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## Keratinocyte-derived TGF $\beta$ is not required to maintain skin immune homeostasis

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### Abstract

**Background**—Transforming growth factor beta 1 (TGF $\beta$ ) is known to be a regulator of autoimmunity. Loss of TGF $\beta$  leads to severe multi-organ autoimmunity in mice. In skin, role of TGF $\beta$  in suppressing autoimmunity is unclear.

**Objective**—Determine whether Keratinocyte (KC)-derived TGF $\beta$  is required for skin immune homeostasis.

**Methods**—We generated *K14-CreER<sup>T2</sup> TGF $\beta$ <sup>fl/fl</sup>* (*TGF $\beta$ <sup>KC</sup>*) mice allowing for tamoxifen-induced deletion of *TGF $\beta$ 1* in KC. The phenotype of skin was analyzed and compared to mice in which epidermal activation of TGF $\beta$  is impaired.

**Results**—KC was the major source of TGF $\beta$  in epidermis. Topical tamoxifen application led to efficient *TGF $\beta$ 1* deletion. The expected acanthosis was observed but no inflammatory infiltrate or altered numbers of resident immune cells were evident. Similarly, *Itgb6<sup>-/-</sup> x K14Cre Itgb8<sup>fl/f</sup>* (*Itgb6<sup>-/-</sup> Itgb8<sup>KC</sup>*) mice lacking both epidermal TGF $\beta$ -activating integrins showed no evidence of cutaneous inflammation.

**Conclusions**—KC-derived TGF $\beta$  and epidermal TGF $\beta$  activation are not required to suppress skin autoimmunity in steady state.

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Conflict of interest

The authors have no conflict of interest to declare

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## 1. Introduction

Transforming growth factor beta 1 (TGF $\beta$ ) is a pleiotropic cytokine belonging to the transforming growth factor superfamily [1]. TGF $\beta$  is secreted as a biologically inactive dimer. It becomes active when mature TGF $\beta$  disassociates from its latency associated peptide (LAP) [2]. Dissociation of LAP from TGF $\beta$  is primarily mediated by the RGD binding integrins  $\alpha_v\beta_6$  and  $\alpha_v\beta_8$  [3–5]. Active TGF $\beta$  binds to a heterodimeric TGF $\beta$  receptor complex and plays an essential role in cell cycle [6], tissue repair [7], and immune homeostasis and tolerance [8].

As a potent regulator of the immune and inflammatory systems, TGF $\beta$  generally exerts an immunosuppressive effect [8]. TGF $\beta^{-/-}$  mice exhibit no gross developmental abnormalities, but develop an acute wasting syndrome accompanied by severe mucosal inflammation due to the requirement of TGF $\beta$  acting on CD4 $^+$  T cells to promote tolerance [9–13]. Mice with ablation of TGF $\beta$  in CD4 $^+$  T cells develop global spontaneous autoimmunity indicating that TGF $\beta$  acts in a paracrine manner on CD4 $^+$  T cells [14]. Similarly, *Itgb6* $^{-/-}$  mice which have defective TGF $\beta$ -activation at barrier surfaces develop inflammatory infiltrates in lung and intestine with age [15]. A similar phenotype has been reported in skin, though it is much more subtle possibly due to the expression of both  $\alpha_v\beta_6$  and  $\alpha_v\beta_8$  in skin but only  $\alpha_v\beta_6$  in lung and intestine [16]. TGF $\beta$  also has a direct effect on KC and inhibits proliferation *in vitro* through regulation of growth-related gene expression [6,17]. Transgenic overexpression in KC of both active and latent TGF $\beta$  results in a severe psoriasis-like skin phenotype in mice [18,19]. Thus, TGF $\beta$  can participate in growth regulation, maintenance of tolerance and induction of inflammation in the skin.

TGF $\beta$  is also required for retention of immune cell in epidermis during steady-state conditions. Autocrine TGF $\beta$  expressed by Langerhans cells (LC) is required for their maintenance in the epidermis as LC-specific ablation of *TGF $\beta$*  or *TGF $\beta$ RII* results in spontaneous migration of LC from the epidermis to regional lymph nodes [20–22]. Similarly, *Itgb6* $^{-/-}$  *Itgb8* $^{KC}$  mice that lack both TGF $\beta$ -activating integrins  $\alpha_v\beta_6$  and  $\alpha_v\beta_8$  in the epidermis have a complete absence of epidermal LC. In addition, epidermal CD8 $^+$  tissue-resident memory T cells (T $_{RM}$ ) require active TGF $\beta$  for their persistence. These cells are absent from *Itgb6* $^{-/-}$  *Itgb8* $^{KC}$  mice and in mice treated with neutralizing mAbs to  $\alpha_v\beta_6$  [5,23,24]. Despite the clear importance of TGF $\beta$  for the maintenance of skin homeostasis, the cellular source of TGF $\beta$  in steady-state skin remains unclear. Herein, we established that KC represent the major cell type expressing TGF $\beta$  in the skin and developed mice with a KC-specific ablation of TGF $\beta$  (*TGF $\beta$*  $^{KC}$  mice). We observed increased KC growth but found that skin immune homeostasis was largely unaffected. From these data, we conclude that KC-derived TGF $\beta$  is not required for skin immune homeostasis.

## 2. Materials and methods

### 2.1. Mice

*TGF- $\beta$*  $^{doxP}$ , *K14Cre*, *K14CreER* $^{T2}$  and huLangerin-DTA mice have been previously described [14,21,25]. *Itgb6* $^{-/-}$  and *Itgb8* $^{doxP}$  mice were provided by D. Sheppard (University of California, San Francisco). We crossed *K14Cre* mice with *Itgb8* $^{doxP}$  and *Itgb6* $^{-/-}$  mice to

obtain *Itgb6*<sup>-/-</sup>*Itgb8*<sup>KC</sup> mice[5]. K14CreER<sup>T2</sup> mice were bred with *TGFβ*<sup>loxP</sup> mice and ROSA26.LSL.YFP (Jackson Laboratories) reporter mice resulting *TGFβ*<sup>KC</sup> mice. All experiments were performed on age- (6–12 week) and sex-matched mice. All mice were maintained under specific-pathogen-free conditions and all animal experiments were approved by University of Pittsburgh Institutional Animal Care and Use Committee.

## 2.2. Tamoxifen treatment

Tamoxifen (T5648; Sigma-Aldrich) was dissolved in 1/10th volume of 200 proof ethanol following incubation at 37 °C for 15–30 min with occasional vortexing. Corn oil (Sigma-Aldrich) was added for a final concentration of Tamoxifen at 10 mg/ml and was administered to mice for 5 consecutive days by intraperitoneal injection at 0.05 mg per g body weight. 5 mg of 4-Hydroxytamoxifen (H7904–5MG; Sigma-Aldrich) was dissolved in 1.25ml 99.5% acetone following incubation at 37 °C for 5–10 min with occasional vortexing. Corn oil (Sigma-Aldrich) was added for a final concentration of 2 mg/ml and was administered to mice for 2 consecutive days by topical application (40 ul/shaved flank and 10 ul/ear).

## 2.3. Cell Sorting by Flow Cytometry and Quantitative PCR

Single-cell suspension of epidermal cells was obtained as below. LC, KC, and DETC were sorted using a FACSAria cell sorter based as CD45<sup>+</sup>MHCII<sup>+</sup>CD207<sup>+</sup>, CD45<sup>-</sup>MHCII<sup>-</sup>, CD45<sup>+</sup>MHCII<sup>-</sup>CD3<sup>+</sup>TCR  $\gamma\delta$ <sup>+</sup>, respectively. RNA was isolated using an RNeasy Mini Kit (Qiagen) and quantified from NanoDrop readings. cDNA was generated using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). TaqMan Gene Expression Master Mix, TaqMan Gene Expression Assays for TGF- $\beta$ 1 and ABI Prism 7900HT (Applied Biosystems) were used for quantitative PCR. All kits were performed according the manufacturer's instructions. All Ct values were normalized to hypoxanthine phosphoribosyltransferase (*Hprt*) or glyceraldehyde phosphate dehydrogenase (*Gapdh*) expression.

## 2.4. Flow cytometry

Preparation of epidermal and dermal single cells suspension from tissues were performed as previously described [25]. For preparing single cells suspension from the skin, shaved skin was harvested and fat tissues was removed mechanically by forceps. Float their dermal side down in the Petri dish containing pre-warmed 37°C 0.3% trypsin (Sigma-Aldrich) solution in 150 mM NaCl, 0.5 mM KCl and 0.5 mM glucose. Incubate in CO<sub>2</sub> incubator at 37° for 45–60 minutes. The epidermis was physically separated from the dermis and disrupted by mincing and vigorous pipetting. Wash dermis with PBS. The dermis was minced finely with scissors and resuspended in RPMI1640 (Gibco, Grand Island, NY) with 2.5 mg/ml collagenase XI (Sigma-Aldrich), 0.25 mg/ml hyaluronidase (Sigma-Aldrich), 0.1 mg/ml DNase (Sigma-Aldrich), 0.01 M HEPES (Sigma-Aldrich), and 10% FBS followed by incubation in a shaking incubator for 1 h at 37°C at 250 rpm. The resulting cells were filtered through a 40 um cell strainer (BD Biosciences). Single-cell suspensions were blocked with 2.4G2 culture supernatant (American Type Culture Collection) and were stained with antibodies to extracellular markers, in PBS with 2% calf serum (Hyclone) and 0.05% Azide for 30 min at 4°C or 37°C. Fluorochrome-conjugated antibodies CD8 (53–6.7),

CD45.2 (104), CD3 (17A2), TCR  $\beta$  (H57–597), Thy1.2 (30-H12), CD69 (H1.2F3), CD103 (2E7), MHCII (M5/114.15.2), and CD11b (M1/70), TCR  $\gamma/\delta$ (GL3), CD 64 (X54–5/7.1), CD34 (HM34), CD24 (M1/69) were purchased from BioLegend. Foxp3(R16–715) and CD4 (GK1.5) were purchased from BD Biosciences. Anti-Langerin (929F3.01) was purchased from Novus Biologicals (Littleton, CO). Sca-1 (D7) was purchased from ebioscience. The fixable viability dye eFluor 780 (eBioscience) was used for live-dead discrimination. Intracellular staining of langerin, foxp3, and green fluorescent protein was performed with a BD Bioscience Cytofix/Cytoperm kit (BD Biosciences) in accordance with the manufacturer's instructions. The intracellular cytokine staining was performed with Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer's instructions. A BD LSRFORTESSA (BD Biosciences) and Flowjo software (TreeStar, Ashland, OR) were used for analysis.

## 2.5. Immunofluorescence of epidermis

Epidermal sheets were prepared as previously described [25]. Shaved defatted flank skin was affixed to slides with double-sided adhesive (3M, St. Paul, MN). Slides were incubated in 10 mM EDTA in PBS for 90 min at 37°C, followed by physical removal of the dermis. The epidermal sheets were fixed in 4% PFA at room temperature (RT) for 30 min. The epidermal sheets were blocked with PBS containing 0.1% tween-20, 2% BSA and 2% rat serum for 1 hours at RT before staining overnight with Alexa Fluor 488–conjugated anti-GFP, Alexa Fluor 594–conjugated anti-mouse I-A/I-E and Alexa Fluor 647–conjugated anti-TCR  $\gamma/\delta$  antibody in PBS containing 0.1% tween-20 and 0.5% BSA. Images were captured on a IX83 fluorescent microscope (Olympus Tokyo, Japan) using a x10 objective; image analysis was performed using cell Sens Dimension software (Olympus). Both LC and DETC in epidermis were counted either by ImageJ software or manually in a blinded fashion.

## 2.6. H&E staining

Mouse skin was embedded in OCT compound and frozen in dry ice. Transverse mouse skin sections that were 8- $\mu$ m thick were prepared from frozen blocks using a cryostat and were fixed for 30 min in 4% PFA. Hematoxylin and eosin staining was performed as follows: 0.1% Mayer's hematoxylin staining for 15 min, two-three quick rinses in distilled water, section under 10 min running tap water and rinses in distilled water. Apply 95% ethanol for 30s, 0.5% eosin staining for 30 s and rinse in 80% ethanol. Then mounting media was used and the section was observed and photographed with a Nikon Eclipse E400 microscope. Acanthosis was quantified by measuring epidermal thickness on the section every 200  $\mu$ m for 5 points and the average was used for the value of the acanthosis.

## 2.7. Statistical analysis

Two groups were compared with Prism software (GraphPad) using the two-tailed unpaired Student's t test. For three groups, Turkey's multiple comparisons was used. Data are presented as mean only or mean  $\pm$  standard error of the mean (s.e.m.).  $p < 0.05$  was considered significant.

### 3 Results

#### 3.1. KC is the major source of TGF $\beta$ in the epidermis

Epidermal cells including KC, dendritic epidermal T cells (DETC) and Langerhans Cells (LC) have all been reported to produce TGF $\beta$  [26,27]. First, we sought to determine which cell type is the dominant source of TGF $\beta$  in the epidermis. Single cell suspensions of epidermis were FACSsorted to obtain pure populations of KC (CD45<sup>-</sup>MHCII<sup>-</sup>), DETC (CD45<sup>+</sup>MHCII<sup>-</sup>CD3<sup>+</sup>TCR $\gamma\delta$ <sup>+</sup>) and LC (CD45<sup>+</sup>MHCII<sup>+</sup>). mRNA was generated and analyzed by qPCR for *Tgfb1* expression levels which were normalized to *Hprt*. On a per cell basis, DETC expressed more TGF $\beta$  than LC and KC (Fig. 1a). However, these cell types account for different proportions of the epidermis. DETC, LC and KC represent approximately 2%, 2% and 95% of total epidermal cells, respectively (unpublished observations). After normalizing for the number of each cell type, TGF $\beta$  contributed by KC was 6 times higher than DETC and 22 times higher than LC (Fig. 1b). Thus, we concluded that KC represented the major cellular source of TGF- $\beta$  in epidermis during steady state conditions.

#### 3.2. Generation and validation of TGF $\beta$ <sup>KC</sup> mice

To determine the requirement of KC-derived TGF $\beta$ , we generated tamoxifen (TAM)-inducible keratinocyte-specific TGF $\beta$ -deficient mice. K14CreER<sup>T2</sup> mice were bred with TGF $\beta$ <sup>f/f</sup> mice and ROSA26.YFP reporter mice resulting in *K14-CreER*<sup>T2</sup> *TGF $\beta$* <sup>f/f</sup> (*TGF $\beta$ <sup>KC</sup>*). We first administered TAM i.p. over five consecutive days to allow for the inducible deletion of TGF $\beta$  in KC. Unexpectedly, the majority of *TGF $\beta$ <sup>KC</sup>* but not TAM treated control mice (*TGF $\beta$ <sup>WT</sup>*) developed severe weight loss necessitating euthanasia first observable on day 5 after the first treatment of TAM (Fig. 2a). Gross and histologic observations of the skin of these mice showed no obvious phenotype (unpublished observations). K14 is reported to be expressed in mucosal epithelia [28]. Thus, the lethal phenotype may result from the inducible deletion of TGF $\beta$  at these sites.

To specifically test the requirement of KC-derived TGF $\beta$  in skin, we next applied 2mg/ml 4OH-Tamoxifen (4OH-TAM) topically on *TGF $\beta$ <sup>KC</sup>* and control mice for two consecutive days (Fig. 2b). After 28 days, the skin was harvested and analyzed by immunofluorescent microscopy for expression of YFP to determine the efficiency of Cre activation. We observed high levels of YFP expression in 4OH-TAM treated *TGF $\beta$ <sup>KC</sup>* but not control mice (Fig. 2c). Flow cytometric analysis revealed a similar high degree of efficiency with >80% of KC expressing YFP (Fig. 2d). Sub-setting of keratinocyte population into interfollicular KC (IFE:CD45<sup>-</sup>MHCII<sup>-</sup>CD34<sup>-</sup>Sca-1<sup>+</sup>), bulge KC (CD45<sup>-</sup>MHCII<sup>-</sup>Sca-1<sup>-</sup>CD34<sup>+</sup>), and isthmus KC (IM:CD45<sup>-</sup>MHCII<sup>-</sup>Sca-1<sup>-</sup>EpCAM<sup>+</sup>) revealed a similar high degree of YFP expression in all populations (Fig. 2e).

To ensure that expression of YFP accurately reflected the absence of TGF $\beta$ , we obtained mRNA from epidermis 28 days following topical tamoxifen application. As expected, levels of *TGF $\beta$ 1* were greatly reduced in 4OH-TAM treated *TGF $\beta$ <sup>KC</sup>* but not control mice (Fig. 2f). In addition, we isolated YFP<sup>+</sup> and YFP<sup>-</sup> KC from 4OH-TAM treated *TGF $\beta$ <sup>KC</sup>* mice and found greatly reduced levels of *TGF $\beta$ 1* in YFP<sup>+</sup> but not YFP<sup>-</sup> KC (Fig. 2g). From these

data, we conclude that topical 4OH-tamoxifen application to  $TGF\beta^{KC}$  mice allows for the efficient deletion of TGF $\beta$  and that expression of YFP accurately reports the loss of TGF $\beta$  expression.

During these experiments, we observed that often some cohorts of  $TGF\beta^{KC}$  would display greatly reduced efficiency of YFP expression. An analysis of mouse age at the time of 4OH-TAM treatment revealed that topical tamoxifen applied only at 6 weeks of age resulted in efficient expression of YFP (Fig. 2h). Even when TAM was applied at 6 weeks of age, some cohorts expressed low YFP expression. In all subsequent experiments, all mice were analyzed for efficiency of YFP expression and only those showing >80% YFP<sup>+</sup> KC were used for analysis (Fig. 2h, red box).

### 3.3. KC-derived TGF $\beta$ is not necessary to maintain skin immune homeostasis

Using the same experimental strategy as above, we first histologically examined back skin 28 days following 4OH-TAM application. Acanthosis (thickened epidermis) was clearly evident on transverse H&E sections from TAM treated  $TGF\beta^{KC}$  but not control mice (Fig. 3a). Quantification determined that the increased acanthosis was significant (Fig. 3b). TGF $\beta$  has been reported to suppress KC proliferation directly *in vitro* [6]. The increased epidermal thickness is consistent with this report and functionally validated the absence of TGF $\beta$  in 4OH-TAM treated  $TGF\beta^{KC}$  mice. Notably, we did not observe evidence of an inflammatory infiltrate on H&E staining.

To determine whether more subtle changes occurred in the cellular composition of skin occurred in 4OH-TAM treated  $TGF\beta^{KC}$  mice, we analyzed single cell epidermal and dermal skin suspensions by flow cytometry. In the epidermis, we did not observe any alterations in the frequency of total CD45<sup>+</sup> cells, LC, TCR $\alpha\beta$  T cells or innate lymphoid cells (ILC) (Fig. 3c). To confirm these results, we examined epidermal whole mounts by immunofluorescent microscopy. The epidermis of  $TGF\beta^{KC}$  mice showed a modest decrease of DETC numbers which was also seen with flow cytometry but no alteration in the number of LC (Fig. 3d, e).

In dermis, the frequency of CD45<sup>+</sup> cells was not altered in 4OH-TAM treated  $TGF\beta^{KC}$  mice (Fig. 3f) compared with control mice. In addition, no changes in the frequency of Treg (CD45<sup>+</sup>Thy1.2<sup>+</sup>TCRb<sup>-</sup>CD3<sup>+</sup>CD4<sup>+</sup>Foxp3<sup>+</sup>),  $\gamma\delta$  T cells (CD45<sup>+</sup>Thy1.2<sup>+</sup>TCRb<sup>-</sup>CD3<sup>mid</sup>), ILC (CD45<sup>+</sup>Thy1.2<sup>+</sup>TCRb<sup>-</sup>CD3<sup>-</sup>), Macrophages (CD45<sup>-</sup>MHCII<sup>+</sup>CD64<sup>+</sup>CD11b<sup>+</sup>) and dendritic cells subsets (dermal cDC2, CD45<sup>-</sup>MHCII<sup>+</sup>CD64<sup>-</sup>CD11b<sup>+</sup>; DN DC, CD45<sup>-</sup>MHCII<sup>+</sup>CD64<sup>-</sup>CD11b<sup>-</sup>; dermal cDC1, CD45<sup>-</sup>MHCII<sup>+</sup>CD64<sup>-</sup>CD207<sup>+</sup>CD103<sup>+</sup>). Taken together, we conclude that the absence of KC-derived TGF $\beta$  has no effect on immune homeostasis in steady state.

### 3.4. KC-mediated activation of TGF $\beta$ is not necessary to maintain skin immune homeostasis

We next sought to determine whether activation of TGF $\beta$  by integrins  $\alpha_v\beta_6$  and  $\alpha_v\beta_8$  expressed by keratinocytes is required to maintain skin immune homeostasis. We used  $Itgb6^{-/-} \times K14Cre Itgb8^{fl/fl}$  ( $Itgb6^{-/-}Itgb8^{KC}$ ) mice that lack both TGF $\beta$ -activating integrins in the epidermis. A prior report has suggested that  $Itgb6^{-/-}$  develop mild spontaneous skin



inflammation at the site of occasional hair loss [15]. We did not observe increased incidence of the hair loss in our mice colony compared to B6 wild type mice (unpublished observations). Histological examination did not reveal an obvious alteration in the cellular infiltrate in *Itgb6*<sup>-/-</sup>*Itgb8*<sup>KC</sup> mice and no evidence of acanthosis (Fig. 4a). As above, we analyzed single cell suspensions of epidermis and dermis for more subtle alterations in the frequency of various immune subsets. We have previously demonstrated, *Itgb6*<sup>-/-</sup>*Itgb8*<sup>KC</sup> mice, have a dramatic absence of LC. To ensure that any observed phenotype derived from the absence of  $\alpha_v\beta_6$  and  $\alpha_v\beta_8$  and not LC, we also examined the cellular composition of skin from huLangerin-DTA that have a constitutive absence of LC. In the epidermis, as expected, we noted the absence of LC in *Itgb6*<sup>-/-</sup>*Itgb8*<sup>KC</sup> and huLangerin-DTA mice (Fig. 4b). We did not observe a significant alteration in the frequency of total CD45<sup>+</sup> cells, TCR  $\alpha\beta$  T cells, DETC or ILC in any group. Notably, unlike *TGF $\beta$* <sup>KC</sup> mice, the frequency of DETC were unaffected. In dermis, the frequency of Treg, TCR  $\gamma\delta$  T cells, ILC, Macrophages and DC was unaltered in *Itgb6*<sup>-/-</sup>*Itgb8*<sup>KC</sup> mice (Fig. 4c). From these data, we conclude that KC-mediated activation of TGF $\beta$  is required for epidermal maintenance of LC and to suppress acanthosis but has no other nonredundant role in skin immune homeostasis during steady state.

#### 4. Discussion

Herein, we have demonstrated that ablation of TGF $\beta$  in keratinocytes does not result in the development of an inflammatory infiltrate or altered numbers of resident immune cells. Similarly, integrins  $\alpha_v\beta_6$  and  $\alpha_v\beta_8$  in the epidermis were not required to maintain skin homeostasis beyond residency of epidermal leukocytes. Taken together, these data support the conclusion that KC-derived TGF $\beta$  and epidermal activation of TGF $\beta$  are not required for maintenance of skin immune homeostasis. The role of TGF $\beta$  from other epidermal sources and dermis remains important to be explored.

Given the known importance of TGF $\beta$  to suppress effector CD4<sup>+</sup> T cells and Tregs which can be found in the skin [29–31], we anticipate that inducible ablation of KC-derived TGF $\beta$  or ablation  $\alpha_v\beta_6$  and  $\alpha_v\beta_8$ -mediated TGF $\beta$ -activation would result in loss of T cell tolerance to skin antigens. The absence of spontaneous inflammation indicates that epidermal TGF $\beta$  is either not required or redundant. It is likely that autocrine/paracrine TGF $\beta$  and  $\alpha_v\beta_8$  expressed by subsets of dendritic cells found in the dermis is sufficient [32,33]. The clear absence of an inflammatory infiltrate in the skin double  $\alpha_v\beta_6$ - and  $\alpha_v\beta_8$ -deficient mice is surprising given on prior observations of mild occasional spontaneous skin inflammation [15]. We speculate that either further back crossing onto the C57BL/6 background or an altered cutaneous microbiome may explain this discrepancy. We previously reported that autocrine/paracrine TGF $\beta$  was required for the maintenance of LC [21,22]. Our data showing that loss of KC-derived TGF $\beta$  did not affect LC number in epidermis further supports this finding and demonstrated that KC-derived TGF $\beta$  is not required.

A caveat for all Cre-mediated loss-of-function studies is whether the achieved reduction of a given gene is sufficiently robust to yield a given phenotype. Topical tamoxifen treatment of *TGF $\beta$* <sup>KC</sup> mice yield >80% efficiency of TGF $\beta$  deletion as measured by direct measure of

TGF $\beta$  mRNA and expression of YFP which we demonstrated efficiently phenocopied levels of TGF $\beta$  mRNA. Importantly, we did observe the expected phenotype of increased acanthosis indicating a functional absence of TGF $\beta$ . We also noted a modest decrease in DETC numbers in tamoxifen treated TGF $\beta$ <sup>KC</sup> mice. We do not think that epidermal retention of DETC is dependent on TGF $\beta$  given the normal numbers of DETC observed in *Itgb6<sup>-/-</sup>Itgb8<sup>KC</sup>* mice and TGF $\beta$ <sup>-/-</sup> mice [20]. Rather, we suspect that reduced DETC numbers is an indirect effect in TGF $\beta$ <sup>KC</sup> mice.

There were two unexpected and unusual observations in the TGF $\beta$ <sup>KC</sup> model. First, the majority of TGF $\beta$ <sup>KC</sup> but not control mice developed severe weight loss shortly after systematic application of tamoxifen. We suspect this phenotype is related to expression of K14 in other tissue such as the oropharynx and esophagus since TGF $\beta$ <sup>-/-</sup> do develop severe esophageal and pharyngeal inflammation [34]. We also noted that highly efficient ablation of TGF $\beta$  occurs following topical application to 6 week old mice with another peak at 10 weeks. This timing is similar to the mouse hair anagen cycle[35]. It is possible that topical tamoxifen targeting genes in KC may have heightened efficiency during anagen. Alternatively, given the importance of TGF $\beta$  and  $\alpha_v\beta_6$  during hair development, this phenomena may only occur when the targeting of TGF $\beta$  [36,37].

In this report, we have focused exclusively on analysis of immune homeostasis under steady-state conditions. An examination of the requirement for KC-derived TGF $\beta$  in the context of autoimmunity, fibrosis and infection remain as exciting future directions.

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## References

- [1]. Massagué J, The transforming growth factor-beta family, *Annu. Rev. Cell Biol* 6 (1990) 597–641. [PubMed: 2177343]
- [2]. Shi M, Zhu J, Wang R, Chen X, Mi L, Walz T, et al., Latent TGF- $\beta$  structure and activation, *Nature*. 474 (2011) 343–349. [PubMed: 21677751]
- [3]. Yang Z, Mu Z, Dabovic B, Jurukovski V, Yu D, Sung J, et al., Absence of integrin-mediated TGF $\beta$ 1 activation in vivo recapitulates the phenotype of TGF $\beta$ 1-null mice, *J. Cell Biol* 176 (2007) 787–793. [PubMed: 17353357]
- [4]. Aluwihare P, Mu Z, Zhao Z, Yu D, Weinreb PH, Horan GS, et al., Mice that lack activity of  $\alpha_v\beta_6$ - and  $\alpha_v\beta_8$ -integrins reproduce the abnormalities of Tgfb1- and Tgfb3-null mice, *J. Cell. Sci* 122 (2009) 227–232. [PubMed: 19118215]
- [5]. Mohammed J, Beura LK, Bobr A, Astry B, Chicoine B, Kashem SW, et al., Stromal cells control the epithelial residence of DCs and memory T cells by regulated activation of TGF- $\beta$ , *Nat. Immunol* 17 (2016) 414–421. [PubMed: 26901152]

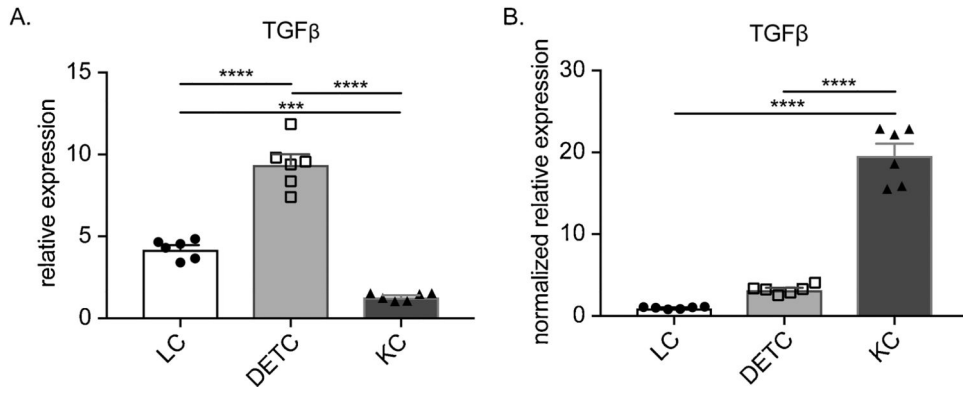


- [6]. Pietenpol JA, Holt JT, Stein RW, Moses HL, Transforming growth factor beta 1 suppression of c-myc gene transcription: role in inhibition of keratinocyte proliferation, Proc. Natl. Acad. Sci. U.S.a 87 (1990) 3758–3762. [PubMed: 2187192]
- [7]. Penn JW, Grobelaar AO, Rolfe KJ, The role of the TGF- $\beta$  family in wound healing, burns and scarring: a review, Int J Burns Trauma. 2 (2012) 18–28. [PubMed: 22928164]
- [8]. Li MO, Wan YY, Sanjabi S, Robertson A-KL, Flavell RA, Transforming growth factor-beta regulation of immune responses, Annu. Rev. Immunol 24 (2006) 99–146. [PubMed: 16551245]
- [9]. Shull MM, Ormsby I, Kier AB, Pawlowski S, Diebold RJ, Yin M, et al., Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease, Nature. 359 (1992) 693–699. [PubMed: 1436033]
- [10]. Kulkarni AB, Huh CG, Becker D, Geiser A, Lyght M, Flanders KC, et al., Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death, Proc. Natl. Acad. Sci. U.S.a 90.2 (1993): 770–774 [PubMed: 8421714]
- [11]. Oh SA, Li MO, TGF- $\beta$ : guardian of T cell function, J Immunol. 191 (2013) 3973–3979. [PubMed: 24098055]
- [12]. Li MO, Sanjabi S, and Flavell RA, Transforming growth factor- $\beta$  controls development, homeostasis, and tolerance of T cells by regulatory T cell-dependent and-independent mechanisms, Immunity. 25.3 (2006): 455–471. [PubMed: 16973386]
- [13]. Marie JC, Liggitt D, Immunity AR, 2006, Cellular mechanisms of fatal early-onset autoimmunity in mice with the T cell-specific targeting of transforming growth factor- $\beta$  receptor, Immunity 2006. 9 1;25(3):455–71.
- [14]. Marie JC, Liggitt D, Rudensky AY, Cellular mechanisms of fatal early-onset autoimmunity in mice with the T cell-specific targeting of transforming growth factor-beta receptor, Immunity. 25 (2006) 441–454. [PubMed: 16973387]
- [15]. Huang XZ, Wu JF, Cass D, Erle DJ, Corry D, Young SG, et al., Inactivation of the integrin beta 6 subunit gene reveals a role of epithelial integrins in regulating inflammation in the lung and skin, J. Cell Biol 133 (1996) 921–928. [PubMed: 8666675]
- [16]. Lowell CA, Mayadas TN, Overview: studying integrins in vivo, Methods Mol. Biol 757 (2012) 369–397. [PubMed: 21909923]
- [17]. Coffey RJ, Bascom CC, Sipes NJ, Graves-Deal R, Weissman BE, Moses HL, Selective inhibition of growth-related gene expression in murine keratinocytes by transforming growth factor beta, Mol. Cell. Biol 8 (1988) 3088–3093. [PubMed: 2463471]
- [18]. Sellheyer K, Bickenbach JR, Rothnagel JA, Bundman D, Longley MA, Krieg T, et al., Inhibition of skin development by overexpression of transforming growth factor beta 1 in the epidermis of transgenic mice, Proc. Natl. Acad. Sci. U.S.a 90 (1993) 5237–5241. [PubMed: 7685120]
- [19]. Li AG, Wang D, Feng X-H, Wang X-J, Latent TGFbeta1 overexpression in keratinocytes results in a severe psoriasis-like skin disorder, Embo J. 23 (2004) 1770–1781. [PubMed: 15057277]
- [20]. Borkowski TA, Letterio JJ, Farr AG, Udey MC, A role for endogenous transforming growth factor beta 1 in Langerhans cell biology: the skin of transforming growth factor beta 1 null mice is devoid of epidermal Langerhans cells, J. Exp. Med 184 (1996) 2417–2422. [PubMed: 8976197]
- [21]. Kaplan DH, Li MO, Jenison MC, Shlomchik WD, Flavell RA, Shlomchik MJ, Autocrine/paracrine TGFbeta1 is required for the development of epidermal Langerhans cells, J. Exp. Med 204 (2007) 2545–2552. [PubMed: 17938236]
- [22]. Bobr A, Igyarto BZ, Haley KM, Li MO, Flavell RA, Kaplan DH, Autocrine/paracrine TGF- $\beta$ 1 inhibits Langerhans cell migration, Proc. Natl. Acad. Sci. U.S.a 109 (2012) 10492–10497. [PubMed: 22689996]
- [23]. Mu D, Cambier S, Fjellbirkeland L, Baron JL, Munger JS, Kawakatsu H, et al., The integrin  $\alpha$ v $\beta$ 8 mediates epithelial homeostasis through MT1-MMP-dependent activation of TGF- $\beta$ 1, J Cell Biol. 157.3 (2002): 493–507. [PubMed: 11970960]
- [24]. Munger JS, Huang X, Kawakatsu H, Griffiths MJ, Dalton SL, Wu J, et al., A mechanism for regulating pulmonary inflammation and fibrosis: the integrin  $\alpha$ v $\beta$ 6 binds and activates latent TGF  $\beta$ 1, Cell. 96.3 (1999): 319–328. [PubMed: 10025398]

- [25]. Kaplan DH, Jenison MC, Saeland S, Shlomchik WD, Shlomchik MJ, Epidermal Langerhans cell-deficient mice develop enhanced contact hypersensitivity, *Immunity*. 23 (2005) 611–620. [PubMed: 16356859]
- [26]. Gruschwitz MS, Hornstein OP, Expression of transforming growth factor type beta on human epidermal dendritic cells, *J. Invest. Dermatol* 99 (1992) 114–116. [PubMed: 1376753]
- [27]. Granucci F, Girolomoni G, Lutz MB, Foti M, Marconi G, Gnocchi P, et al., Modulation of cytokine expression in mouse dendritic cell clones, *Eur. J. Immunol* 24 (1994) 2522–2526. [PubMed: 7925580]
- [28]. Smith CM, Hayamizu TF, Finger JH, Bello SM, McCright IJ, Xu J, et al., The mouse Gene Expression Database (GXD): 2019 update, *Nucleic Acids Res.* 47 (2019) D774–D779. [PubMed: 30335138]
- [29]. Marie JC, Letterio JJ, Gavin M, Rudensky AY, TGF-beta1 maintains suppressor function and Foxp3 expression in CD4+CD25+ regulatory T cells, *J. Exp. Med* 201 (2005) 1061–1067. [PubMed: 15809351]
- [30]. Viglietta V, Baecher-Allan C, Weiner HL, Hafler DA, Loss of functional suppression by CD4+CD25+ regulatory T cells in patients with multiple sclerosis, *J. Exp. Med* 199 (2004) 971–979. [PubMed: 15067033]
- [31]. Ali N, Rosenblum MD, Regulatory T cells in skin, *Immunology*. 152 (2017) 372–381. [PubMed: 28699278]
- [32]. Travis MA, Reizis B, Melton AC, Masteller E, Tang Q, Proctor JM, et al., Loss of integrin alpha(v)beta8 on dendritic cells causes autoimmunity and colitis in mice, *Nature*. 449 (2007) 361–365. [PubMed: 17694047]
- [33]. Melton AC, Bailey-Bucktrout SL, Travis MA, Fife BT, Bluestone JA, Sheppard D, Expression of  $\alpha v \beta 8$  integrin on dendritic cells regulates Th17 cell development and experimental autoimmune encephalomyelitis in mice, *J. Clin. Invest* 120 (2010) 4436–4444. [PubMed: 21099117]
- [34]. Yaswen L, Kulkarni AB, Fredrickson T, Mittleman B, Schiffman R, Payne S, et al., Autoimmune manifestations in the transforming growth factor-beta 1 knockout mouse, *Blood*. 87 (1996) 1439–1445. [PubMed: 8608234]
- [35]. Hou C, Miao Y, Wang X, Chen C, Lin B, Hu Z, Expression of matrix metalloproteinases and tissue inhibitor of matrix metalloproteinases in the hair cycle, *Exp Ther Med*. 12 (2016) 231–237. [PubMed: 27429651]
- [36]. Foitzik K, Lindner G, Mueller-Roeber S, Maurer M, Botchkareva N, Botchkarev V, et al., Control of murine hair follicle regression (catagen) by TGF- $\beta 1$  in vivo, *The FASEB Journal*. 14.5 (2000): 752–760. [PubMed: 10744631]
- [37]. Xie Y, McElwee KJ, Owen GR, Häkkinen L, and Larjava HS, Integrin  $\beta 6$ -deficient mice show enhanced keratinocyte proliferation and retarded hair follicle regression after depilation, *J Invest Dermatol*. 132.3 (2012): 547–555. [PubMed: 22113470]

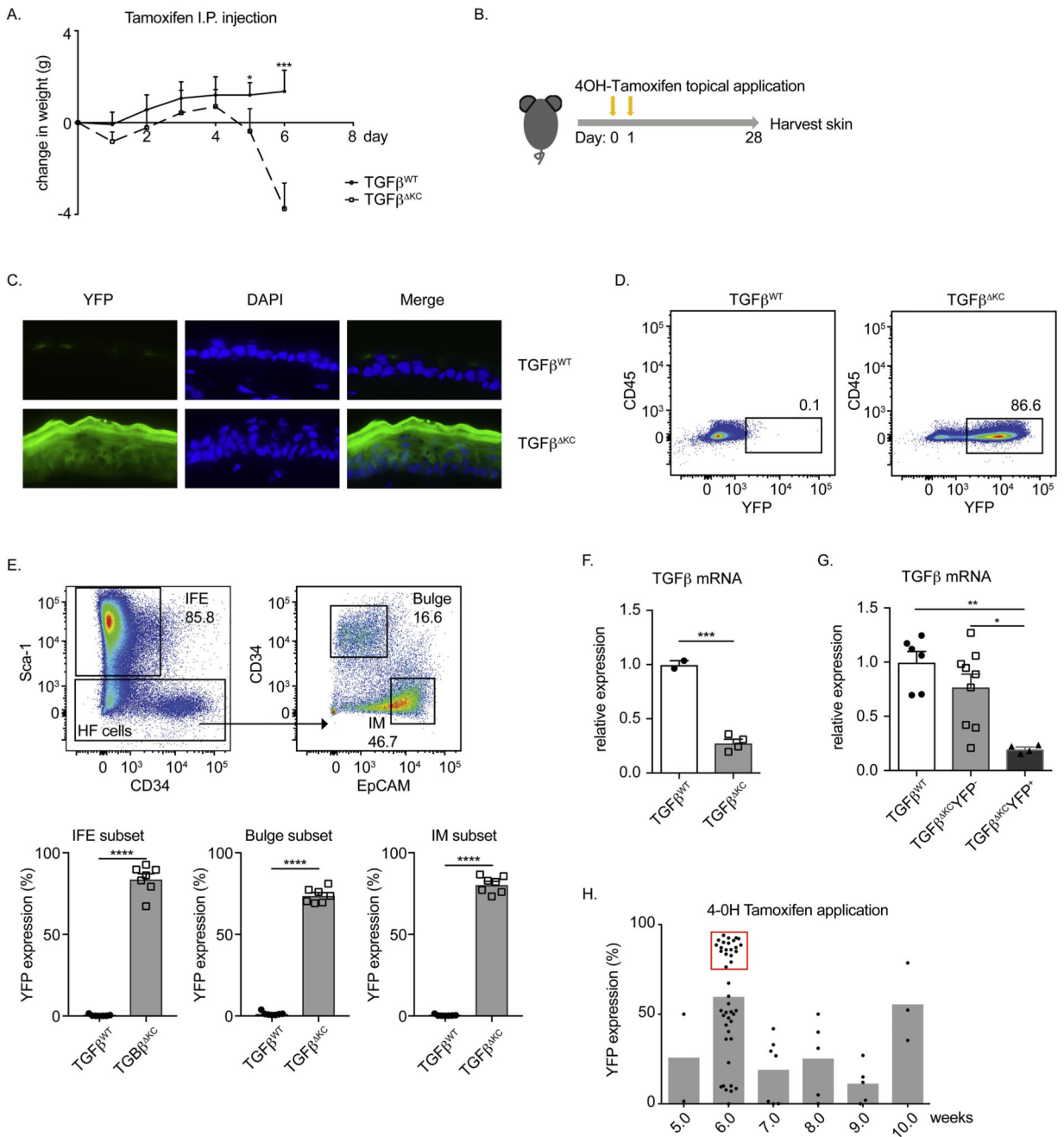
**Highlights**

- *K14-CreER<sup>T2</sup> TGFβ1<sup>fl/fl</sup>* mice develop acanthosis
- *K14-CreER<sup>T2</sup> TGFβ1<sup>fl/fl</sup>* mice do not develop spontaneous skin inflammation
- *Itgb6<sup>-/-</sup> x K14Cre Itgb8<sup>fl/fl</sup>* mice do not develop spontaneous skin inflammation



**Fig. 1. Keratinocytes are the major source of TGFβ in the epidermis**

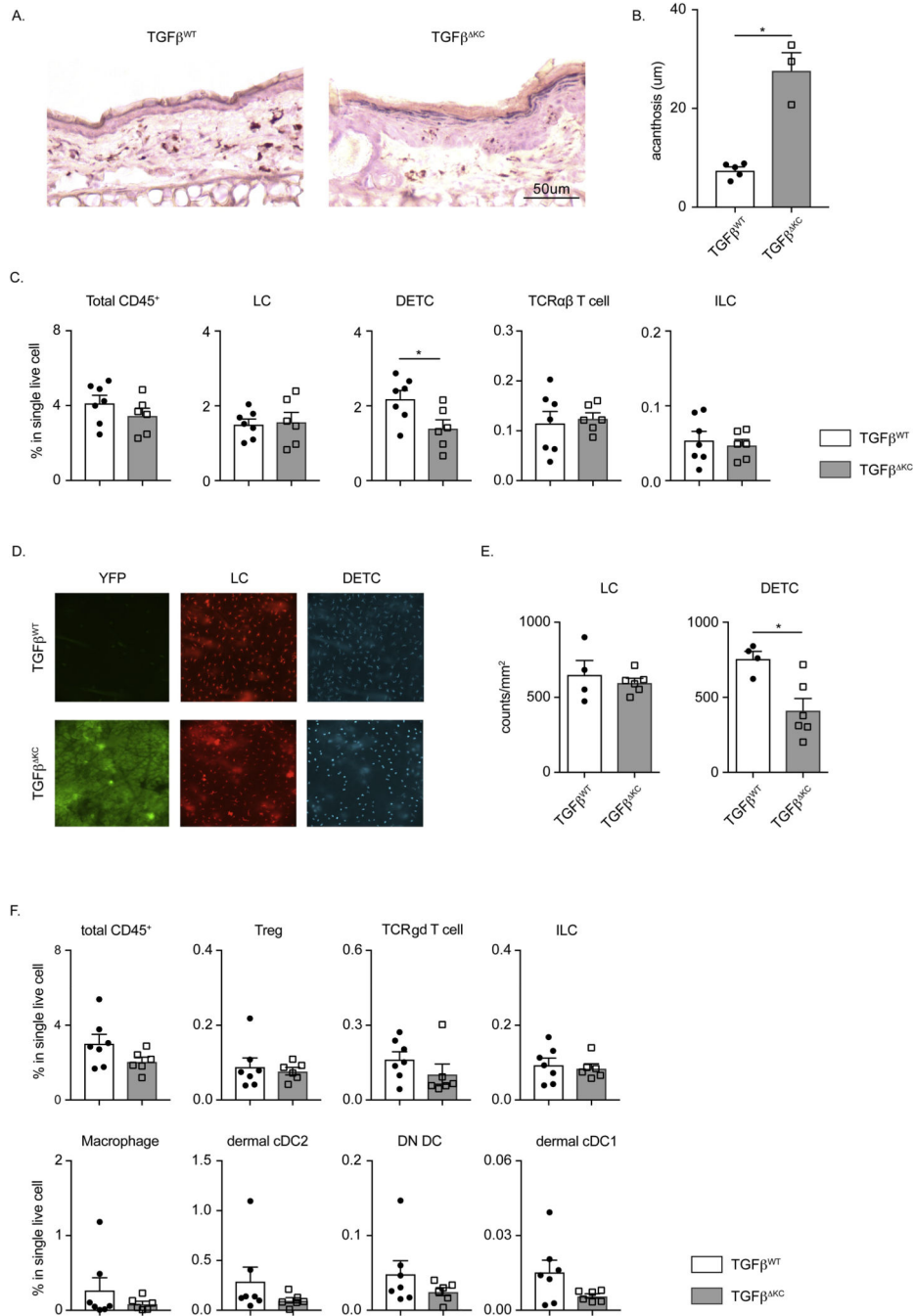
(A) TGFβ1 mRNA expression levels from FACSsorted LC, DETC and KC as analyzed by qRT-PCR normalized to *Hprt*. (B) TGFβ1 mRNA expression as in (A) normalized to relative percentage of each cell type in epidermis. Each symbol represent data from an individual animal. Data are presentative of at least 2 independent experiments. Data are means ± SEM. \*\*\* p<0.001; \*\*\*\* p<0.0001.



**Fig. 2. Generation and validation of TGFβ<sup>KC</sup> mice**  
 (A) 10mg/ml tamoxifen was injected i.p. at 5ul per g animal weight for 5 consecutive days (day 0–4). Time course of weight change during/after i.p. tamoxifen is shown for control TGFβ<sup>WT</sup> (solid line) and TGFβ<sup>KC</sup> (dashed line) mice. (B) 4OH-Tamoxifen topical application protocol: 2mg/ml 4OH-Tamoxifen was applied to shaved skin on two consecutive days and the skin was analyzed 28 days after the first 4OH-tamoxifen. (C) Immunofluorescent microscopic visualization of TGFβ<sup>WT</sup> and TGFβ<sup>KC</sup> flank skin illustrates epidermal expression of YFP (green) and DAPI nuclear label (blue). (D) Flow cytometry staining of YFP in KC (live single CD45<sup>-</sup>). (E) Gating strategy of KC population:  
 IFE subset: YFP expression (%) is significantly higher in TGFβ<sup>ΔKC</sup> (\*\*\*\*).  
 Bulge subset: YFP expression (%) is significantly higher in TGFβ<sup>ΔKC</sup> (\*\*\*\*).  
 IM subset: YFP expression (%) is significantly higher in TGFβ<sup>ΔKC</sup> (\*\*\*\*).  
 H: YFP expression (%) over 10 weeks after 4-OH Tamoxifen application, showing a peak at 6.0 weeks.

interfollicular: IFE-, Bulge- and isthmus: IM-KC subsets and frequency of YFP expression in each KC subsets. Cells were gated on live single CD45<sup>-</sup> cells. (F) *Tgfb1* mRNA relative expression level normalized to *Gapdh* in epidermis from TGFβ<sup>WT</sup> and TGFβ<sup>KC</sup> mice. The average gene expression level in control mice was taken as 1. (G) Relative *Tgfb1* mRNA expression normalized to *Gapdh* in KC sorted from TGFβ<sup>WT</sup> mice and YFP<sup>+</sup> and YFP<sup>-</sup> KC from TGFβ<sup>KC</sup> mice. The average gene expression level in KC from TGFβ<sup>WT</sup> mice was taken as 1. (H) An analysis of YFP induction efficiency in KC isolated in 4OH-tamoxifen treated mice of the indicated age. The red box indicated mice used in subsequent experiments. n=4-5 mice (A) and each symbol represents data from an individual animal (E-H). Data are representative of at least 2 independent experiments. Data are means ± SEM. \* p<0.05; \*\* p<0.01; \*\*\*p<0.001, \*\*\*\* p<0.0001.





**Fig. 3. KC-derived TGF $\beta$  is not necessary to maintain skin immune homeostasis**  
 (A) Representative H&E staining of transverse ear section of  $TGF\beta^{WT}$  and  $TGF\beta^{KC}$  mice.  
 (B) Epidermal thickness in  $TGF\beta^{WT}$  and  $TGF\beta^{KC}$  mice is shown. (C) Flow cytometry analysis of epidermal single cell suspension from  $TGF\beta^{WT}$  and  $TGF\beta^{KC}$  mice. Frequency of the indicated population in total live single cells are shown. (D) Immunofluorescent microscopic visualization whole mounted epidermis from  $TGF\beta^{WT}$  and  $TGF\beta^{KC}$  for YFP (green), LC (red, MHCII) and DETC (blue, TCR $\gamma\delta$ ) is shown. (E) Quantification of cell numbers in (D) is shown. (F) Flow cytometry analysis of dermal single cell suspension from

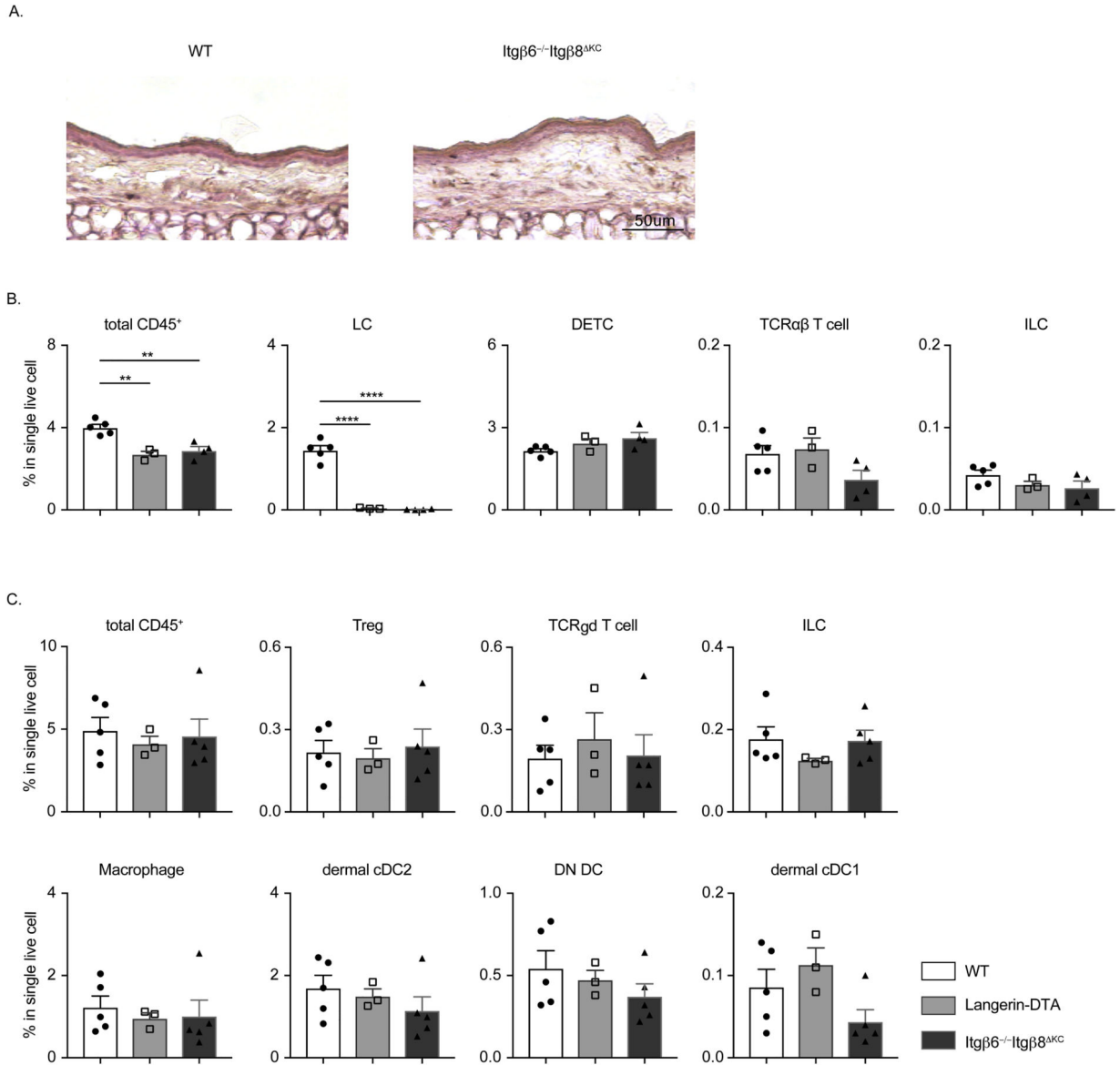
TGF $\beta$ <sup>WT</sup> and TGF $\beta$ <sup>KC</sup> mice. The percentages of the indicated population in single live cells are shown. Each symbol represents data from an individual animal. Data are representative of at least 2 independent experiments. Data are means  $\pm$  SEM. \* p<0.05.

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**Fig. 4. KC-mediated activation of TGFβ is not necessary to maintain skin immune homeostasis**  
 (A) Representative H&E staining of transverse ear section of WT and *Itgb6*<sup>-/-</sup>*Itgb8*<sup>KC</sup> mice. (B and C) Flow cytometry analysis of epidermal (B) and dermal (C) single cell suspension from WT, huLangerin-DTA and *Itgb6*<sup>-/-</sup>*Itgb8*<sup>KC</sup> mice. Frequency of the indicated population is shown. Data are representative of at least 2 independent experiments. Data are means ± SEM. \*\* p<0.01; \*\*\*\* p<0.0001.

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