



Original Article

Keratinocyte-derived TGFβ is not required to maintain skin immune homeostasis

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ARTICLE INFO

Article history:

Received 9 April 2019

Received in revised form 24 April 2019

Accepted 25 April 2019

Keywords:

TGFβ

Keratinocyte

Skin

 $\alpha_v\beta_6$ $\alpha_v\beta_8$

ABSTRACT

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

Objective: Determine whether Keratinocyte (KC)-derived TGFβ is required for skin immune homeostasis. **Methods:** We generated *K14-CreER^{T2} TGFβ1^{fl/fl} (TGFβ^{ΔKC})* mice allowing for tamoxifen-induced deletion of *TGFβ1* in KC. The phenotype of skin was analyzed and compared to mice in which epidermal activation of TGFβ is impaired.

Results: KC was the major source of TGFβ in epidermis. Topical tamoxifen application led to efficient *TGFβ1* deletion. The expected acanthosis was observed but no inflammatory infiltrate or altered numbers of resident immune cells were evident. Similarly, *Itgb6^{-/-} x K14Cre Itgb8^{fl/fl} (Itgb6^{-/-}Itgb8^{ΔKC})* mice lacking both epidermal TGFβ-activating integrins showed no evidence of cutaneous inflammation.

Conclusions: KC-derived TGFβ and epidermal TGFβ activation are not required to suppress skin autoimmunity in steady state.

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1. Introduction

Transforming growth factor beta 1 (TGFβ) is a pleiotropic cytokine belonging to the transforming growth factor superfamily [1]. TGFβ is secreted as a biologically inactive dimer. It becomes active when mature TGFβ disassociates from its latency associated peptide (LAP) [2]. Dissociation of LAP from TGFβ is primarily mediated by the RGD binding integrins $\alpha_v\beta_6$ and $\alpha_v\beta_8$ [3–5]. Active TGFβ binds to a heterodimeric TGFβ 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, TGFβ generally exerts an immunosuppressive effect [8]. TGFβ^{-/-} mice exhibit no gross developmental abnormalities, but develop an acute wasting syndrome accompanied by severe mucosal inflammation due to the requirement of TGFβ acting on CD4⁺ T cells to promote tolerance [9–13]. Mice with ablation of TGFβ in CD4⁺ T

cells develop global spontaneous autoimmunity indicating that TGFβ from CD4⁺ T cells is essential [14]. Similarly, *Itgb6^{-/-}* mice which have defective TGFβ-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]. TGFβ 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 TGFβ results in a severe psoriasis-like skin phenotype in mice [18,19]. Thus, TGFβ can participate in growth regulation, maintenance of tolerance and induction of inflammation in the skin.

TGFβ is also required for retention of immune cell in epidermis during steady-state conditions. Autocrine TGFβ expressed by Langerhans cells (LC) is required for their maintenance in the epidermis as LC-specific ablation of *TGFβ* or *TGFβRII* results in spontaneous migration of LC from the epidermis to regional lymph nodes [20–22]. Similarly, *Itgb6^{-/-} Itgb8^{ΔKC}* mice that lack both TGFβ-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 TGFβ for their persistence. These cells are absent

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from *Itgb6*^{-/-}*Itgb8*^{ΔKC} mice and in mice treated with neutralizing mAbs to $\alpha_v\beta_6$ [5,23,24]. Despite the clear importance of TGF β for the maintenance of skin homeostasis, the cellular source of TGF β in steady-state skin remains unclear. Herein, we established that KC represent the major cell type expressing TGF β in the skin and developed mice with a KC-specific ablation of TGF β (*TGF β* ^{ΔKC} mice). We observed increased KC growth but found that skin immune homeostasis was largely unaffected. From these data, we conclude that KC-derived TGF β is not required for skin immune homeostasis.

2. Materials and methods

2.1. Mice

TGF- β ^{loxP}, *K14Cre*, *K14-CreER*^{T2} and *huLangerin-DTA* mice have been previously described [14,21,25]. *Itgb6*^{-/-} and *Itgb8*^{loxP} mice were provided by D. Sheppard (University of California, San Francisco). We crossed *K14-Cre* mice with *Itgb8*^{loxP} and *Itgb6*^{-/-} mice to obtain *Itgb6*^{-/-}*Itgb8*^{ΔKC} mice [5]. *K14CreER*^{T2} mice were bred with *TGF β* ^{loxP} mice and *ROSA26.LSL.YFP* (Jackson Laboratories) reporter mice resulting *TGF β* ^{ΔKC} 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 g body weight. 5 mg of 4-Hydroxytamoxifen (H7904-5MG; Sigma-Aldrich) was dissolved in 1.25 ml \geq 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 μ l/shaved flank and 10 μ l/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⁺MHCII⁺, CD45⁻ MHCII⁻, CD45⁺MHCII⁻CD3⁺TCR $\gamma\delta$ ⁺, 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- β 1 and ABI Prism 7900 HT (Applied Biosystems) were used for quantitative PCR. All kits were performed according the manufacturer's instructions. All Ct values were normalized to hypoxanthine phosphoribosyl-transferase (*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 CO₂ incubator at 37 °C 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 μ m 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 β (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 (3 M, 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 h 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- μ 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 30s 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 μ 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 β in the epidermis

Epidermal cells including KC, dendritic epidermal T cells (DETC) and Langerhans Cells (LC) have all been reported to produce TGF β [26,27]. First, we sought to determine which cell type is the dominant source of TGF β in the epidermis. Single cell suspensions of epidermis were FACSsorted to obtain pure populations of KC (CD45⁻MHCII⁻), DETC (CD45⁺MHCII⁻CD3⁺TCR $\gamma\delta$ ⁺) and LC (CD45⁺MHCII⁺). 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 β 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 β 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- β in epidermis during steady state conditions.

3.2. Generation and validation of TGF $\beta^{\Delta KC}$ mice

To determine the requirement of KC-derived TGF β , we generated tamoxifen (TAM)-inducible keratinocyte-specific TGF β -deficient mice. K14CreER^{T2} mice were bred with TGF $\beta^{fl/fl}$ mice and ROSA26.YFP reporter mice resulting in K14-CreER^{T2} TGF $\beta^{fl/fl}$ (TGF $\beta^{\Delta KC}$). We first administered TAM i.p. over five consecutive days to allow for the inducible deletion of TGF β in KC. Unexpectedly, the majority of TGF $\beta^{\Delta KC}$ but not TAM treated control mice (TGF β^{WT}) 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 β at these sites.

To specifically test the requirement of KC-derived TGF β in skin, we next applied 2 mg/ml 4OH-Tamoxifen (4OH-TAM) topically on TGF $\beta^{\Delta KC}$ 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 $\beta^{\Delta KC}$ 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⁻MHCII⁻CD34⁻Sca-1⁺), bulge KC (CD45⁻MHCII⁻Sca-1⁻CD34⁺), and isthmus KC (IM:CD45⁻MHCII⁻Sca-1⁻EpCAM⁺) 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 β , 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 $\beta^{\Delta KC}$ but not control mice (Fig. 2f). In addition, we isolated YFP⁺ and YFP⁻ KC from 4OH-TAM treated TGF $\beta^{\Delta KC}$ mice and found greatly reduced levels of TGF $\beta 1$ in YFP⁺ but not YFP⁻ KC (Fig. 2g). From these data, we conclude that topical 4OH-tamoxifen application to TGF $\beta^{\Delta KC}$ mice allows for the efficient deletion of TGF β and that expression of YFP accurately reports the loss of TGF β expression.

During these experiments, we observed that often some cohorts of TGF $\beta^{\Delta KC}$ 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⁺ KC were used for analysis (Fig. 2h, red box).

3.3. KC-derived TGF β 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 $\beta^{\Delta KC}$ but not control mice (Fig. 3a). Quantification determined that the increased acanthosis was significant (Fig. 3b). TGF β 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 β in 4OH-TAM treated

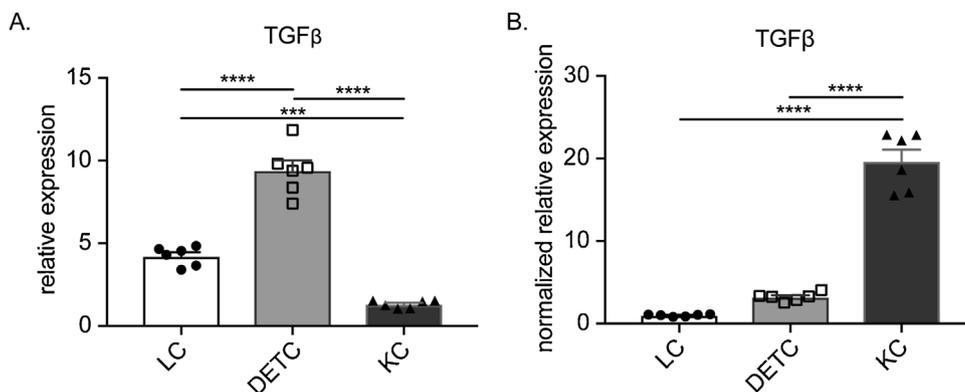


Fig. 1. Keratinocytes are the major source of TGF β in the epidermis.

(A) TGF $\beta 1$ mRNA expression levels from FACSsorted LC, DETC and KC as analyzed by qRT-PCR normalized to *Hprt*. (B) TGF $\beta 1$ 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 representative of at least 2 independent experiments. Data are means \pm SEM. *** $p < 0.001$; **** $p < 0.0001$.

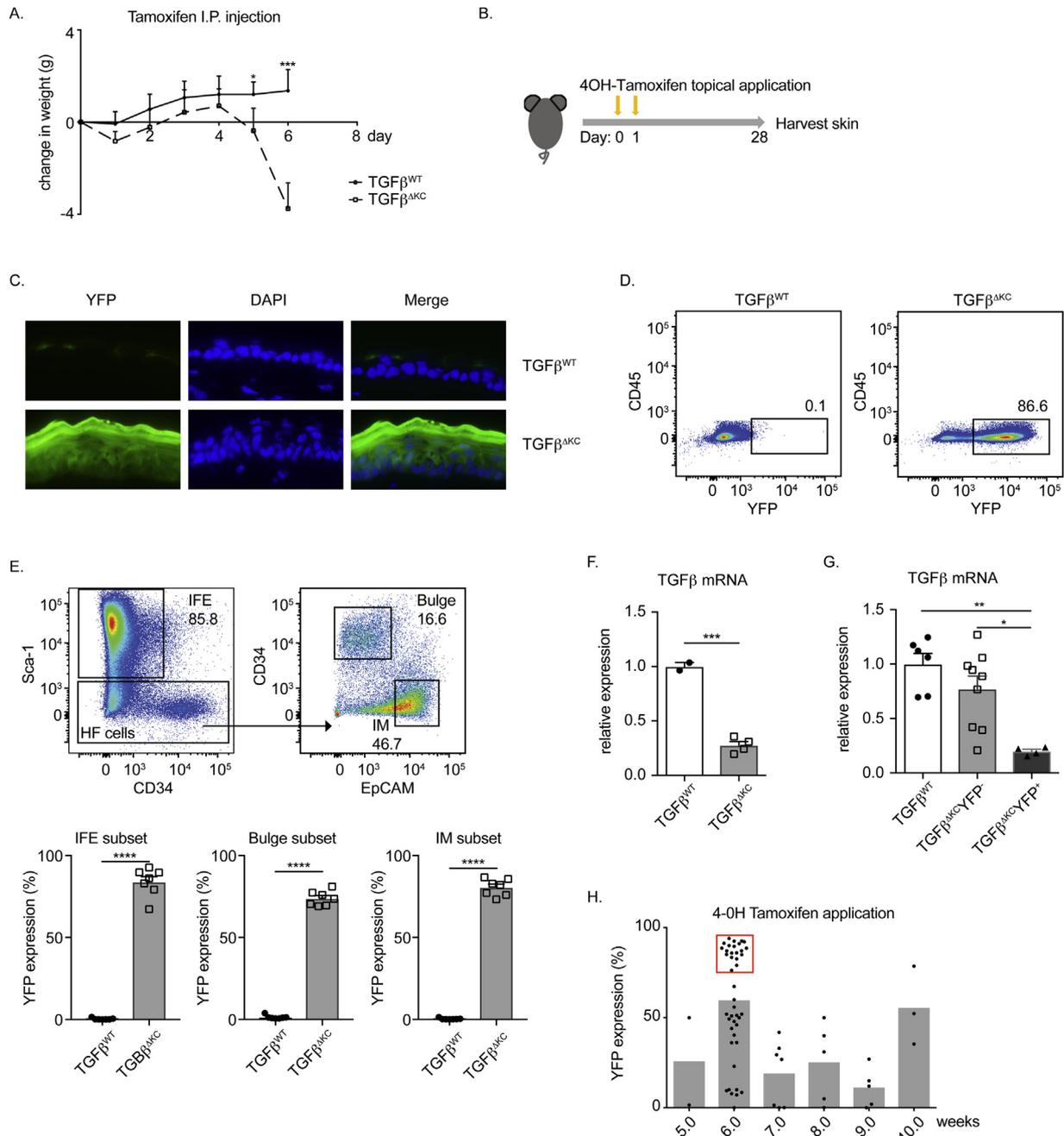


Fig. 2. Generation and validation of $TGF\beta^{\Delta KC}$ mice.

(A) 10 mg/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 $TGF\beta^{WT}$ (solid line) and $TGF\beta^{\Delta KC}$ (dashed line) mice. (B) 4OH-Tamoxifen topical application protocol: 2 mg/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 $TGF\beta^{WT}$ and $TGF\beta^{\Delta KC}$ flank skin illustrates epidermal expression of YFP (green) and DAPI nuclear label (blue). (D) Flow cytometry staining of YFP in KC (live single $CD45^{-}$). (E) Gating strategy of KC population: interfollicular: IFE-, Bulge- and isthmus: IM-KC subsets and frequency of YFP expression in each KC subsets. Cells were gated on live single $CD45^{-}$ cells. (F) *Tgfb1* mRNA relative expression level normalized to *Gapdh* in epidermis from $TGF\beta^{WT}$ and $TGF\beta^{\Delta KC}$ 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 $TGF\beta^{WT}$ mice and YFP⁺ and YFP⁻ KC from $TGF\beta^{\Delta KC}$ mice. The average gene expression level in KC from $TGF\beta^{WT}$ 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 \pm SEM. * p < 0.05; ** p < 0.01; *** p < 0.001, **** p < 0.0001.

$TGF\beta^{\Delta KC}$ 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 $TGF\beta^{\Delta KC}$ 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 $CD45^{+}$ cells, LC,

TCR $\gamma\delta$ T cells or innate lymphoid cells (ILC) (Fig. 3c). To confirm these results, we examined epidermal whole mounts by immunofluorescent microscopy. The epidermis of $TGF\beta^{\Delta KC}$ 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 $CD45^{+}$ cells was not altered in 4OH-TAM treated $TGF\beta^{\Delta KC}$ mice (Fig. 3f) compared with control mice. In

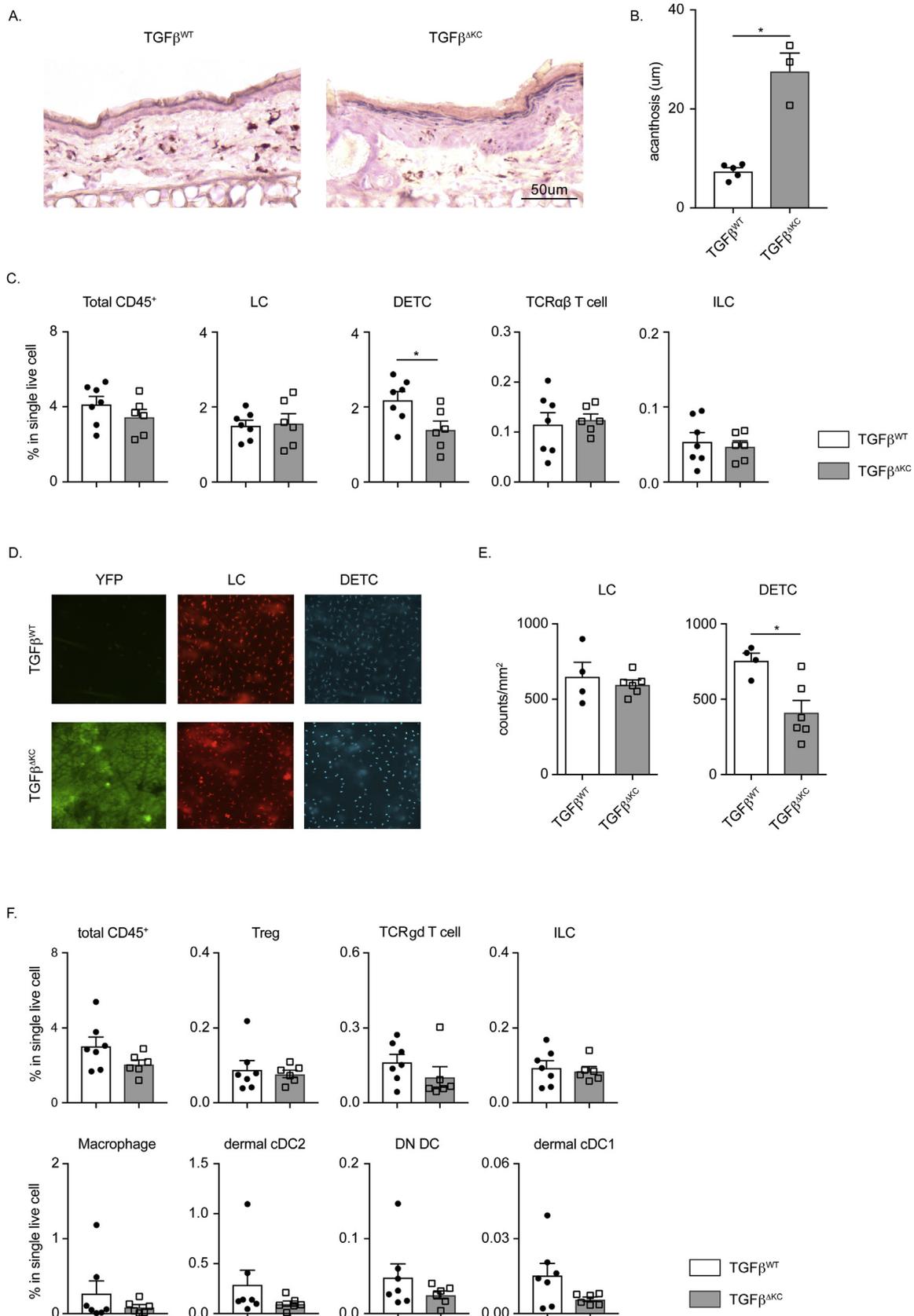


Fig. 3. KC-derived TGF β is not necessary to maintain skin immune homeostasis.

(A) Representative H&E staining of transverse ear section of TGF β^{WT} and TGF $\beta^{\Delta KC}$ mice. (B) Epidermal thickness in TGF β^{WT} and TGF $\beta^{\Delta KC}$ mice is shown. (C) Flow cytometry analysis of epidermal single cell suspension from TGF β^{WT} and TGF $\beta^{\Delta KC}$ mice. Frequency of the indicated population in total live single cells are shown. (D) Immunofluorescent microscopic visualization whole mounted epidermis from TGF β^{WT} and TGF $\beta^{\Delta KC}$ 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 β^{WT} and TGF $\beta^{\Delta KC}$ 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. * $p < 0.05$.

addition, no changes in the frequency of Treg (CD45⁺Thy1.2⁺TCRb⁻CD3⁺CD4⁺Foxp3⁺), TCRγδ T cells (CD45⁺Thy1.2⁺TCRb⁻CD3^{mid}), ILC (CD45⁺Thy1.2⁺TCRb⁻CD3⁻), Macrophages (CD45⁺MHCII⁺CD64⁺CD11b⁺) and dendritic cells subsets (dermal cDC2, CD45⁺MHCII⁺CD64⁻CD11b⁺; DN DC, CD45⁺MHCII⁺CD64⁻CD11b⁻; dermal cDC1, CD45⁺MHCII⁺CD64⁻CD207⁺CD103⁺). Taken together, we conclude that the absence of KC-derived TGFβ has no effect on immune homeostasis in steady state.

3.4. KC-mediated activation of TGFβ is not necessary to maintain skin immune homeostasis

We next sought to determine whether activation of TGFβ by integrins α_vβ6 and α_vβ8 expressed by keratinocytes is required to maintain skin immune homeostasis. We used *Itgb6*^{-/-} × *K14Cre Itgb8*^{fl/fl} (*Itgb6*^{-/-}*Itgb8*^{ΔKC}) mice that lack both TGFβ-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*^{-/-}*Itgb8*^{ΔKC} 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*^{-/-}*Itgb8*^{ΔKC} mice, have a dramatic absence of LC. To ensure that any observe phenotype derived from the absence of α_vβ6 and α_vβ8 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*^{-/-}*Itgb8*^{ΔKC} and huLangerin-DTA mice (Fig. 4b). We did not observe a significant alteration in the frequency of total CD45⁺ cells, TCRαβ T cells, DETC or ILC in any group. Notably, unlike *TGFβ*^{ΔKC} mice, the frequency of DETC were unaffected. In dermis, the frequency of Treg, TCRγδ T

cells were unaffected. In dermis, the frequency of Treg, TCRγδ T

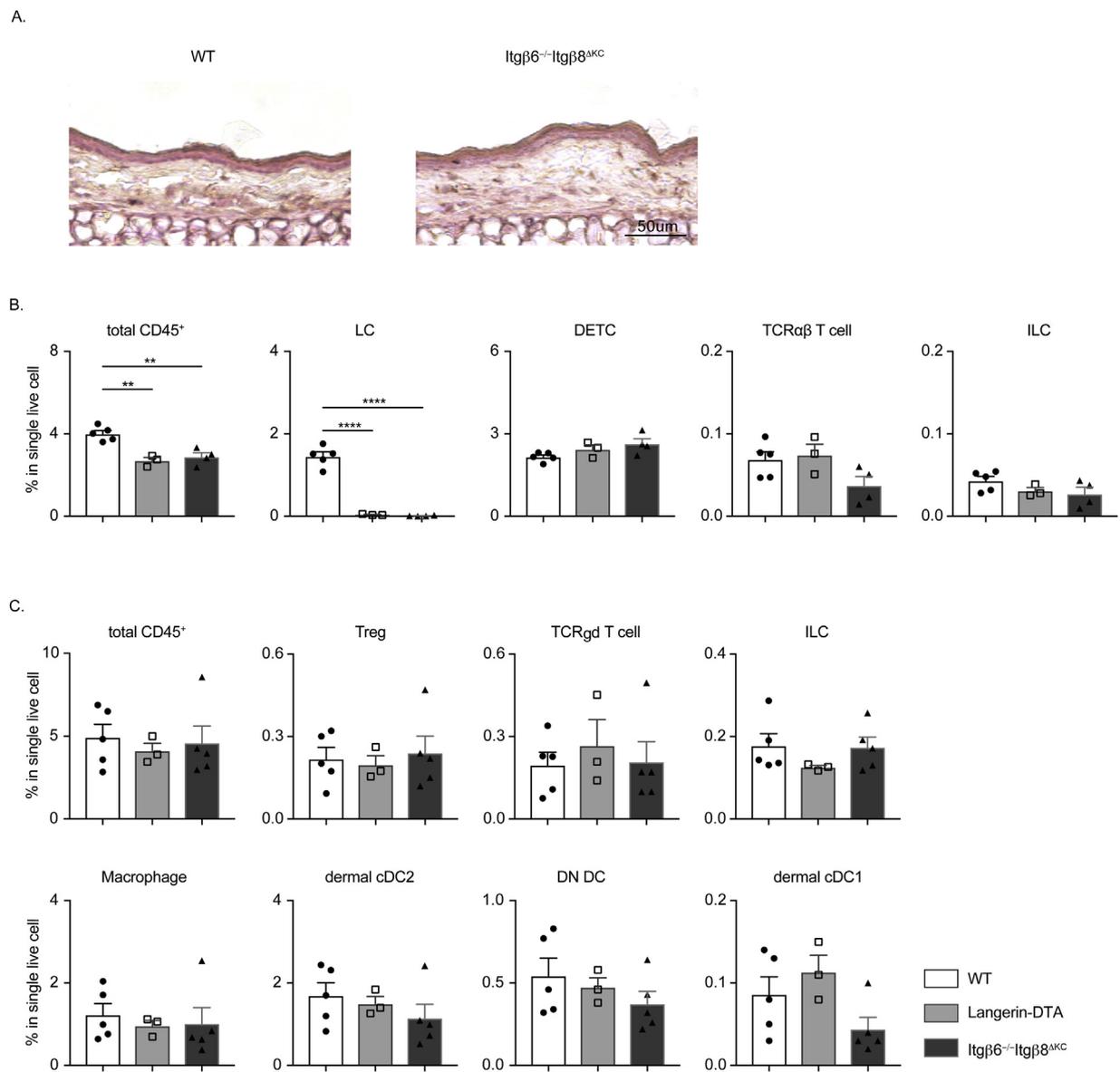


Fig. 4. KC-mediated activation of TGFβ is not necessary to maintain skin immune homeostasis. (A) Representative H&E staining of transverse ear section of WT and *Itgb6*^{-/-}*Itgb8*^{ΔKC} mice. (B and C) Flow cytometry analysis of epidermal (B) and dermal (C) single cell suspension from WT, huLangerin-DTA and *Itgb6*^{-/-}*Itgb8*^{ΔKC} 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.

cells, ILC, Macrophages and DC was unaltered in *Itgb6^{-/-}Itgb8^{ΔKC}* mice (Fig. 4c). From these data, we conclude that KC-mediated activation of TGFβ 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 TGFβ 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 TGFβ and epidermal activation of TGFβ are not required for maintenance of skin immune homeostasis. The role of TGFβ from other epidermal sources and dermis remains important to be explored.

Given the known importance of TGFβ to suppress effector CD4⁺ T cells and Tregs which can be found in the skin [29–31], we anticipate that inducible ablation of KC-derived TGFβ or ablation $\alpha_v\beta_6$ and $\alpha_v\beta_8$ -mediated TGFβ-activation would result in loss of T cell tolerance to skin antigens. The absence of spontaneous inflammation indicates that epidermal TGFβ is either not required or redundant. It is likely that autocrine/paracrine TGFβ 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 TGFβ was required for the maintenance of LC [21,22]. Our data showing that loss of KC-derived TGFβ did not affect LC number in epidermis further supports this finding and demonstrated that KC-derived TGFβ 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 *TGFβ^{ΔKC}* mice yield >80% efficiency of TGFβ deletion as measured by direct measure of TGFβ mRNA and expression of YFP which we demonstrated efficiently phenocopied levels of TGFβ mRNA. Importantly, we did observe the expected phenotype of increased acanthosis indicating a functional absence of TGFβ. We also noted a modest decrease in DETC numbers in tamoxifen treated *TGFβ^{ΔKC}* mice. We do not think that epidermal retention of DETC is dependent on TGFβ given the normal numbers of DETC observed in *Itgb6^{-/-}Itgb8^{ΔKC}* mice and *TGFβ^{-/-}* mice [20]. Rather, we suspect that reduced DETC numbers is an indirect effect in *TGFβ^{ΔKC}* mice.

There were two unexpected and unusual observations in the *TGFβ^{ΔKC}* model. First, the majority of *TGFβ^{ΔKC}* 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 *TGFβ^{-/-}* do develop severe esophageal and pharyngeal inflammation [34]. We also noted that highly efficient ablation of TGFβ 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 TGFβ and $\alpha_v\beta_6$ during hair development, this phenomena may only occur when the targeting of TGFβ [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 TGFβ in the context of

autoimmunity, fibrosis and infection remain as exciting future directions.

Funding

This work benefitted from SPECIAL BD LSRFORTESSATM funded by NIH1S10OD011925-01. YY was financially supported by China Scholarship Council (CSC) No 201706370257, T.H. by JSPS Overseas Research Fellowships and DHK by R01AR060744.

Conflict of interest

The authors have no conflict of interest to declare

Acknowledgments

We thank the members of the Kaplan laboratory and members throughout the departments of Dermatology and Immunology for helpful discussions. We also thank the Division of Laboratory Animal Resources of the University of Pittsburgh for excellent animal care.

References

- [1] J. Massagué, The transforming growth factor-beta family, *Annu. Rev. Cell Biol.* 6 (1990) 597–641.
- [2] M. Shi, J. Zhu, R. Wang, X. Chen, L. Mi, T. Walz, et al., Latent TGF-β structure and activation, *Nature*. 474 (2011) 343–349.
- [3] Z. Yang, Z. Mu, B. Dabovic, V. Jurukovski, D. Yu, J. Sung, et al., Absence of integrin-mediated TGFβ1 activation in vivo recapitulates the phenotype of TGFβ1-null mice, *J. Cell Biol.* 176 (2007) 787–793.
- [4] P. Aluwihare, Z. Mu, Z. Zhao, D. Yu, P.H. Weinreb, G.S. Horan, et al., Mice that lack activity of alphavbeta6- and alphavbeta8-integrins reproduce the abnormalities of *Tgfb1*- and *Tgfb3*-null mice, *J. Cell. Sci.* 122 (2009) 227–232.
- [5] J. Mohammed, L.K. Beura, A. Bobr, B. Astry, B. Chicoine, S.W. Kashem, et al., Stromal cells control the epithelial residence of DCs and memory T cells by regulated activation of TGF-β, *Nat. Immunol.* 17 (2016) 414–421.
- [6] J.A. Pietenpol, J.T. Holt, R.W. Stein, H.L. Moses, 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.
- [7] J.W. Penn, A.O. Grobbelaar, K.J. Rolfe, The role of the TGF-β family in wound healing, burns and scarring: a review, *Int. J. Burns Trauma* 2 (2012) 18–28.
- [8] M.O. Li, Y.Y. Wan, S. Sanjabi, A.-K.L. Robertson, R.A. Flavell, Transforming growth factor-beta regulation of immune responses, *Annu. Rev. Immunol.* 24 (2006) 99–146.
- [9] M.M. Shull, I. Ormsby, A.B. Kier, S. Pawlowski, R.J. Diebold, M. Yin, et al., Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease, *Nature* 359 (1992) 693–699.
- [10] A.B. Kulkarni, C.G. Huh, D. Becker, A. Geiser, M. Lyght, K.C. Flanders, 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.
- [11] S.A. Oh, M.O. Li, TGF-β: guardian of T cell function, *J. Immunol.* 191 (2013) 3973–3979.
- [12] M.O. Li, S. Sanjabi, R.A. Flavell, Transforming growth factor-β controls development, homeostasis, and tolerance of T cells by regulatory T cell-dependent and-independent mechanisms, *Immunity* 25 (3) (2006) 455–471.
- [13] J.C. Marie, D. Liggitt, A.R. Immunity, Cellular mechanisms of fatal early-onset autoimmunity in mice with the T cell-specific targeting of transforming growth factor-β receptor, *Immunity* 25 (3) (2006) 441–454.
- [14] M.O. Li, Y.Y. Wan, R.A. Flavell, T cell-produced transforming growth factor-beta 1 controls T cell tolerance and regulates Th1- and Th17-cell differentiation, *Immunity* 26 (5) (2007) 579–591.
- [15] X.Z. Huang, J.F. Wu, D. Cass, D.J. Erle, D. Corry, S.G. Young, 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.
- [16] C.A. Lowell, T.N. Mayadas, Overview: studying integrins in vivo, *Methods Mol. Biol.* 757 (2012) 369–397.
- [17] R.J. Coffey, C.C. Bascom, N.J. Sipes, R. Graves-Deal, B.E. Weissman, H.L. Moses, Selective inhibition of growth-related gene expression in murine keratinocytes by transforming growth factor beta, *Mol. Cell. Biol.* 8 (1988) 3088–3093.
- [18] K. Sellheyer, J.R. Bickenbach, J.A. Rothnagel, D. Bundman, M.A. Longley, T. Krieg, 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.
- [19] A.G. Li, D. Wang, X.-H. Feng, X.-J. Wang, Latent TGFβ1 overexpression in keratinocytes results in a severe psoriasis-like skin disorder, *Embo J.* 23 (2004) 1770–1781.

- [20] T.A. Borkowski, J.J. Letterio, A.G. Farr, M.C. Udey, 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.
- [21] D.H. Kaplan, M.O. Li, M.C. Jenison, W.D. Shlomchik, R.A. Flavell, M.J. Shlomchik, Autocrine/paracrine TGFbeta1 is required for the development of epidermal Langerhans cells, *J. Exp. Med.* 204 (2007) 2545–2552.
- [22] A. Bobr, B.Z. Igyarto, K.M. Haley, M.O. Li, R.A. Flavell, D.H. Kaplan, Autocrine/paracrine TGF- β 1 inhibits Langerhans cell migration, *Proc. Natl. Acad. Sci. U. S. A.* 109 (2012) 10492–10497.
- [23] D. Mu, S. Cambier, L. Fjellbirkeland, J.L. Baron, J.S. Munger, H. Kawakatsu, et al., The integrin α v β 8 mediates epithelial homeostasis through MT1-MMP-dependent activation of TGF- β 1, *J. Cell Biol.* 157 (3) (2002) 493–507.
- [24] J.S. Munger, X. Huang, H. Kawakatsu, M.J. Griffiths, S.L. Dalton, J. Wu, et al., A mechanism for regulating pulmonary inflammation and fibrosis: the integrin α v β 6 binds and activates latent TGF β 1, *Cell* 96 (3) (1999) 319–328.
- [25] D.H. Kaplan, M.C. Jenison, S. Saeland, W.D. Shlomchik, M.J. Shlomchik, Epidermal Langerhans cell-deficient mice develop enhanced contact hypersensitivity, *Immunity* 23 (2005) 611–620.
- [26] M.S. Gruschwitz, O.P. Hornstein, Expression of transforming growth factor type beta on human epidermal dendritic cells, *J. Invest. Dermatol.* 99 (1992) 114–116.
- [27] F. Granucci, G. Girolomoni, M.B. Lutz, M. Foti, G. Marconi, P. Gnocchi, et al., Modulation of cytokine expression in mouse dendritic cell clones, *Eur. J. Immunol.* 24 (1994) 2522–2526.
- [28] C.M. Smith, T.F. Hayamizu, J.H. Finger, S.M. Bello, I.J. McCright, J. Xu, et al., The mouse Gene Expression Database (GXD): 2019 update, *Nucleic Acids Res.* 47 (2019) D774–D779.
- [29] J.C. Marie, J.J. Letterio, M. Gavin, A.Y. Rudensky, TGF-beta1 maintains suppressor function and Foxp3 expression in CD4+CD25+ regulatory T cells, *J. Exp. Med.* 201 (2005) 1061–1067.
- [30] V. Viglietta, C. Baecher-Allan, H.L. Weiner, D.A. Hafler, Loss of functional suppression by CD4+CD25+ regulatory T cells in patients with multiple sclerosis, *J. Exp. Med.* 199 (2004) 971–979.
- [31] N. Ali, M.D. Rosenblum, Regulatory T cells in skin, *Immunology* 152 (2017) 372–381.
- [32] M.A. Travis, B. Reizis, A.C. Melton, E. Masteller, Q. Tang, J.M. Proctor, et al., Loss of integrin alpha(v)beta8 on dendritic cells causes autoimmunity and colitis in mice, *Nature* 449 (2007) 361–365.
- [33] A.C. Melton, S.L. Bailey-Bucktrout, M.A. Travis, B.T. Fife, J.A. Bluestone, D. Sheppard, Expression of α v β 8 integrin on dendritic cells regulates Th17 cell development and experimental autoimmune encephalomyelitis in mice, *J. Clin. Invest.* 120 (2010) 4436–4444.
- [34] L. Yaswen, A.B. Kulkarni, T. Fredrickson, B. Mittleman, R. Schiffman, S. Payne, et al., Autoimmune manifestations in the transforming growth factor-beta 1 knockout mouse, *Blood* 87 (1996) 1439–1445.
- [35] C. Hou, Y. Miao, X. Wang, C. Chen, B. Lin, Z. Hu, Expression of matrix metalloproteinases and tissue inhibitor of matrix metalloproteinases in the hair cycle, *Exp. Ther. Med.* 12 (2016) 231–237.
- [36] K. Foitzik, G. Lindner, S. Mueller-Roeber, M. Maurer, N. Botchkareva, V. Botchkarev, et al., Control of murine hair follicle regression (catagen) by TGF- β 1 in vivo, *FASEB J.* 14 (5) (2000) 752–760.
- [37] Y. Xie, K.J. McElwee, G.R. Owen, L. Häkkinen, H.S. Larjava, Integrin β 6-deficient mice show enhanced keratinocyte proliferation and retarded hair follicle regression after depilation, *J. Invest. Dermatol.* 132 (3) (2012) 547–555.