2012). Explorations of the genomic state of LCs after different types of migratory stimuli represent an exciting future avenue to explore this question.

**Figure 4. TNF-α does not suppress integrin *Itgb6* and *Itgb8* mRNA expression by KC in vivo.** RT-qPCR analysis of *Itgb6* and *Itgb8* mRNA from wild-type mouse sorted epidermal cells that had been treated with an intradermal injection. (a, b) TNF-α for 4 or 24 h. IFE KCs were gated as CD45.2<sup>-</sup>, CD207<sup>-</sup>, MHCII<sup>-</sup>, CD34<sup>-</sup>, EpCAM<sup>-</sup>, and Sca1<sup>+</sup> and IM KCs as CD45.2<sup>+</sup>, CD207<sup>+</sup>, MHCII<sup>+</sup>, CD34<sup>+</sup>, EpCAM<sup>+</sup>, and Sca1<sup>-</sup>; (a, b) results are presented relative to *Gapdh*. (c) Microscopy of flank skin transverse sections from WT mice for 72 h after intradermal injection. TNF-α (100 ng) or PBS stained with Langerin (red), EpCAM (cyan), and DAPI. Data are representative of (a, b) two independent experiments or (c) three independent experiments. Bar = 50 μm. (a, b) Each symbol represents data from an individual mouse. \*\**P* < 0.01. EpCAM, epithelial cell adhesion molecule; h, hour; IFE, interfollicular; IM, infundibulum/isthmus; KC, keratinocyte; MHC, major histocompatibility complex; ns, not significant; Rel Expr, relative expression; WT, wild type.



**MATERIALS AND METHODS**

**Mice**

HuLangerin-CreER<sup>T2</sup> and TGFβRCA mice have been previously described (Bartholin et al., 2008; Bobr et al., 2012). *Itgb6*<sup>-/-</sup> and *Itgb8*<sup>loxP</sup> mice were kindly provided by D. Sheppard (University of California, San Francisco). HuLangerin-CreER<sup>T2</sup> mice were bred with TGFβRCA and ROSA26.LSL.YFP (Jackson Laboratory, Bar Harbor, ME) reporter mice, resulting in TGFβRCA<sup>LC</sup> mice (Mohammed et al., 2016). C57BL/6 (wild type) and Tg(KRT14-cre)1Amc/J (K14-Cre) mice were purchased from Jackson Laboratory. We crossed K14-Cre mice with *Itgb8*<sup>loxP</sup> and *Itgb6*<sup>-/-</sup> mice to obtain *Itgb6*<sup>-/-</sup> and *Itgb8*<sup>ΔKC</sup> mice (Mohammed et al., 2016). We used age- and sex-matched mice that were aged between 6 and 12 weeks in all experiments. All mice were maintained under specific pathogen-free conditions, and all mouse experiments were approved by the University of Pittsburgh Institutional Animal Care and Use Committee (Pittsburgh, PA).

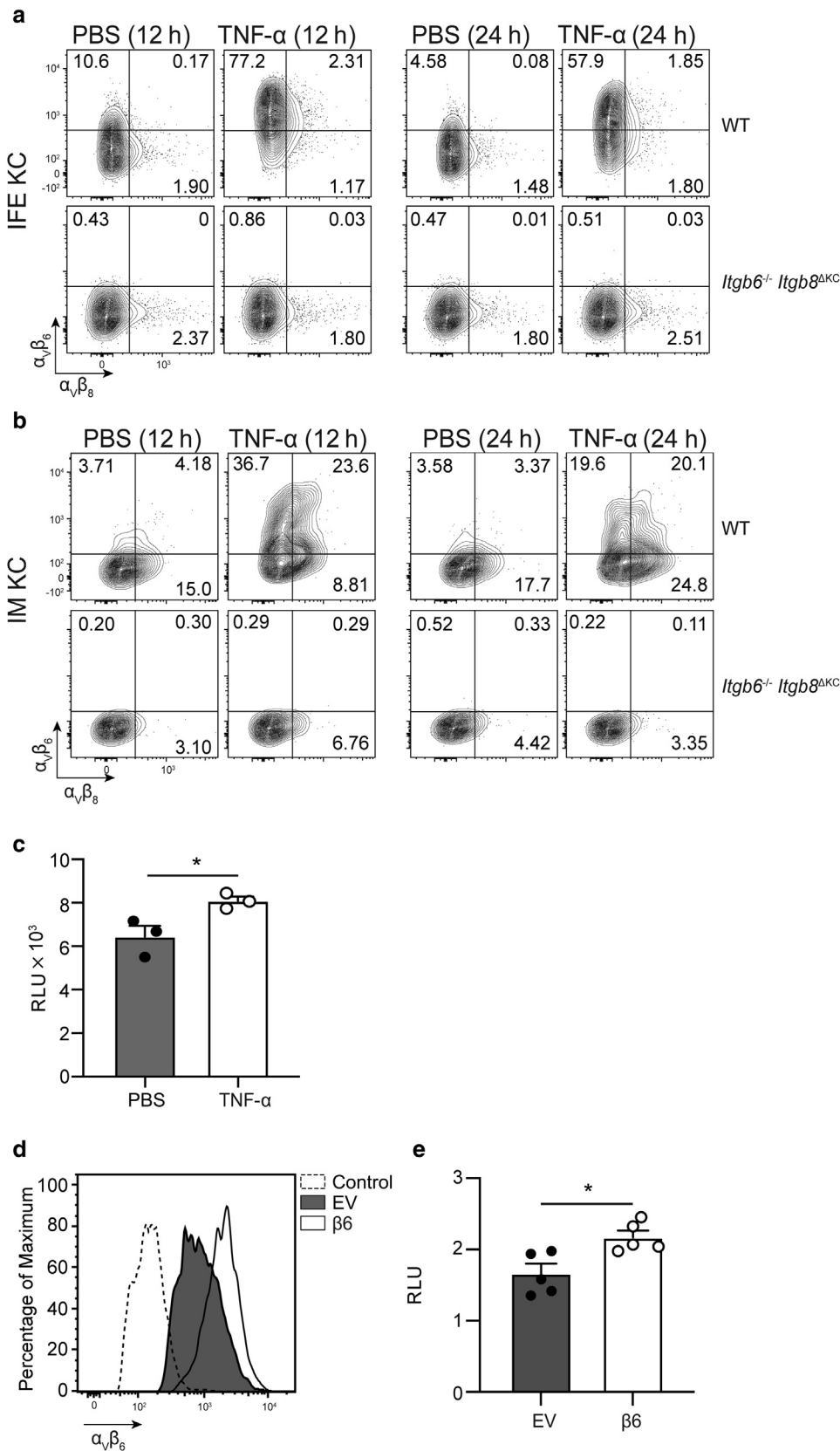
**Reagents and treatments**

TNF-α (315-01A) or IL-1β (211-11B) recombinant mouse peptides were purchased from PeproTech (Rocky Hills, NJ) and were resuspended in PBS. For UVB experiments, we used two TL 20W/12RS lamps (Philips, Amsterdam, The Netherlands). We used UVB doses previously described as 20 mJ/cm<sup>2</sup> for pKCs or experiments, (Mohammed et al., 2016).

DMBA (57-97-6; Sigma-Aldrich, St. Louis, MI) was applied at 0.1mM in DMSO for pKC experiments. For in vivo mouse experiments, 10 mM of DMBA was resuspended in DMSO-to-ethanol-to-glycerol solution (1:1:3) and was applied to the flank skin of shaven mice. Antibodies directly conjugated to different fluorophores were used for flow cytometry and immunofluorescence. Anti-CD11c(N418)-PerCp5.5, CD11b(M1/70)-PeCy7, I-A/I-E/MHCII (M5/114.15.2)-AF700, Langerin(4c7)-phycoerythrin, CD103(2E7)-AF647, CD45.2(104)-BV605, Sca-1(E13-161.7)-PerCp5.5, EpCAM(G8.8)-PeCy7, and CD34(HM34)-PE/Dazzle594 were purchased from BioLegend (San Diego, CA). Viability Dye eFluor 780 (eBioscience; Invitrogen, Carlsbad, CA) was used for live-dead discrimination. Anti-αvβ6 (6.3g9) and anti-αvβ8 (C6D4)-phycoerythrin were kindly provided by Dean Sheppard and Stephen Nishimura, respectively. Anti-αvβ6 (6.3g9) was directly conjugated to Alexa Fluor 647 (A20186; Thermo Fischer Scientific, Waltham, MA).

**Tamoxifen treatment**

Tamoxifen (T5648; Sigma-Aldrich) was dissolved in 1/10 volume of 200 proof ethanol with repeated incubations at 55 °C (15–30 seconds) and vortexing. The tamoxifen-ethanol mixture was diluted with corn oil (C8267; Sigma-Aldrich) to a final concentration of 10 mg/ml. Before treatments, TGFβRCA<sup>LC</sup> mice received 5 consecutive days of intraperitoneal injection of tamoxifen at 0.05 mg/g of mouse weight.



**Figure 5. TNF- $\alpha$  increases the surface expression of integrins  $\alpha v\beta 6$  and  $\alpha v\beta 8$  on epidermal KCs.**

Representative flow plots of integrins  $\alpha v\beta 6$  and  $\alpha v\beta 8$  on (a) IFE and (b) IM KC populations from WT or *Itgb6*<sup>-/-</sup> *Itgb8*<sup>ΔKC</sup> mice that had received the intradermal injection. TNF- $\alpha$  (100 ng) for 12 or 24 h; (a) IFE KCs were gated as CD45.2<sup>-</sup>, CD207<sup>-</sup>, MHCII<sup>-</sup>, CD34<sup>-</sup>, EpCAM<sup>-</sup>, and Sca1<sup>+</sup>, and (b) IM KCs were gated as CD45.2<sup>-</sup>, CD207<sup>-</sup>, MHCII<sup>-</sup>, CD34<sup>-</sup>, EpCAM<sup>+</sup>, and Sca1<sup>-</sup>. Luciferase activity in tMLEC TGF $\beta$  reporter cells cocultured with bulk epidermal cells from WT mice was harvested 24 h after in vivo intradermal injection of TNF- $\alpha$  (100 ng); (c) data are presented as RLU. Integrin  $\alpha v\beta 6$  expression in a KC cell line transfected with an EV or with an integrin  $\beta 6$  plasmid construct ( $\beta 6$ ). (d) Control-indicated EV cells stained with FMO. Luciferase activity in tMLEC TGF $\beta$  reporter cells cocultured with either EV or  $\beta 6$  transfected KC cell line. (e) Flow cytometry analysis of  $\alpha v\beta 6$  expression on KC cells that been transfected with either EV or  $\beta 6$  vector. Data are presented as RLU, normalized to control cells. (a, b, e) Data are representative of two independent experiments with a cohort size of 3–4. (c) Each symbol represents data from an individual mouse. \**P* < 0.05. EpCAM, epithelial cell adhesion molecule; EV, empty plasmid construct; h, hour; FMO, fluorescence minus 1; IFE, interfollicular; IM, infundibulum/ isthmus; KC, keratinocyte; MHC, major histocompatibility complex; RLU, relative light unit; tMLEC, thymic mink lung epithelial reporter cell; WT, wild type.

### Immunofluorescence and imaging

Epidermal sheets were prepared as previously described (Mohammed et al., 2016). Briefly, skin fat was mechanically

removed and subsequently mounted on microscopy slides that had been precoated with double-sided adhesive tape (3M, St. Paul, MN). Slides were incubated in 10 mM EDTA at 37 °C for 45–90 minutes.



The dermis was peeled away from the epidermis with standard forceps. Skin-whole mounts were prepared from skin samples embedded in optimal cutting temperature compound, and 8  $\mu$ m transverse skin slice sections were prepared. Epidermal sheets and skin-whole mounts were fixed in 4% paraformaldehyde at room temperature for 30 minutes and blocked for 1 hour at room temperature in PBS buffer containing 0.1% tween-20, 2% BSA, and 2% rat serum. Immunostaining of skin samples was done overnight in PBS containing 0.1% tween-20 and 0.5% BSA. Skin samples were stained with anti-EpCAM(G8.8)-AF647, anti-A1-E/MHCII-AF488, and anti-Langerin(CD207)-phycoerythrin, followed by anti-phycoerythrin-AF555 and DAPI. Images were captured on an IX83 fluorescent microscope (Olympus, Tokyo, Japan) using a  $\times$ 10 objective; image analysis was performed using cellSens Dimension software (Olympus).

### Flow cytometry

Single-cell suspension from tissues was prepared as previously described (Mohammed et al., 2016). Epidermal single-cell suspensions were prepared from shaved mouse skin that was incubated for 2 hours at 37 °C in 0.3% in 150 mM sodium chloride, 0.5 mM potassium chloride, and 0.5 mM glucose. The skin was minced finely with scissors and resuspended in RPMI 1640 media (Gibco, Grand Island, NY) containing 2.5 mg/ml Collagenase XI (Sigma-Aldrich), 0.1 mg/ml DNase (Sigma-Aldrich), 0.01 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Sigma-Aldrich), and 10% fetal bovine serum, followed by incubation in a shaking incubator for 30 minutes at 37 °C. The resulting cell mesh was filtered through a 40- $\mu$ m cell strainer (BD Biosciences, San Jose, CA). LNs (axillary and inguinal) were incubated in 400 U/ml Collagenase D (Roche Applied Science, Penzberg, Germany) and 0.1 mg/ml DNase in RPMI 1640 with 10% fetal bovine serum for 40 minutes at 37 °C and then minced through a 40- $\mu$ m cell strainer. Single-cell suspensions were blocked with 2.4G2 culture supernatant (ATCC, Manassas, VA). Surface staining was performed in standard FACS buffer for 30 minutes at 4 °C. For intracellular cytokine staining of Langerin (CD207), cells were first fixed and permeabilized with Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer's instructions. LSRFortessa flow cytometers (Becton Dickinson, Franklin Lakes, NJ) and Flowjo software (TreeStar, Ashland, OR) were used for analysis. Epidermal KC subsets were sorted on FACSAria cell sorter (BD Biosciences) and gated as IFE KCs: CD45.2<sup>+</sup>, Sca1<sup>+</sup>, CD34<sup>+</sup>, and EpCAM<sup>-</sup> and IM KCs: CD45.2<sup>-</sup>, Sca1<sup>-</sup>, CD34<sup>-</sup>, and EpCAM<sup>+</sup>.

### RT-qPCR

Total RNA from flow cytometry-sorted epidermal cells and pKC cultures were extracted with Thermo Fischer's Trizol-LS (10296028, Carlsbad, CA) or RNeasy Mini extraction kit (Qiagen, Hilden, Germany) following the manufacturer's instructions and was quantified using Nanodrop (NanoDrop Technologies, Wilmington, DE). cDNA was generated using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) and was subjected to RT-qPCR using TaqMan Gene Expression Master Mix and TaqMan Gene Expression Assays for *Gapdh*, *Itgb6*, *Itgb8*, *Itgav*, *Fermt1* (Kindlin1), and *Tln1*.

### Cell culture

Newborn mice (aged 1–4 days) were used to culture pKCs as previously described (Dlugosz et al., 1995; Mohammed et al., 2016). TMLECs that had been previously transfected with a plasmid

containing the luciferase cDNA downstream of a TGF $\beta$ -responsive portion of the PAI-1 promoter were cultured as originally described (Abe et al., 1994).

### KC transfection

We have generated a stable KC cell line from pKCs by spontaneous immortalization. These cells were grown at 37 °C, at 5% carbon dioxide, and in EMEM media complemented with 8% chelex serum and 1% penicillin/streptomycin for 8 weeks, followed by limited dilution cloning. The resulting cell line was transfected with a pCDNA3.1+/C-(K)-DYK-ITGB6 ( $\beta$ 6) plasmid construct or with an empty pCDNA3.1+/C-(K)-DYK (empty vector) that were purchased from GenScript Biotech (Piscataway, NJ). Transfections were performed in 4-mm-gap cuvettes using a BTX ECM 830 square wave electroporator. Transfection was accomplished by a single pulse of 300 volts and 10 ms, with 5–10  $\mu$ g of plasmid DNA per  $1.0 \times 10^6$  cells to  $2.0 \times 10^6$  cells. Four days after transfection, selection reagent G418 (0.1–2.5 mg/ml; Sigma-Aldrich) was added to the medium to select the stably transfected cells for 15 days, with the medium being refreshed every other day. The cells were cloned by limiting dilution.

### TGF $\beta$ -activation reporter assay

The ability of KCs to transactivate TGF $\beta$  was determined by coculture of KCs with thymic mink lung epithelial reporter cells as previously described (Mohammed et al., 2016). Briefly, thymic mink lung epithelial reporter cells were grown in DMEM media containing 10% fetal calf serum and were plated at  $2.0 \times 10^4$  cells per well in a 96-well cell culture-treated plate for 3 hours at 37 °C and 5% carbon dioxide. TNF- $\alpha$ , IL-1 $\beta$ , UVB, DMBA, or nontreated KCs were harvested with 0.25% trypsin and 2.21 mM EDTA (25 053-Cl; Corning, Corning, NY) for 5 minutes at 37 °C and 5% carbon dioxide. Experimental pKCs were cocultured with thymic mink lung epithelial reporter cells at a density of  $4.0 \times 10^4$  cells in 1% chelexed serum EMEM media as previously described (Abe et al., 1994; Dlugosz et al., 1995). The cells were cultured for 16–20 hours, after which the reporter cells were lysed and assayed for luciferase activity using Bright Glo Luciferase Assay System (Promega, Madison, WI).

### Statistical analysis

Groups were compared with Prism software (GraphPad Software, San Diego, CA) using the two-tailed unpaired Student's *t*-test. Data are presented as mean only or mean  $\pm$  SEM. *P* < 0.05 was considered significant.

### Data availability statement

No datasets were generated or analyzed during this study.

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## CONFLICT OF INTEREST

The authors state no conflict of interest.

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