The role of epidermal TGFβ activating integrins in Langerhans cell migration

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In addition to providing a physical barrier, the epidermis provides a niche for the long-term residency of Langerhans cells (LC). LC provide protection by priming elements of the adaptive immune system, but they can also participate in the maintenance and development of autoimmune and inflammatory diseases. Within the epidermis, LC possess the ability to migrate from the skin epidermis to regional lymph nodes (LN). At steady-state, LC migrate to LN transporting self and potentially commensal-derived antigen to promote the development of peripheral tolerance. During infection, LC transport potential pathogen-derived antigens and prime naïve adaptive immune responses. The mechanisms that allow for the long-term retention of these leukocytes has not been extensively studied. Hence, elucidating the mechanisms by which LC maintain their epidermal residency and mediate migration is of great therapeutic interest and will extend our knowledge in LC biology.

Epidermal integrins αvβ6 and αvβ8 on keratinocytes (KCs) cleave LAP-TGFβ1 to its active form TGFβ1 which acts on the TGFβ receptor of LCs to maintain their long-term residency within the skin epidermis. We have also demonstrated that selective expression of a ligand independent constitutively active form of TGFβRI, inhibits LC migration during homeostasis and in response to UVB exposure. Interestingly, UVB treatment resulted in the loss of epidermal integrins αvβ6 and αvβ8 on KCs. However, whether inflammation induced LC migration acts through a similar
mechanism remains unclear. Thus, we propose to test the hypothesis that inflammatory stimuli mediate LC migration from the epidermis through the inhibition of KC-integrin mediated TGF-β activation.

Here we demonstrate that LC migration in response to inflammatory stimuli was also inhibited by ligand independent TGFβRI signaling. Unlike UVB stimulation which reduced KCs expression of avβ6, in vitro and in vivo exposure to TNF-α or IL-1β increased avβ6 mRNA and protein expression by KCs. As a result, KC increase transactivation of latent TGFβ. Expression of avβ8 was largely unchanged. These findings demonstrate that ligand independent TGFβRI signaling in LC can overcome inflammatory migration stimuli, but reduced KCs-mediated transactivation of latent TGFβ by KCs may only drive LC migration during homeostasis and in response to UVB stimulation.
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Abbreviations

LC: Langerhans Cell
LN: Lymph Nodes
TGFβ: Transformative Growth Factor βeta
UVB: Ultraviolet Light in the B Spectrum
KC: Keratinocytes
pKC: primary Keratinocytes
DC: Dendritic Cells
TCRγδ T-cells: gamma delta T-cells
CD8+ T RM: CD8+ resident memory T-cells
CD4+ T RM: CD4+ resident memory T-cells
ILC: Innate Lymphoid Cell
T FH: CD4+ Follicular Helper T-cells
T reg: CD4+ Regulatory T-cells
Th17: CD4+ Helper 17 T-cells
CHS: Contact Hypersensitivity
DTR: Diptheria Toxin Receptor
DTA: Diptheria Toxin Subunit A
MHCII: Major Histocompatibility Complex II
LAP: Latency Associated Peptide
DNTB: Dinotrothiocyanobenzene
DNFB: Dinitrofluorobenzene
DMBA: 7,12-Dimethylbenz[a]anthracene

TAM: Tamoxifen

PRR: Pattern Recognition Receptors

TLR: Toll-Like Receptors

CLR: C-type Lectin Receptors

NOD: Nucleotide binding Oligomerization Domain

NLRs: NOD-Like Receptors (NLRs)

TGFβRCA: Constitutively Active TGFβRI

IFE KC: Interfollicular KC

IM KC: Isthmus KC

I.D.: Intradermal

Tln1: Talin1

Fermt1: Kindlin1

tMLECS: thymic Mink Lung Epithelial Cells

FT- β6: Integrin β6 reporter fluorescent timer mouse

RLU: Relative Light Units
1.0 Introduction and Background

1.1 The epidermal niche for cells of the immune system

The skin is the largest organ in the human body, providing both physical and immunological protection. Anatomically, the skin consists of three layers: the hypodermis (also known as the subcutaneous layer), the dermis, and the epidermis. Each cutaneous layer is uniquely populated with a plethora of immune and non-immune cells such as fibroblasts, myoblast, keratinocytes (KCs), dendritic cells (DC), macrophages, mast cells, B cells, T-cells, etc (Raziyeva et al., 2021; Yousef, Alhajj, & Sharma, 2021). Together, these cells maintain steady-state tissue homeostasis and orchestrate efficient immune responses against a wide range of biologic and non-biologic stimuli.

The skin epidermis is predominantly composed of KCs of ectodermal origin, merkel cells, and melanocytes (T. Kobayashi, Naik, & Nagao, 2019). KCs provide a niche to house cells of the immune system such as gamma delta T-cells (TCRγδ T-cells) and Langerhans cells (LC). As the skin begins to experience pathogenic insults, CD8+ resident memory T-cells (CD8+ T_{RM}) and CD4+ resident memory T-cells (CD4+ T_{RM}) also establish epidermal residency (Clark, 2015). It has been suggested that the epidermis also contains different type of innate lymphoid cells (ILCs) (Ricardo-Gonzalez et al., 2018).

Notably, the skin epidermis of mice is quite distinct from human epidermis. In humans, the epidermis is about six to ten cells layers of KCs, while mouse epidermis is only composed of
three layers of KCs (T. Kobayashi et al., 2019). There are also distinct immune cell populations in mouse versus human epidermis. The mouse epidermis is home to TCRγδ T-cells, LC, and CD8+ T_{RM}. In contrast to mouse epidermis, human epidermis lack TCRγδ T-cell, but is home to LC, CD8+ T_{RM}, and CD4+ T_{RM} (Clark, 2015). Another key difference, is that human LC expresses a distinct set of antigen-presenting molecules such as CD1a, CD1b, CD1c, which enables them to present lipid derived antigens to naïve T-cells (Collin & Bigley, 2018). Despite these differences, we have discovered many fundamental principles in mouse skin immunology that have improve our understanding of human skin diseases.

In terms of host-immune function, TCRγδ T-cells are thought to participate in wound healing, while LC are key in initiating adaptive immune responses by constantly capturing, processing, and delivering antigen to the draining lymph nodes (LN) (Kaplan, 2017; Nielsen, Witherden, & Havran, 2017). Meanwhile, CD8+ T_{RM} and CD4+ T_{RM} re-activate in response to previously encountered stimuli (Muller, Dandie, Ragg, & Woods, 1993). Although, LC and CD8+T_{RM} provide distinct host immune responses, they both require Transforming Growth Factor βeta (TGFβ) signaling for their long-term epidermal residency (Mohammed et al., 2016). Notably, TCRγδ T-cells epidermal numbers are unaffected in the absence of TGFβ (Borkowski, Letterio, Farr, & Udey, 1996). It remains unclear whether epidermal human CD8+ T_{RM} or CD4+ T_{RM} required TGFβ for their long-term epidermal residency. However, human skin treated with Losartan, an antagonist to the angiotensin II type I receptor that also suppresses TGFβ signaling, results in a significant reduction in LC numbers when compared to control skin (Mohammed et al., 2016). Thus, TGFβ signaling is key for mouse and potentially human LC epidermal retention.
1.2 LC function in skin immunology and ontogeny

At steady-state, LC is the only antigen-presenting cell type in the epidermis. These epidermal antigen-presenting cells were first observed by Paul Langerhans in 1868 and were uniquely distinguished by their dendritic-like morphology. The identity of LC remains elusive, since by basis of ontogeny and cell-programming they are closely related to macrophages, however LC displays classical DC functions and constitutively migrate to the draining LNs to interact with naïve T-cells(Kaplan, 2017). Thus, expanding our knowledge on LC biology is imperative to gain a better understand of how LC integrate microenvironment signals to elicit unique adaptive immunological responses.

1.2.1 Migratory properties of LC and initiation of adaptive immune responses

Given their location at the interface between the skin and the external environment, LC are the first line of immunological defense. LC have the ability to acquire and process foreign and self-antigens(Schuler & Steinman, 1985; Steinman, 1991). LC then transport these antigens to the regional lymph nodes where they initiate antigen specific adaptive immune responses or promote peripheral self-tolerance(Kaplan, 2017). The ability of LC to migrate into the draining LN to initiate T-cell mediated adaptive immune responses was the foundation of what is now known as dendritic cell/LC paradigm. Egress from the epidermal compartment requires LC to disengage from surrounding KCs by suppressing E-cadherin -a cell adhesion molecule that is expressed by both KCs and LC(Tang, Amagai, Granger, Stanley, & Udey, 1993). Then, LC cross the epidermal
basal membrane by breaking down the extracellular matrix via expression of metalloprotease 2 and 9 (Ratzinger et al., 2002). Once in the dermal compartment, LC begin migration into the afferent lymphatic vessels by engaging LC-CCR7 receptor with chemokines CCL19 and CCL21 that are produced by lymphatic endothelial cells (Ohl et al., 2004; Tal et al., 2011). Once in the draining LNs, LC accumulate in the inner paracortex where they interact with naïve T-cells to innate T-cell mediated responses (Kissenpfennig et al., 2005).

1.2.2 The role of LC in Th17 T-cell differentiation and humoral responses

LC play a significant role in the differentiation of CD4+ T helper 17 cells (Th17) and CD4+ follicular helper T-cells (T_{FH}) humoral responses. It has been demonstrated that LC are required for the development of Th17 responses against epicutaneous C. albicans infection that results in the establishment of dermal antigen specific CD4+T_{RM} that clear future encounters with cutaneous infection with C. albicans (Igyarto et al., 2011; Kashem et al., 2015; Mathers et al., 2009). Th17 differentiation in response to C. albicans is dependent on LC engaging with ligands for the C-type lectin receptor Dectin-1. Engagement of the Dectin-1 receptor in LC results production of IL-6, a cytokine that is required for Th17 differentiation. Moreover, mice with specific KCs ablation of the protease ADAM17 results in spontaneous cutaneous dysbiosis follow by an overgrowth in S. aureus infection. Interestingly, the absence of ADAM17 in KCs prevented the generation of IL-17 producing CD4+ and TCR\(\gamma\delta\) T-cells (T. Kobayashi et al., 2015).
LC also play an important role in the development of humoral immunity. In response to cutaneous antigen, LC promote the development of T\textsubscript{FH} that in turn support the development of plasma cells and germinal centers (Levin et al., 2017). In a model of intradermal immunization and infection with \textit{L. major}, LC deficient mice had reduced germinal centers and T\textsubscript{FH} responses (Yao et al., 2015; Zimara et al., 2014). It has also been demonstrated that antigen targeted selectively to LC results in the expansion of T\textsubscript{FH} populations and supports germinal center formation that in turn results in the production of IgG1 (Lahoud et al., 2011). Altogether, these studies demonstrate a key role for LC in the differentiation of Th\textsubscript{17} cells and their ability to promote the development of humoral responses.

1.2.3 LC and contact hypersensitivity

Hapten-induced contact hypersensitivity (CHS) is a mouse model of allergic contact dermatitis. In this model, a sensitizing hapten is used to immunize mice in the skin, and the T-cell mediated response is evaluated by application of the same hapten at a different anatomical site. The role that LC play in CHS is controversial due to different results using different mouse models of LC ablation. Depletion of LC using a muLangerin-DTR mouse does not affect CHS responses (Honda et al., 2010). However, in huLangerin-DTA mice, which constitutively lack LC, CHS responses are significantly exacerbated (Kaplan, Jenison, Saeland, Shlomchik, & Shlomchik, 2005). Acute ablation of LC using a huLangerin-DTR also increase CHS responses, however the CHS response was not as severe when compared to huLangerin-DTA mice (Bobr et al., 2010). Interestingly, constitutive depletion of the major histocompatibility complex II (MHCII) in LC, but not Myd88 increased CHS response (Haley et al., 2012; Igyarto et al., 2009). These findings
demonstrate a key role for LC in CHS responses, however it remains unclear how specifically LC mediates hapten-induced inflammatory responses.

### 1.2.4 Induction of tolerance by LC

LC migration into the draining LNs occurs homeostatically or in response to inflammatory stimuli. Similar to immature classical DC, immature LC migrate homeostatically into the draining LNs and promote peripheral tolerance. It has previously been shown that resting human LC activate and induce proliferation of skin-resident regulatory T-cells (T\(_{\text{reg}}\)) \textit{in vitro} (Seneschal, Clark, Gehad, Baecher-Allan, & Kupper, 2012). Interestingly, targeting antigen to LC in mice promotes T\(_{\text{reg}}\) proliferation that was only observed with self-antigen and not non-self-antigen (Idoyaga et al., 2013). Epicutaneous application of dinotrothiocyanobenzene (DNTB) induces tolerance against a similar compound dinitrofluorobenzene (DNFB), a stronger sensitizer, that is mediated by LC and CD8+ T-cell tolerance and activation of T\(_{\text{reg}}\) (Gomez de Aguero et al., 2012). Early studies demonstrated that LC loaded with melanin, long polymer chains made of derivatives of the amino acid tyrosine, are able to cross the basal membrane during steady state (Tobin, 1998). It remains unclear as to how LC participate in the maintenance or development of peripheral tolerance. However, these findings suggests that LC have a significant role in maintaining peripheral tolerance.
1.2.5 LC ontogeny

During ontogeny, myeloid progenitor cells derived from the yolk sac and to a lesser extent from the fetal liver, populate the skin epidermis where most likely they receive tissue-specific cues for their differentiation and maintenance (Hoeffel et al., 2012). Two days after birth, these myeloid progenitors proliferate and begin to differentiate into mature LC to establish the epidermal LC network (Chorro et al., 2009). At steady-state, LC continuously migrate into the dLNs, and their ability to self-renew replenishes a mature epidermal LC network (Merad et al., 2002).

Development and differentiation of LC are dependent on transcription factors Runx3, Id2, PU.1, Axl and the activation of the receptor CSFR-1 with IL-34-derived from KCs. In the absence of Runx3, Id2, PU.1, or Axl, there is profound reduction or full depletion of epidermal LC (T. Bauer et al., 2012; Chopin et al., 2013; Fainaru et al., 2004; Greter et al., 2012; Hacker et al., 2003; Y. Wang et al., 2012). Interestingly, these transcription factors and the tyrosine kinase receptor Axl, have been associated to TGFβ signaling. LC can be considered a constituent of the macrophage family based on their ontogeny and for their ability to self-renew in tissue at steady state. However, LC display DC functions and constitutively migrate into the draining LNs. Despite our progress in understanding LC identity and biology, it remains unclear how LC interact with other epidermal leukocytes and epidermal KCs. In this dissertation, we focus on further understanding of the intimate relationship between LC and KCs by exploring the requirement for TGFβ signaling for LC epidermal retention and migration.
1.3 TGFβ signaling in LC is required for epidermal retention

The epidermis of mice deficient of the cytokine TGFβ1 (TGFβ1−/−) is completely devoid of LC, but entry of myeloid precursors into the epidermis during ontogeny is not affected (Borkowski et al., 1996; Kel, Girard-Madoux, Reizis, & Clausen, 2010). Specific conditional ablation of TGFβRI or TGFβRII in LC recapitulates the spontaneous emigration from the skin into the regional LNs (Bobr et al., 2012; Borkowski et al., 1996; Kel et al., 2010). This phenomenon has also been observed with genes associated with the TGFβ1 pathway, such as Lamtor2. Conditional ablation of Lamtor2 results in LC egress from the epidermal compartment to accumulate into the draining LNs (Sparber et al., 2015). Additionally, genetic deletion of LC-specific TGFβ1 also results in their spontaneous migration into the LNs (Kaplan et al., 2007). We have previously shown that the conditional expression of a constitutively-active form of TGFβRI prevents LC homeostatic migration (Mohammed et al., 2016). These studies suggest that autocrine TGFβ1 is not required for LC differentiation, but instead for their long-term persistence in the epidermis, and that the loss of TGFβ1 results in LC migration into the draining LNs. Altogether, these findings clearly highlight the different role for TGFβ in LC long-term maintenance and migration.

1.4 TGFβ biology and activation

TGFβ is a pleiotropic cytokine that is produced by nearly all cell types and is secreted into the cellular matrix in its inactive form. Upon synthesis and secretion, TGFβ1 is bound to the
latency associated peptide (LAP) resulting in inactive TGFβ (LAP-TGFβ1)(Travis & Sheppard, 2014). Removal of the LAP protein from TGFβ1 can be accomplished through in vitro methods such as low pH or heat treatment(Worthington, Klementowicz, & Travis, 2011). Physiologically, the release of LAP from TGFβ1 is mediated through integrin-mediated cleavage, interaction with reactive oxygen species, mechanical stress, and enzymatic processes through thrombospondin-1, plasmin, cathepsin D, and metalloproteases MMP9 and MMP14(Crawford et al., 1998; Travis & Sheppard, 2014).

In mammals, there are three members of the TGFβ family of cytokines: TGFβ1, TGFβ2, and TGFβ3(Batlle & Massague, 2019). TGFβ1 is the predominant isoform that is expressed in the immune system. Mice with global ablation of TGFβ1 died 3-4 weeks of age due to multiorgan inflammation, while ablation of TGFβ2 and TGFβ3 also results in lethality due to development defects(Memon, Anway, Covert, Uzumcu, & Skinner, 2008; Travis & Sheppard, 2014). Interestingly, TGFβ1 deficient mice that were crossed with mice lacking MHCII or β2-microglobulin did not developed multi-organ inflammation, suggesting a significant role for adaptive immunity in mediating multiorgan inflammation(S. Kobayashi et al., 1999; Letterio et al., 1996). Indeed, it has been demonstrated that TGFβ1 is crucial in suppressing T-cell and dendritic cell function that results in autoimmunity(Travis & Sheppard, 2014).
1.5 LC requires transactivation of TGFβ through KCs-integrins avβ6 and avβ8

In the epidermis, release of active TGFβ1 from LAP is accomplished through an integrin mediated process. Integrins are a family of cell adhesion receptors that engage with extracellular matrix ligands, adjacent and self-surface ligands, and soluble ligands (Takada, Ye, & Simon, 2007). In humans, there are 18 α- and 8 β-subunits that generate 24 αβ transmembrane heterodimers that mediate different biological functions (Shimaoka & Springer, 2003). Integrins that activate TGFβ1 bind to the RGD (Arg-Gly-Asp) motif on LAP peptide. Although, there are several αβ heterodimers that bind to the RGD motif, integrins avβ6 and avβ8 are the most efficient at releasing active TGFβ1 from its LAP protein (Shi et al., 2011). Of importance, integrin avβ6 is exclusively expressed epithelial cells such as the lung, gut/intestines, skin, and kidney (Koivisto, Bi, Hakkinen, & Larjava, 2018). In comparison, integrin avβ8 is predominantly expressed on hematopoietic cells and follicular KC (Mohammed et al., 2016; Worthington, Fenton, Czajkowska, Klementowicz, & Travis, 2012). These data raised the possibility that activation of TGFβ1 that is required for LC epidermal persistence, could be mediated through an integrin mediated process. Indeed, global genetic ablation of Itgb6 or specific ablation of KCs-Itgb8 in mice results in partial depletion of epidermal LC. Mice lacking both integrins Itgb6 and Itgb8 are completely void of epidermal LC (Mohammed et al., 2016). Notably, LC specific expression of a constitutively active, ligand independent form of the TGFβRI (TGFβRCA) prevents spontaneous LC migration in Itgb6−/− mice (Mohammed et al., 2016). Hence, TGFβ1 produced by LC is transactivated by integrins avβ6 and avβ8 on KCs to regulate long-term epidermal residency.
1.6 KCs actively participate in immunogenic responses

KCs are the predominant cell type in the epidermis (up to 96% of the total epidermal cell population) that actively participate in the recruitment and localization of leukocytes into the epidermis (Y. Yang, Zenke, Hirai, & Kaplan, 2019). However, these functions are not equivalent to all KCs in the epidermis. KCs can be categorized based on their relative spatial relationship to the hair follicle as interfollicular (IFE), Infundibulum/Isthmus (IM), and Bulge KCs (Nagao et al., 2012) (Figure 1). IM KCs express CCL2 and CCL20 chemokine gradients, while CCL8 is exclusively expressed by bulge KCs. During inflammation, recruitment of LC precursors (i.e., bone marrow-derived monocytes) requires the engagement of chemokine receptors CCR2 and CCR6 by CCL2 and CCL20 gradients, respectively. Engagement of CCL8 to CCR8 on leukocytes prevents them from establishing residency near the bulge region (Nagao et al., 2012). In the case of CD8+ T<sub>RM</sub>, antigen-specific effector CD8+ T-cells gain entry into the epidermis through a combination of adhesion molecules and cytokine and chemokine gradients (Schon, Zollner, & Boehncke, 2003; Thomsen, Nansen, Madsen, Bartholdy, & Christensen, 2003). KCs and other stromal cells secrete signaling gradients made of CCL2, CCL3, CCL4, CCL5, CXCL9 and CXCL10 that directly bind to CCR2, CCR5, CXCR3 on effector CD8+ T-cells. Once in the epidermis, epidermal leukocytes anchor to surrounding KCs through adhesion molecules such as EpCAM and E-cadherin (Tang et al., 1993; Watanabe et al., 2015).

Given their location at the interface between the skin and the external environment, KCs constantly respond to a wide range of damaging stimuli such as physical wounding, pathogenic
insults, UV irradiation, chemical irritants, and allergens. KCs respond to these damaging stimuli through constitutively expressed pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), C-type lectin receptors (CLR) and nucleotide binding oligomerization domain (NOD)-like receptors (NLRs) (Kawai & Akira, 2009). Engagement of PRRs results in the production of pro-inflammatory molecules such as IL-1α, IL-1β, TNF-α, IL-18, CXCL9, and CXCL10 (Albanesi, 2010), which participate in inflammatory responses. Hence, KCs significantly contribute to and participate in epidermal immune responses.

**Figure 1: Epidermal KCs subsets.** Microscopy of whole mounts of back epidermis from wild-type mice demonstrating the different epidermal KCs subsets. White arrow points at IM KCs.

### 1.7 Synthesis and hypothesis statement

LC provide host immunity, but they can also participate in the maintenance and development of autoimmune and inflammatory diseases such as psoriasis, mycosis fungoides, vitiligo, and alopecia areata (Atmatzidis, Lambert, & Lambert, 2017; Clark, 2015). Thus,
mechanisms that have the potential to deplete pathogenic epidermal leukocytes are of great therapeutic interest.

Inflammatory cytokines including TNF-α and IL-1β are well known to trigger LC migration and are thought to directly interact with LC (Cumberbatch, Dearman, & Kimber, 1997; Cumberbatch, Fielding, & Kimber, 1994). A direct effect on LC is unlikely, at least for IL-1β, as LC lacking Myd88 which is required for signaling through TLR and IL-1R family members, migrate normally in response to IL-1β injection (Didovic, Opitz, Holzmann, Forster, & Weighardt, 2016; Haley et al., 2012). LC migration is also unaffected in response to C. albicans infection, DNFB application and during homeostasis. These findings are reproduced in mice deficient in Myd88−/−, however, specific expression of Myd88 in KCs in Myd88−/− mice rescues LC migration in response to migratory stimuli (Didovic et al., 2016). Thus, IL-1β likely triggers LC migration through a LC-extrinsic mechanism that is dependent on reduced TGFβ transactivation.

Here, we aim to explore a model where KCs are the sensors for danger signals and in turn control LC migration. In our model, integrins αvβ6 and αvβ8 on KCs cleave LAP-TGFβ1 to its active form (TGFβ1), which acts on the TGF-β receptor of LC in an autocrine manner to maintain long-term residency within the skin epidermis (Figure 2). In previous studies, we have demonstrated that UVB and homeostatic LC migration can be hindered by ligand independent TGFβR. Interestingly, we also saw a suppression of integrins β6 and β8 in vivo KCs after UVB treatment. However, whether pro-inflammatory stimuli regulate expression and function of αvβ6/8 in a similar manner remains to be elucidated. We aim to determine the signals that act on KCs that regulate integrin mediated TGFβ1 activation and thus changes in epidermal leukocyte residency.
Thus, we propose to test the hypothesis that inflammatory stimuli mediate LC migration from the epidermis through the inhibition of KCs-integrin mediated TGF-β activation. By exploring this hypothesis, we will gain a further understanding of LC biology and function, but through its intimate relationship with KCs.

In the next chapters, we demonstrate that enforced TGFβR signaling in LC is sufficient to prevent homeostatic and inflammation induced LC migration. In contrast to our expectations, pro-inflammatory stimuli did not decrease but rather increased Itgb6 and αvβ6 expression in both pKC and in vivo 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.

**Figure 2: TGFβ1 mediates Retention of epidermal LC.** Inactive TGFβ1 (LAP-TGFβ1) gets cleaved via integrins αvβ6 and αvβ8 situated on the surface of KCs. The newly activated TGFβ1 binds directly to the TGFβR1 on LC providing the signal for epidermal retention.
2.0 Intrinsic Enforced TGFβRI Signaling Prevents Inflammation-Induced LC Migration

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All experimental data presented in this chapter were collected by Jacinto S. De La Cruz Diaz.

2.1 Introduction

The ability of LC to migrate into the draining LNs is the bridge between the innate and immune system, also known as the LC paradigm. As described in the introduction chapter, LC play a significant role in initiating Th17 responses, support humoral responses, mediate contact hypersensitivity responses, and developed peripheral tolerance. Hence, it is crucial to expand our knowledge on how LC migrate in response to a plethora of migratory stimuli to mediate different adaptive inflammatory responses.

LC migration occurs at steady-state, in response to UVB and inflammatory stimuli such as TNF-α, IL-1β, epicutaneous C. albicans infection, and small chemical haptens. The inflammatory cytokines TNF-α and IL-1β are thought to be responsible for inflammation-induced LC migration, since LC in mice that lack either Il1β or Tnrf2 are unable to exit the epidermis. In parallel with these observations, antibody-based neutralization of TNF-α or IL-1β prevents inflammation-
induced LC migration (Cumberbatch et al., 1997; Cumberbatch et al., 1994; Eaton, Roberts, Kimber, Dearman, & Metryka, 2015; Shornick, Bisarya, & Chaplin, 2001; B. Wang et al., 1996).

Another key cytokine involved in LC migration is TGFβ; however, TGFβ acts as a repressor of LC migration. LC-specific genetic ablation of \( TGF\beta1, TGF\beta R1 \) (ALK5), \( TGF\beta R2 \) or constitutive ablation of \( TGF\beta1 \) results in fully differentiated LC that spontaneously migrate from the epidermis into regional LNs (Borkowski et al., 1996; Kaplan, 2017; Kaplan et al., 2007; Kel et al., 2010). These studies demonstrate the requirement of autocrine TGFβ signaling in LC maintenance and migration. TGFβ1 signals through a heterodimeric serine/threonine kinase receptor which is composed of two subunits, TGFβR1 and TGFβR2. TGFβ receptor activation induces the kinase activity of the intracellular domain from TGFβR2, which in turn phosphorylates the intracellular domain of TGFβR1 (Derynck & Budi, 2019; Massague et al., 1992). This latter step has been shown to be key in mediating TGFβ signal transduction, since replacing TGFβR1-serine/threonine residues with non-phosphorylatable amino acids abolishes TGFβ signaling. Conversely, substituting these residues with non-cleavable phosphomimetic amino acids (e.g., aspartic acid) results in constitutive TGFβ signaling that is independent of ligand engagement or TGFβR2 activation (Wieser, Wrana, & Massague, 1995; Wrana, Attisano, Wieser, Ventura, & Massague, 1994). Engagement of the TGFβ receptor by TGFβ1 activates the canonical TGFβ1-SMAD signaling pathway (SMAD2/3/4) (Derynck & Budi, 2019; Hata & Chen, 2016).

We have previously demonstrated that interruption of TGFβ signaling contributes to hapten-induced LC migration (Bobr et al., 2012). Moreover, ligand independent TGFβRI signaling in LC was sufficient to prevent LC migration during homeostasis and in response to
UVB (Mohammed et al., 2016). Hence, these studies suggest a role for intrinsic TGFβ signaling in steady-state and inflammation-induced LC migration. However, whether loss of TGFβ signaling occurs in response to other inflammatory stimuli remains unclear. In these studies, we demonstrate that conditional expression of a constitutively-active form of TGFβR in LC prevents TNF-α, IL-1β, epicutaneous *C. albicans* infection, and chemical hapten-induced migration from the epidermis into the draining LNs.

2.2 Methods

2.2.1 Mice

HuLangerin-CreERT2 and TGFβRICA mice have been previously described (Bartholin et al., 2008; Bobr et al., 2012). HuLangerin-CreERT2 mice were bred with TGFβRICA and ROSA26.LSL.YFP (Jackson Laboratories) reporter mice resulting in LC\textsuperscript{TGFβRCA} mice (Mohammed et al., 2016). We used age- and sex-matched mice that were between 6 and 12 weeks of age in all experiments. 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.
2.2.2 Reagents and treatments

Recombinant murine TNF-α and IL-1β were purchased from Peprotech (Cranbury, NJ) and resuspended in PBS. 12-Dimethylbenz[a]anthracene (DMBA, 57-97-6; sigma) was resuspended in DMSO:ethanol:glycerol solution (1:1:3) and applied at a concentration of 10mM in a 1.5cm² x 1.5cm² mouse shaven flank skin. Antibodies directly conjugated to different fluorophores were used for flow cytometry and immunofluorescence. Anti-pSMAD2 (A5S; Sigma) Anti-CD11c(N418)-PerCp5.5, CD11b(M1/70)-PeCy7, I-A/I-E/MHCII (M5/114.15.2)-AF700, Langerin(4c7)-PE, CD103(2E7)-AF647 were purchased from Biolegend, (San Diego, CA). Viability dye eFluor 780 (eBioscience; Invitrogen, Carlsbad, CA) was used for live-dead discrimination.

2.2.3 Tamoxifen treatment

Tamoxifen (T5648; Sigma-Aldrich) was dissolved in 1/10th volume of 200 proof ethanol with repeated incubations at 55°C (15–30 secs) -samples were roughly vortexed in between incubations. The tamoxifen-ethanol mixture was diluted with corn oil (C8267; Sigma-Aldrich) to a final concentration of 10 mg/ml. Prior to treatments, LC⁺TGFβRICA mice received five consecutive days of intraperitoneal injection of tamoxifen at 0.05mg/g of mouse weight.
2.2.4 Immunofluorescence and imaging

Epidermal sheets were prepared as previously described (Mohammed et al., 2016). Briefly, skin fat was mechanically removed and the skin was subsequently mounted on microscopy slides that had been pre-coated with double-sided adhesive tape (3M, St. Paul, MN). Slides were incubated in 10mM EDTA at 37°C for 45-90mins. Dermis was peeled away from the epidermis with standard forceps. Epidermal sheets were fixed in 4% PFA at RT for 30 min and blocked for 1hr at RT 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-A/I-E/(MHCII)-AF488 and the nucleus with DAPI. Images were captured on a IX83 fluorescent microscope (Olympus Tokyo, Japan) using a x10 objective; image analysis was performed using cellSens Dimension software (Olympus).

2.2.5 Flow cytometry

Epidermal single-cell suspensions were prepared from shaved mouse skin that was incubated for 2 h at 37 °C in 0.3% in 150 mM NaCl, 0.5 mM KCl and 0.5 mM glucose. The skin was minced finely with scissors and resuspended in RPMI1640 media (Gibco, Grand Island, NY) containing 2.5 mg/ml collagenase XI (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 30mins at 37°C. The resulting cell mesh was filtered through a 40um cell strainer (BD
Biosciences). Lymph nodes (axillary and inguinal) single-cell suspensions were prepared by first mechanically dissociating the tissue. The resulting cell mesh was incubated in 400 U/mL Collagenase D (Roche Applied Science) and 0.1 mg/ml DNase in RPMI1640 with 10% FBS for 40 min at 37°C. The resulting cell suspension was filtered through a 40um cell strainer (BD Biosciences). Single-cell suspensions from the epidermis were blocked with 2.4G2 culture supernatant (American Type Culture Collection). Surface staining was performed in standard FACS buffer for 30 min at 4°C. For intracellular cytokine staining of Langerin (CD207), and pSMAD2 cells were first fixed and permeabilized with Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer’s instructions. Intracellular staining of pSMAD2 was followed with anti-rabbit-PE. LSR Fortessa flow cytometers (Becton Dickinson, Franklin Lakes, NJ) and Flowjo software (TreeStar, Ashland, OR) were used for analysis.

2.2.6 Statistical analysis

Groups were compared with Prism software (GraphPad) using the two-tailed unpaired Student’s t test. Data are presented as mean only or mean ± standard error of the mean (s.e.m.). p < 0.05 was considered significant.
2.3 Results

2.4 Constitutive TGFβ signaling in LC prevents LC migration in response to TNF-α and IL-1β.

We have previously observed that conditional expression of ligand independent TGFβR signaling in LC 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 inflammatory stimuli could also be overcome by ligand independent TGFβR signaling, we utilized our hulangerinCreERT2 x lox-stop-lox TGFβRCA x ROSA26.lox-stop-lox-YFP mice (LC\textsuperscript{TGFβRCA}). In these mice, tamoxifen (TAM) treatment results in the dual expression of a constitutively active TGFβRI receptor (TGFβRCA) and YFP as a faithful reporter of Cre activity (Bartholin et al., 2008; Mohammed et al., 2016). On the day after the last TAM treatment, we intradermally (I.D.) administered TNF-α or PBS. The number of LC in the epidermis at 72 hours post injection was determined by immunofluorescent microscopic evaluation of epidermal whole mounts. LN LC were uniquely identified and quantified by flow cytometry by gating as CD11c\textsuperscript{+}, MHC-II\textsuperscript{high}, CD11b\textsuperscript{+}, CD207\textsuperscript{+}, CD103\textsuperscript{-} (Figure 3). As expected, TNF-α significantly reduced LC density in the skin epidermis with a concomitant increase in the LN of WT mice. However, TNF-α was unable to reduce the number of epidermal LC which prevented LC accumulation in the draining LN of LC\textsuperscript{TGFβRCA} mice (Figure 4a, c, d). We verified that LC in LC\textsuperscript{TGFβRCA} mice uniquely expressed YFP, indicative of efficient expression of TGFβRCA in LC (Figure 4g).
We then tested whether forced TGFβ signaling in LC could also prevent IL-1β induced migration. Using the same experimental approach, we analyzed the absolute numbers of epidermal LC and LN LC after intradermal administration of IL-1β in wild-type and LC\textsuperscript{TGFβRCA} mice. IL-1β reduced the number of epidermal LC in control mice but numbers in LC\textsuperscript{TGFβRCA} mice did not change. As expected, wild type LC accumulated in the draining LNs in response to IL-1β; however, LC numbers in the LN of LC\textsuperscript{TGFβRCA} mice were unaffected (Figure 4b, d, f). It has previously been shown that the chemical hapten 0.1mM 7,12-Dimethylbenz[a]anthracene (DMBA) also elicits LC migration (Muller et al., 1993). To determine whether LC migration in response to DMBA is also inhibited by constitutive TGFβR signaling in LC, we applied a single epicutaneous application of DMBA to the shaven flank skin of LC\textsuperscript{TGFβRCA} and control LC\textsuperscript{YFP} mice. Although visualization of epidermal LC in epidermal whole mounts was technically not feasible during DMBA treatment, we did observe increased numbers of LC in the draining LN of control LC\textsuperscript{YFP} mice + 3 days post-infection (Figure 5e). Similar to our findings with proinflammatory cytokines, LC numbers were not increased in LC\textsuperscript{TGFβRCA} mice demonstrating an absence of LC migration in response to DMBA. From these experiments, we verified that TNF-α, IL-1β, and chemical haptens such as DMBA strongly elicit LC migration from the epidermis into the draining LNs. More importantly, LC migration in response to these inflammatory stimuli can be inhibited by conditionally expressing a constitutively active form of TGFβR in LC. These data suggest a key role for TGFβ signaling in mediating LC migration in response to inflammatory stimuli.
Figure 3: Gating strategy for the identification of migratory cutaneous DC in LNs. Gating strategy to identify different populations of migratory cutaneous DC from the draining LNs of wild-type.
Figure 4: Enforced intrinsic TGFβ-mediated signaling is sufficient to prevent TNF-α or IL-1β induced LC migration. LC<sup>TGFB<sub>RCA</sub></sup> or control LC<sup>YFP</sup> mice received five-daily doses of tamoxifen (0.05mg/g) to induce constitutive TGFβ-mediated signaling in LC. The ear pinnae of these mice were subject to i.d. injection of either PBS, TNF-α or IL-1β (100ng). Representative immunofluorescence (a,b) and summary data of epidermal sheets (c,e) stained for...
MHCI (green) 72hrs post treatment with TNF-α or IL-1β; autofluorescence shows hair shafts. Absolute number of LC in draining LNs after TNF-α or IL-1β treatment (d,f). The number of LC in LN was evaluated by flow cytometry gating LC as CD11c+, MHC-II$^\text{high}$, CD11b+, CD207+, CD103$^\text{neg}$ (f, e). The number of LC that expressed YFP after I.P. TAM treatment on LC$^{\text{TGFβRCA}}$ control mice. Data are representative of two (d,f) or at least three separate experiments (a-c,e). Each symbol represents data from an individual animal. *p < 0.05 and ****p < 0.0001. Scale bar represents 100um.

2.5 Constitutively active TGFβR in LC prevents epicutaneous C. albicans induced LC migration.

We have previously demonstrated that LC are required to initiate a CD4+ Th$_{17}$ immune response against the fungal pathogen C. albicans(Igyarto et al., 2011). To test whether constitutive TGFβR signaling can inhibit LC migration in response to C. albicans, we epicutaneously infected LC$^{\text{TGFβRCA}}$ and control LC$^{\text{YFP}}$ mice with C. albicans. Epicutaneous C. albicans infection also prevented the visualization of epidermal LC in epidermal whole mounts, and thus we instead quantify LC migration by enumerating the absolute number of LC in the draining LNs. C. albicans significantly induced the influx of LC into the draining LNs of wild-type mice. However, LC numbers in the LN of LC$^{\text{TGFβRCA}}$ mice were unaffected (Figure 5a). Based on these results, we hypothesize that LC$^{\text{TGFβRCA}}$ mice would have a dampened adaptive immune response. As predicted, we found that the total numbers of CD4+ T-cells in the draining LNs of LC$^{\text{TGFβRCA}}$ mice
were significantly reduced. Notably, there was also a significant reduction in RORγt+ CD4+ T-cells (Figure 6).

To verify that these findings were solely due to the inability of LC to migrate into the draining LNs, we examined the migration of other cutaneous dendritic cells. Notably, migration of cDC1 (CD11c+, MHC-II\textsuperscript{high}, CD207+, CD103+, CD11b−), cDC2 (CD11c+, MHC-II\textsuperscript{high}, CD207−, CD103−, CD11b+), and double negative DC (CD11c+, MHC-II\textsuperscript{high}, CD207−, CD103−, CD11b+) were equivalent in both control and LC\textsuperscript{TGFβRCA} mice thereby demonstrating the failure to migrate in LC\textsuperscript{TGFβRCA} mice is selective to LC (Figure 5b, c, d). In summary, these findings demonstrate that conditional expression of the constitutively active form of TGFβR in LC is sufficient to prevent \textit{C. albicans} induced migration, and that the effectiveness of the adaptive response is dependent on LC migration to dLN.
mice were treated i.p. tamoxifen as described above. Flank skin of mice was then treated with either epicutaneous *C. albicans* or Dimethylbenz[a]anthracene (DMBA) and PBS or vehicle controls, respectively.

Inguinal LC were identified by flow cytometry by gating as CD11c*, MHC-II*high*, CD11b*, CD207*, CD103*neg*(a,e). Different cutaneous dendritic cell subsets from *C. albicans* infected mice were detected by gating for cDC1 as CD11c*, MHC-II*high*, CD207*, CD103*, CD11b*, cDN2 as CD11c*, MHC-II*high*, CD207*, CD103*, CD11b*, and dnDC as CD11c*, MHC-II*high*, CD207*, CD103*, CD11b*. Data are representative of two separate experiments (a-e). Each symbol represents data from an individual animal. *p < 0.05 and ****p < 0.0001.
Figure 6: Expansion of RORγ+ CD4+ T-cells due to epicutaneous *C. albicans* infection is hindered in LCTGFβR1CA mice. LCTGFβRCA or control LCYFP mice were treated with i.p. tamoxifen as described above. Flank skin of mice was then treated with either epicutaneous *C. albicans* or PBS. Data are representative of two separate experiments (a-e). Each symbol represents data from an individual animal. **p < 0.01 and ****p < 0.0001.

### 2.6 TNF-α interrupts TGFβ signaling in activated epidermal LC but not in LN LC

We have previously reported that hapten induced LC migration results in the interruption of TGFβ signaling in activated LC (Bobr et al., 2012). Based on our findings that enforced TGFβ signaling was sufficient to prevent a wide range of inflammation induced LC migration, we sought to investigate whether TNF-α also inhibits TGFβ signaling in activated LC. Phosphorylation of the intracellular protein pSMAD2 is part of the canonical TGFβ pathway (Moustakas, Souchelnytskyi, & Heldin, 2001). To detect intracellular protein pSMAD2, we validated that the monoclonal anti-pSMAD2(A5S) had increased staining in our LCTGFβRCA mice. Staining of pSMAD2 by Clone A5S was proven to be successful in our control mice (LCTGFβRCA +/−) and LCTGFβRCA/f mice. However, LC with enforced TGFβ signaling had higher staining for pSMAD2...
than its WT counterparts (LC\textsuperscript{TGFβRCA+/+}), suggesting that clone A5S was staining the TGFβ SMAD pathway (Figure 7). Notably, WT LC showed a range of staining for pSMAD2, which is consistent with previously published results (Bobr et al., 2012). We next examined the expression of pSMAD2 in LC from WT mice after 24h of I.D. administration of TNF-α or PBS. In the epidermis, mice treated with PBS had an overall increase in pSMAD2 among MHCII\textsuperscript{low} LC, while TNF-α reduced pSMAD2 staining in activated LC (MHCII\textsuperscript{high}) (Figure 8a). Based on this observation, we predicted that LC in the dermis and the draining LNs would have significant reduced TGFβ signaling. Similar to our findings in the epidermis, TNF-α reduced the overall expression of pSMAD2 in migratory dermal LC (Figure 8b). Interestingly, LC that had recently migrated into the draining LNs after TNF-α treatment had increased pSMAD2 staining (Figure 8c). Thus, these data suggest that activated epidermal and dermal LC suppressed pSMAD2 in response to TNF-α. But once LC arrived at the draining LNs, they regain TGFβ signaling sensitivity. Thus, the loss of TGFβ signaling in LC mediates hapten and TNF-α induced LC migration. However, the loss of TGFβ signaling in migratory LC is not sustained in the draining LNs.
Figure 7: Validation of anti-pSMAD2 antibody using LC^{TGFβRCA}. Expression of pSMAD2 in epidermal LCs (CD45^+, MHC-II^+, Langerin^+), from LC^{TGFβRCA \text{f/f}} and control mice (LCTGFβRCA ^+/+) on day +5 after tamoxifen treatment.
Figure 8: TGFβ signaling (pSMAD2) in Migratory LC. Expression of MHC II vs pSMAD2 is shown in LCs isolated from the epidermis, dermis, and draining LNs of wild-type mice after I.D. TNF-α (100ng). Epidermal and dermal LC were harvested after 24h of treatment, while LC LNs were harvest after 72h of I.D. treatment with TNF-α. Data represent two independent experiments with groups of three mice each.
2.7 Discussion

TGFβ is a cytokine that plays a key role in the maintenance and migration of epidermal LC. The experimental data presented in this chapter further extends our knowledge on the role of TGFβ in inflammation induced LC migration. Prior to these studies, it had been shown that UVB-induced and homeostatic LC migration can be inhibited by enforced TGFβ signaling in LC. Herein, we demonstrate that LC migration induced by TNF-α, IL-1β, epicutaneous *C. albicans* infection, and chemical-hapten, DMBA can also be inhibited through forced TGFβR1 signaling in LC. We also demonstrated that preventing LC migration in response to epicutaneous *C. albicans* infection results in a dampened immune response against this pathogen. Here we also provide evidence that TNF-α, in addition to hapten stimuli, also suppresses TGFβ signaling in epidermal and dermal LC. Interestingly, we also saw that LC begin to response to TGFβ once they arrived at the draining LNs after cutaneous TNF-α treatment. Altogether, conditional expression of a constitutive form of TGFβR1 in LC significantly prevents both steady-state and inflammation induced LC migration. Additionally, TNF-α and hapten stimuli suppresses TGFβ signaling in LC to mediate migration, but once arrived in the LN TGFβ responsiveness in LC appears to recover.

One important observation is that epidermal steady-state LC showed a wide range of pSMAD2 staining. This heterogeneity in TGFβ signaling can be attributed to the transient feedback inhibition of TGFβ signaling by SMAD7(Moustakas et al., 2001). Nonetheless, it is unclear whether those LC experiencing transient low TGFβ signaling are more susceptible to migration. However, this observation could explain the partial depletion of epidermal LC after
TNF-α and IL-1β treatment (Figure 4). It is possible that those LC experiencing high levels of TGFβ signaling are less likely to migrate and thus are retained in the epidermis even in the presence of strong migratory cues. This latter observation fits well with our findings that enforced TGFβ signaling is capable of preventing a wide range of migratory stimuli in LC.

It is important to highlight that activation of the TGFβR1 and TGFβR2 through engagement of TGFβ1 has been shown to also activate SMAD-independent pathways, such as Erk, JNK, and p38 MAPK Kinase (Derynck & Zhang, 2003; Zhang, Gu, Yu, Zhou, & Mi, 2016). Thus, we cannot discard the possibility that artificial expression of constitutive active TGFβR1 may be activating TGFβ non-canonical pathways. This raises the possibility that artificial induction of constitutive TGFβ1 signaling may impact the responsiveness of LC to inflammatory stimuli through a non-canonical pathway.

Overall, our data are consistent with a model in which artificial ablation of TGFβ signaling results in spontaneous LC migration into the draining LNs. These findings suggest that steady-state, UVB, and inflammatory stimuli require the loss of intrinsic TGFβ signaling for LC migration. Unexpectedly, LC that had recently migrated into the draining LNs re-gained sensitivity to TGFβ signaling. Nonetheless, the mechanism that leads to the loss of TGFβ signaling in LC remains unclear. One prediction is that inflammatory stimuli disrupt upstream proteins involved in TGFβ signaling (e.g., TGFβ1, TGFβR1 TGFβR2) or extrinsic proteins involved in the activation of the cytokine TGFβ1 such as TGFβ-activating integrins. As previously mentioned, a mechanism that directly disrupts intrinsic TGFβ signaling pathway in LC is unlikely. In the next
chapter, we focus on deciphering the role of TGFβ-activating integrins in inflammation induced LC migration.
3.0 Inflammatory Stimuli Do Not Suppress Expression of TGFβ-Activating Integrins αvβ6 or αvβ8 on KCs

Portions of this chapter were compiled and submitted to the *Journal of Investigative Dermatology (JID): Innovations*:


All experimental data presented in this chapter were collected by Jacinto S. De La Cruz Diaz, except for data associated with figure 10. This data was provided by the Transgenic and Gene Targeting Core at the University of Pittsburgh.

3.1 Introduction

The skin is responsible for formulating robust immune responses against a wide range of biological and non-biological stimuli. KCs and LC work in harmony to maintain barrier integrity and to orchestrate effective immune responses (T. Kobayashi et al., 2019). Inflammatory cytokines including TNF-α and IL-1β are well known to trigger LC migration and are thought to directly interact with LC (Cumberbatch et al., 1997; Cumberbatch et al., 1994). However, a direct effect on LC is unlikely, at least for IL-1β, as LC lacking *Myd88* which is required for signaling through TLR and IL-1R family members, migrate normally in response to IL-1β injection (Didovic et al., 2016; Haley et al., 2012). LC migration is also unaffected in response to *C. albicans* infection, DNFB application and during homeostasis. These observations suggest that LC migration is contextual and dependent on surrounding cells such as KCs.
As previously described, TGFβ1 is key in mediating LC maintenance and migration. Upon synthesis and secretion, TGFβ1 is bound to the latency associated peptide (LAP) resulting in inactive TGFβ (LAP-TGFβ1) (Travis & Sheppard, 2014). Removal of the LAP protein from TGFβ1 can be accomplished through low pH, proteases, mechanical stress, reactive oxygen species, and integrin mediated processes (Worthington et al., 2011). In the epidermis, activation of LAP-TGFβ1 is accomplished via functionally-active forms of the integrins αvβ6 and αvβ8 expressed on KCs (Aluwihare et al., 2009; Z. Yang et al., 2007). Integrin activity is positively regulated through the cytoplasmic adaptor proteins Talin1 (Tln1) and Kindlin1 (Fermt1) (Kerr & Byzova, 2018; Qin, Vinogradova, & Plow, 2004; Takada et al., 2007). Genetic ablation or inhibition of αvβ6 or αvβ8 on KCs reduced active TGF-β resulting in loss of LC from the epidermis. For comparison, constitutive TGF-β signaling in LC prevented the loss of epidermal LC in β6−/− mice. Therefore, the regulated expression of αvβ6 and αvβ8 on KCs appears to directly control epidermal residence of LC.

Epidermal KCs can be categorized based on their relative spatial relationship to the hair follicles as IFE, IM, and Bulge KCs (Nagao et al., 2012) (Figure 1). At steady-state, LC reside intercalated with IFE and IM KCs but are actively excluded from the bulge (Nagao et al., 2012). IFE KCs express integrin αvβ6 but not αvβ8 and IM KCs express αvβ8 but not αvβ6 (Mohammed et al., 2016). Epidermal exposure to UVB is sufficient to reduce KCs expression of integrins αvβ6 or αvβ8 and induce efficient LC migration that can be inhibited by LC-specific expression of TGFβRCA (Mohammed et al., 2016). These observations support a model where, under homeostatic conditions autocrine LC-derived LAP-TGFβ is transactivated either by αvβ6 or αvβ8
expressed by KCs and then acts directly on LC to prevent spontaneous migration. Moreover, it suggests that reduced TGFβ transactivation following UVB irradiation is a trigger for LC migration. Whether a similar mechanism occurs with inflammatory stimuli remains unexplored.

Here, we report that UVB efficiently reduced expression of avβ6 and avβ8 on pKC resulting in reduced TGFβ transactivation. In contrast, TNF-α and IL-1β increased avβ6 expression in both pKC and epidermal KCs resulting in increased TGFβ transactivation. Thus, loss of TGFβ transactivation is associated with homeostatic and UVB-induced LC migration, but not with inflammation-induced migration. We also describe the methods that were developed to study TGFβ activating integrins *in vitro* and *in vivo*.

### 3.2 Methods

**3.2.1 Mice**

Itgb6<sup>−/−</sup> and Itgb8<sup>loxP</sup> mice were kindly provided by D. Sheppard (University of California, San Francisco). C57BL/6 (WT) and Tg(KRT14-cre)1Amc/J (K14-Cre) mice were purchased from Jackson Laboratories. We crossed K14-Cre mice with Itgb8<sup>loxP</sup> and Itgb6<sup>−/−</sup> mice to obtain Itgb6<sup>−/−</sup> and Itgb8<sup>ΔKCS</sup> mice (Mohammed et al., 2016). We used age- and sex-matched mice that were between 6 and 12 weeks of age in all experiments. 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.
3.2.2 Development of FT- β6 reporter mouse

Phenotypic analysis of FT- β6 reporter BAC transgenic mouse is described in the results section (3.2.2).

To achieve the knock-in of fluorescent timer protein through Crispr/Cas9 technology, we injected zygotes with a mixture of Cas9mRNA (100ng/ul), Itgb6-3sgRNA (50ng/ul) and Itgb6-Timer-Antisense (10ng/ul). The Itgb6-The Itgb6- is a sgRNA targeting the region surrounding the start codon of Itgb6. Timer is a long single stranded oligonucleotides and was used as template for homology repair. Itgb6-3: AGACCAGCTCAATCCCTTATT, Itgb6-Timer-Antisense was order from IDT as Megamer; DNA oligo. Founder animals with the correct knock-in were identified by genomic PCR for the Timer insertion at the 5’end: F TGCATAAGTCTCCCCACCT, R CTCCAGATGCACCTTGACCT; 3’ end: F CCTGGAGCCTACAACGTC, R TCCCCGTGAAGCCTTTGTGT; WT locus/Insertion: F TGCATAAGTCTCCCCACCT, R TCCCCGTGAAGCCTTTGTGT.

3.2.3 Reagents and treatments

Recombinant murine TNF-α and IL-1β were purchased from Peprotech (Cranbury, NJ) and resuspended in PBS. For UVB experiments we used two TL 20W/12RS lamps (Philips). We used UVB doses previously described as 20 mJ/cm² for pKC experiments(Mohammed et al., 2016). DMBA (57-97-6; sigma) was applied at 0.1mM in DMSO for pKC experiments. Antibodies directly conjugated to different fluorophores were used for flow cytometry and immunofluorescence. I-A/I-E/MHCII (M5/114.15.2)-AF700, Langerin(4c7)-PE, CD45.2(104)-
BV605, Sca-1(E13-161.7)-PerCp5.5, EpCAM(G8.8)-PeCy7, 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. Polyclonal anti-mCherry (PA5-34974; ThermoFischer) was used to detect FT-Itgb6 fluorescent protein. Intracellular staining of mCherry was followed with anti-rabbit-PE. Anti-αvβ6(6.3g9) and anti-αvβ8(C6D4)-PE were kindly provided by Drs. Dean Sheppard and Stephen Nishimura, respectively. Anti-αvβ6(6.3g9) was directly conjugated to Alexa Fluor 647 (ThermoFischer, A20186).

3.2.4 Immunofluorescence & imaging

Skin whole mounts were prepared from skin samples embedded in OCT compound and 8um transverse skin slice sections were prepared. Epidermal sheets and skin whole mounts were fixed in 4% PFA at RT for 30 min and blocked for 1hr at RT 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-A/I-E/MHCII-AF488, anti-Langerin(CD207)-PE and followed by anti-PE-AF555, and DAPI. Images were captured on a IX83 fluorescent microscope (Olympus Tokyo, Japan) using a x10 objective; image analysis was performed using cellSens Dimension software (Olympus).
3.2.5 Flow cytometry

Single cell suspension from tissues were prepared as previously described (Mohammed et al., 2016). Epidermal single-cell suspensions were prepared from shaved mouse skin that was incubated for 2 h at 37 °C in 0.3% in 150 mM NaCl, 0.5 mM KCl and 0.5 mM glucose. The skin was minced finely with scissors and resuspended in RPMI1640 media (Gibco, Grand Island, NY) containing 2.5 mg/ml collagenase XI (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 30mins at 37°C. The resulting cell mesh was filtered through a 40um cell strainer (BD Biosciences). Single-cell suspensions were blocked with 2.4G2 culture supernatant (American Type Culture Collection). Surface staining was performed in standard FACS buffer for 30 min 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 KCs subsets were sorted on FACSaria cell sorter(BD Biosciences) and gated as IFE KCs: CD45.2−, Sca1+, CD34−, EpCAM−; and IM KCs: CD45.2−, Sca1−, CD34+, and EpCAM+. 
3.2.6 qRT-PCR

Total RNA from flow cytometry–sorted epidermal cells and pKC cultures was extracted with Thermo’s Trizol-LS (10296028) or RNeasy Mini extraction kit (Qiagen) following the manufacturer's instructions and was quantified using Nanodrop (NanoDrop). cDNA was generated using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and was subjected to quantitative PCR using TaqMan Gene Expression Master Mix and TaqMan Gene Expression Assays for Gapdh, Itgb6, Itgb8, Itgav, Fermt1 (Kindlin1), and Tln1.

3.2.7 Cell culture

Newborn mice (1-4 days old) were used to culture pKC as previously described (Dlugosz, Glick, Tennenbaum, Weinberg, & Yuspa, 1995; Mohammed et al., 2016). Thymic mink lung epithelial reporter cells (tMLECS) that had been previously transfected with a plasmid containing the luciferase cDNA downstream of a TGFβ responsive portion of the plasminogen activator inhibitor 1 promoter were cultured as originally described (Abe et al., 1994).

3.2.8 Keratinocyte cell transfection

We have generated a stable KCs cell line from pKC by spontaneous immortalization. These cells were grown at 37°C, 5% CO₂, 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 (β6) plasmid construct or with empty pCDNA3.1+/C-(K)-DYK (EV) that were purchased from Genscript USA inc. Transfections were performed in 4 mm-gap cuvettes using a BTX ECM 830 square wave electroporator. Transfection were accomplished by a single pulse of 300 volts and 10 ms, with 5-10 μg of plasmid DNA per 1.0 x 10⁶ to 2.0 x 10⁶ cells. Four days after transfection, selection reagent G418 (0.1mg/ml to 2.5mg/ml, Sigma) 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.

3.2.9 TGF-β-activation reporter assay

The ability of KCs to transactivate TGFβ was determined by co-culture of KCs with tMLECs as previously described (Mohammed et al., 2016). Briefly, tMLEC were grown in DMEM media containing 10% FCS and were plated at 2.0 x 10³ cells/well in a 96-well cell culture treated plate for 3 h at 37°C and 5% CO₂. TNF-α, IL-1β, UVB, DMBA, or non-treated KCs were harvest with 0.25% trypsin and 2.21 mM EDTA (25 053-CI; Corning Cellgro) for 10mins at 37 °C and 5% CO₂. Experimental pKC were co-culture with tMLEC reporter cells at a density of 4.0x10⁴cells in 1% chelexed serum EMEM media as previously described (Abe et al., 1994;
Dlugosz et al., 1995). The cells were cultured for 16–20 h, after which the reporter cells were lysed and assayed for luciferase activity using Bright Glo Luciferase Assay System (Promega).

3.2.10 Statistical analysis

Groups were compared with Prism software (GraphPad) using the two-tailed unpaired Student’s t test. Data are presented as mean only or mean ± standard error of the mean (s.e.m.). p < 0.05 was considered significant.

3.3 Results

3.3.1 Methods developed to study mouse integrins αvβ6/αvβ8

3.3.1.1 Development and validation of integrin β6 timer reporter mouse

To investigate integrin β6 expression during inflammation vs. steady state, we attempted to develop an integrin β6 reporter timer mouse (FT- β6). Our FT-β6 mice, is based on the fluorescent protein mCherry that has been modified to emit a fluorescent blue signal for the first ten hours of its life (464nm), and then subsequently undergoing a self-spontaneous conformational reaction that results in the emission of a red fluorescent signal (600nm)(Terskikh et al., 2000). To drive reporter expression, we employed bacterial artificial chromosome (BAC) transgenic mice. We utilized the BAC clone RP24-96C15 that is derived from mouse chromosome 2 that contains
all promoter and regulatory sequences necessary for accurate endogenous expression. RP24-96C15 is 194kb and contains integrin β6 with 26kb of sequence 5’ to the promoter and 44kb 3’ to the polyA. The BAC clone was modified using homologous recombination in *e.coli* to insert FT. The FT sequence was inserted after the *ITGB6* ATG codon and was followed by a STOP cassette. The BAC transgenic will not result in a null allele for *ITGB6*. The successful recombination of BAC- FT- β6 was confirmed by PCR and restriction digestion (data not shown). Recombined genomic DNA was separated from the BAC vector by NotI digestion, which was then purified by gel electrophoresis. The purified product was microinjected into C57Bl-6 zygotes. However, despite several rounds of injection, this approach did not yield any viable pups. We then decided to do a knock-in of the reporter protein by using clustered regularly interspaced palindromic repeats (CRISPR-Cas9) technology with the help of the Transgenic and Gene Targeting Core. One hundred and seventy C57Bl/6J fertilized embryos were injected with a mixture of Cas9mRNA, sgRNA, and FT- β6 reporter sequence- antisense. From the injected zygotes, sixty-five embryos developed and were transferred to the oviducts of two pseudopregnant female receipts. Three out of seven pups were born in good conditions, and toes biopsies were taken to identify potential founders through PCR analysis. Our PCR analysis concluded that two pups had homozgyous insertions of the FT-β6 knock in (Figure 9). We took our founders and bred them to wild-type black C57BL/6J to create heterozygous FT-β6 progeny. The genomic insertion of the two founders were sequenced, and we found that FT-β6 founder #2 had a sequence truncation at the 5’ end. This truncation most likely prevent it from expressing a functional FT-Itgb6 protein (data not shown). Expression our fluorescent protein from FT-Itgb6 founder #3 was not detected via fluorescence microscopy (Figure 10) or by using the Aurora CyTek flow cytometry (data not shown).
determine whether the FT-β6 protein was expressed, we directly stained for mCherry protein via flow cytometry. However, were unable to detect protein expression of FT-β6 on founder #3 (Figure 11) or its progeny (data not shown). Thus, none of our original founders for FT-β6 successfully expressed our timer fluorescent protein due to a truncation in the 5’ end or probably due to a lack in FT-β6 mRNA synthesis.

![Image](image.png)

**Figure 9: Potential FT-β6 founder mice were screened via PCR analysis.** From the seven potential founders only three were viable, and two were homozygous for the fluorescent protein insertion. We verified the insertion by amplifying the 3’ and 5’ end of knock-in sequence that overlap with the endogenous locus (a) we also amplify the wild-type locus/ knock-in insertion (b). Bands should appear as follow: 5’= 397bp, 3’= 399 (a) WT allele= 598, and knock-in = 1306bp.
Figure 10: Detection of FT-β6 via fluorescence microscopy. Fluorescence Microscopy of gut transverse sections from wild-type mice, FT-β6 founder#2 and FT-β6 founder#3. To detect red and blue fluorescence, we used a filter that range from 420-475nm and 580-620nm, respectively.
Figure 11: Detection of fluorescent protein mCherry in FT-β6. Flow plots of bulk epidermal KC that were stained with different amounts of anti-mCherry (PA5-34974) in wild type (WT) and FT-β6 mice.

3.3.1.2 Validation and optimization of antibodies that detect mouse integrins αvβ6 and αvβ8

The development of monoclonal antibodies to detect integrins αvβ6/αvβ8 has been imperative for the understanding of TGFβ activation in mouse biology. We have previously shown that anti-αvβ6 (6.3g9) and anti-αvβ8 (ADWA-21) efficiently prevented integrin αvβ6 and αvβ8 functional activity in both in vivo and in vitro models. We obtained 6.3g9 and ADWA-21 through a collaboration with Dean Sheppard. In order to evaluate changes in integrin expression in the presence of inflammatory stimuli, we sought to optimize staining of anti-αvβ6 (6.3g9) and anti-αvβ8 (ADWA-21) for flow cytometry detection. Anti-αvβ6 (6.3g9) and anti-αvβ8 (ADWA-21) were directly conjugated to different fluorescent dyes and tags such as biotin. We then titered these conjugated antibodies to determine the optimal concentration for the specific detection of integrins.
αvβ6 and αvβ8 on KCs. Staining of KCs isolated from $\text{Itgb}6^{-/-}\text{Itgb}8^{\Delta\text{KCS}}$ mice was included as a specificity control. Anti-αvβ6 (6.3g9) specifically detected mouse integrin αvβ6 on epidermal KCs at an optimal concentration of 0.2 ug/test (Figure 12). However, flow cytometry detection of integrin αvβ8 by anti-αvβ8 (ADWA-21) was not feasible (data not shown). Next, we sought to test anti-αvβ8 (C6D4)-PE mouse antibody that was recently developed by Stephen Nishimura at UCSF. C6D4 has been shown to block integrin αvβ8-TGFβ transactivation in in vitro models (Takasaka et al., 2018). Flow cytometry detection of mouse integrin αvβ8 on epidermal KCs was proven to be successful at an optimal antibody concentration of 1.0ug/test (Figure 12). Notably, anti-αvβ6 (6.3g9) and anti-αvβ8 (C6D4)-PE preferably stained the IFE and IM KCs, respectively. Flow cytometry detection of mouse integrin αvβ8 has not yet been reported in the literature. Thus, anti-αvβ6 (6.3g9) and anti-αvβ8 (C6D4)-PE not only prevent TGFβ transactivation, but they serve as tool for the detection of mouse integrins αvβ6 and αvβ8 on KC.
Figure 12: Flow cytometry detection of integrins of the αvβ6 and αvβ8 on epidermal KCs. Representative flow plots of the expression of integrins αvβ6 and αvβ8 on IFE and IM KCs populations from wild-type or Ilgb6ΔKCs mice; IFE KCs were gated as CD45.2−, Sca1+, CD34−, EpCAM−; and IM KCs: CD45.2−, Sca1−, CD34−, and EpCAM+.

3.3.1.3 Development of pKC and KCs cell cultures

We were interested in using pKC to study the expression and function of integrins αvβ6 and αvβ8 in response to a wide range of migratory stimuli. Although, there are a few methods to obtain pKC, their isolation and culture can be challenging and requires significant optimization (Dlugosz et al., 1995). We decided to derived our pKC from the skin of mouse neonates, since adult mouse dorsal skin leads to a low yield of viable cells (Li, Adase, & Zhang, 2017). To obtain pKC, we sacrifice post-natal +4 day neonates from wild-type mice and carefully peel off the whole skin from the body. We then floated the skin (epidermis facing upwards) in
several digestion buffers at different concentrations (dispase and trypsin) to separate the dermis from the epidermis. We found that 4.0 mg/ml of dispase was an optimal concentration to efficiently separate the epidermis at 4C overnight. The epidermis was separated from the dermis and resuspended in KC growth media and mince to create a single cell suspension. The single cell mixture was then plated into 6x6 culture media plates. We then change the KC growth media every two days until pKC had reached confluency. To determine whether our pKC cultures express functionable αvβ6 and αvβ8, we co-culture our pKC with a tMLECs that synthesizes luciferase in response to TGF-β signaling (Annes, Chen, Munger, & Rifkin, 2004). Thus, changes in luciferase activity reflect changes in integrin mediated TGF-β activation. By antibody treatment with anti-αvβ6, anti- αvβ8, or a combination of both, we have verified that our pKC activate TGF-β through integrin αvβ6 and αvβ8 (Figure 13).

We also used our pKC cultures to create stable KCs cell lines using the spontaneous immortalization method. Once our pKC had reached confluency from initial seeding, passage of the cells with trypsin digestion resulted in ~95% cell mortality. After 21 days, small adherent clusters of cells were observed. With prolonged culture, the clusters became large enough to form a confluent monolayer. Subsequent cell passages of the cells revealed stable growth that resemble the typical cobblestone morphology, a characteristic of KCs morphology (Figure 14). Through this method we developed five stable immortalized KCs cell lines. We then co-culture our KCs cell lines with our tMLEC cells - a reporter cell line for TGFβ is transactivation. We found that our KCs cell lines derived from pKC had different efficiencies to transactivate LAP-TGFβ (Figure 15). Thus, our pKC cultures and stable KCs cell lines are able to transactivate latent-TGFβ through
integrins αvβ6 and αvβ8. These cell lines can now be used to interrogate integrins αvβ6 and αvβ8 expression and function in vitro.

Figure 13: pKC transactivate LAP-TGF-β through integrins αvβ6 and αvβ8. pKC cultures were tested for their ability to activate TGF-β with a tMLEC cell line that respond to TGF-β signaling fused to a luciferase reporter. pKC cultures were pre-incubated with anti-αvβ6 and αvβ8 antibodies prior to examination of their ability to transactivate LAP-TGF-β. RLU: Relative Light Units. Two-way anova, ****<0.001p. Data represent two independent experiments.
Figure 14: Immortalized KCs cell lines developed normal morphology. Phase contrast images at 10x magnification of stable KCs cell lines derived from pKC cultures. KCs developed the typical KCs-cobblestone morphology.

Figure 15: Immortalized KCs cell lines transactivate TGF-β. KCs cell lines that were derived from pKC cultures were co-culture with MLECs to evaluate their ability to transactivate LAP-TGF-β. pKC from β6+ mice were used as a negative control. RLU: Relative Light Units. Data is representative of two different independent experiments.
3.3.2 Migratory stimuli increase integrin avβ6 and active TGFβ bioavailability in pKC

We previously reported that exposure of KCs to UVB reduced expression of the TGFβ-activating integrins *Itgb6* and *Itgb8* in vivo and in vitro (Mohammed et al., 2016). Coupled with the observation that forced TGFβR signaling prevented UVB-induced LC migration, we concluded that LC migration in response to UVB resulted from reduced epidermal bioavailability of TGFβ. To test whether inflammatory stimuli also suppress KCs expression of *Itgb6* and *Itgb8*, we treated *in vitro* pKC with UVB, TNF-α, IL-1β, and DMBA and evaluated mRNA expression by RT-qPCR at 24 h post-treatment. As expected, pKC expression of *Itgb6* and *Itgb8* were reduced after UVB exposure (Figure 16 a, b). Similarly, expression of transcripts for the integrin-associated proteins *Itgav* (αv), *Tln1* (Talin1), and *Fermt1* (Kindlin1) were coordinately reduced suggesting a broad reduction in the pathway responsible for transactivating latent TGFβ (Figure 16c-e). Contrary to our expectations, we observed that pKC incubated with TNF-α, IL-1β, or DMBA consistently increased expression of *Itgb6* (Figure 16a). Expression of *Itgav*, *Tln1*, and *Fermt1* remained relatively unchanged, with expression of *Itgb8* reduced only after DMBA treatment (Figure 16b-e). Analysis of protein expression by flow cytometry revealed reduced expression of avβ6 following UVB treatment and modest but statistically significant increased expression following TNF-α and DMBA treatment (Figure 16f). Expression of avβ8 by pKC was largely unchanged with only a minor decrease following DMBA treatment (Figure 16g). Finally, we tested the capacity of pKC to activate latent TGFβ after being treated with UVB, TNF-α, IL-1β and DMBA. We then co-culture them with our reporter cell line that expresses luciferase when
autocrine TGFβ is transactivated by treated cells. Consistent with the expression data, pKC treated with UVB showed reduced capacity to transactivate TGFβ while pKC treated with TNF-α, IL-1β, or DMBA showed enhanced TGFβ transactivation (Figure 16h). From these data, we conclude that the three inflammatory stimuli we have examined all promote increased expression of integrin αvβ6 and the capacity of \textit{in vitro} pKC to transactivate TGFβ.
Figure 16: Migratory stimuli increase the ability of pKC to activate latent-TGFβ. pKC were treated with 100ng of TNF-α, IL-1β, or 0.1 mM DMBA for 24h or 20 mj UVB. Quantitative RT-PCR analysis of Itgb6, Itgb8, Itgav, Fermt1, and Tln1 is shown (a-e). Flow cytometry analysis of the expression of integrins αβ6 and αβ8 on treated pKC reported ad MFI (f,g). Luciferase activity in tMLEC TGFβ reporter cells co-cultured with pKC pretreated with the indicated stimulus is shown. Data is presented as relative light units (RLU), normalized to control cells; PBS or
vehicle treated pKC(h). Data are representative of two independent experiments (a-e) or three independent experiments (h). Each symbol represents data from an individual animal (a-h). *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001. MFI, geometric mean fluorescence intensity.

### 3.3.3 KCs expression of Itgb6 is increased by TNF-α in vivo

We have previously demonstrated that subsets of KCs express different levels of Itgb6 and Itgb8. In the steady state, IFE KCs primarily express Itgb6 but not Itgb8, while IM KCs primarily express Itgb8 with minimal traces of Itgb6, and bulge KCs express both (Mohammed et al., 2016). We noted that pKC expressed high levels of both Itgb6 and Itgb8 suggesting that pKC may be more representative of bulge KCs rather than IFE or IM KCs. Since LC are actively excluded from the bulge and reside in the IFE and IM (Nagao et al., 2012), we next examined expression of Itgb6 and Itgb8 in IFE and IM KCs by RT-qPCR. WT mice were injected i.d. with 100ug of TNF-α, or PBS on flank skin. After 4 or 24 h, single cell epidermal suspensions were FACS sorted as IFE (CD45-2, CD207-, MHCII-, CD34-, EpCAM+, Sca1+) or IM (CD45-2, CD207-, MHCII-, CD34-, EpCAM+, Sca1-). As expected, IFE KCs expressed higher levels of Itgb6 than IM KCs (Figure 17a). Administration of TNF-α transiently increased expression of Itgb6 at 4 h, which returned to baseline by 24 h 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 17b). Administration of TNF-α resulted in a transient non-significant reduction of Itgb8 expression in IM KCs that returned to baseline by 24 h. Since expression of Itgb8 only trended
downward in TNF-α-treated IM KCs, we examined whether TNF-α might preferentially drive migration of LC out the follicular ostia. TNF-α was administered i.d. and flank skin was harvested 48 hours later to visualize LC by immunofluorescence imaging. Follicular isthmus and LC were identified by staining with α-EpCAM and α-Langerin antibodies, respectively. We observed equivalent LC migration from both the IFE and IM (Figure 17c). Taken together, these data demonstrate that TNF-α transiently increase expression of Itgb6 mRNA in IFE and IM KCs in vivo while expression of Itgb8 remain largely unchanged.
Figure 17: TNF-α does not suppress integrin Itgb6 and Itgb8 mRNA expression by KCs in vivo. Quantitative RT-PCR analysis of Itgb6 and Itgb8 mRNA from wild-type mouse sorted epidermal cells that had been treated with i.d. TNF-α for 4 or 24h. IFE KCs were gated as CD45^2^, CD207^-, MHCII^-, CD34^-, EpCAM^-, Sca1^+^) or IM (CD45^-, CD207^-, MHCII^-, CD34^+, EpCAM^+, Sca1^-); results are presented relative to GAPDH (a,b). Microscopy of flank skin transverse sections from wild-type mice for 72 h following i.d. treatment with TNF-α (100ng) or PBS; stained with Langerin (red), EpCAM (cyan), and DAPI (c). Data are representative of two independent experiments (a,b) or three independent experiments (c). scale bar = 50μm Each symbol represents data from an individual animal (a,b). **p < 0.01
3.3.4 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 surface expression of integrins αvβ6 and αvβ8 proteins by flow cytometry using recently developed and optimized 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 18a, b). Staining of KCs isolated from Itgb6-/- Itgb8ΔKCS mice was included as a specificity control. Treatment with TNF-α increased expression of αvβ6 in IFE KCs at 12 h that largely persisted at 24 h. IM KCs also increased expression of αvβ6, though less robustly than IFE KCs. Expression of αvβ8 was evident in PBS treated IM KCs and modestly increased following 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 i.d. to WT mice and bulk epidermal cells were isolated 12 h later and incubated in vitro with a reporter cell line to detect active TGFβ, as described above. Epidermal cells from TNF-α treated skin showed an enhanced capacity to transactivate latent TGFβ compared with epidermal cells from PBS treated skin (Figure 18c). Finally, to determine whether increased expression of integrin β6 is sufficient to increase surface expression of αvβ6 and to increase TGFβ activation, we overexpressed integrin β6 in cell line generated from immortalized primary murine keratinocytes. Cells were electroporated with either empty vector (EV) or a vector containing integrin β6 under the control of the CMV promoter (β6) followed by antibiotic selection and cloning by limited dilution. Surface expression of integrin of αvβ6 was clearly increased in KCs transfected with β6 compared with
empty vector (EV) (Figure 18d). As expected, KCs transfected with β6 showed increased activation of latent TGFβ (Figure 18d). Thus, increased integrin β6 expression is sufficient to augment TGFβ activation. These data demonstrate that overexpression of integrin β6 in KCs or administration of TNF-α in the skin drives increased expression of avβ6 and avβ8, which is sufficient to increase epidermal bioavailability of active TGFβ.
Figure 18: TNF-α increases surface expression of integrins αvβ6 and αvβ8 on epidermal KCs. Representative flow plots of integrins αvβ6 and αvβ8 on IFE(a) and IM(b) KCs populations from wild-type or Itgb6−/−Itgb8ΔKCS mice that had received i.d. TNF-α (100ng) for 12 or 24hrs; IFE KCs were gated as CD45−, CD207−, MHCI−, CD34+, EpCAM+, Sca1+) or IM (CD45−, CD207−, MHCI−, CD34+, EpCAM+, Sca1−b). Luciferase activity in tMLEC TGFβ reporter cells co-cultured with bulk epidermal cells from wild-type mice were harvested 24hrs after in vivo i.d. TNF-α (100ng); data is presented as relative light units (RLU) (c). Integrin αvβ6 expression in a KCs cell line transfected with an empty plasmid construct (EV) or with an integrin β6 plasmid construct (β6). Control indicated EV cells stained with fluorescence minus 1 (FMO) (d). Luciferase activity in tMLEC TGFβ reporter cells co-cultured with either EV or β6 transfected KCs cell line. Data is presented as relative light units (RLU), normalized to control cells. Data are representative of two independent experiments with a cohort size of 3-4 (a,b,e). Each symbol represents data from an individual animal (c). *p < 0.05

3.4 Discussion

Herein, we detailed the in vitro and in vivo methods developed to study the role of TGFβ-activating integrins αvβ6 and αvβ8 in response to LC migratory stimuli. We attempted the development of an integrin β6 reporter mouse (FT-β6) Although we obtained viable founders, we were unable to detect protein expression of our fluorescent protein. Next, we focused on the development of antibodies that specifically detect mouse integrins αvβ6 and αvβ8. By using Itgb6−/−Itgb8ΔKCS mice, we were able to validate that anti-αvβ6(6.3g9) and anti-αvβ8(C6D4) specifically detect integrins αvβ6 and αvβ8 on mouse KCs. To study the effects of a wide range of migratory
stimuli on integrin expression, we also developed protocols for the efficient isolation of pKC from mouse neonates. We then subject our pKC cultures to spontaneous immortalization to establish several KCs cell lines that had different efficiencies to transactivate LAP-TGFβ.

These tools allowed us to test our hypothesis of whether inflammatory stimuli suppressed the expression of TGFβ-activating integrins αvβ6 and αvβ8 on KCs. Here, we demonstrate that the loss in TGFβ bioavailability through the transactivation of integrins αvβ6 and αvβ8 on KCs does not occur in response to inflammatory stimuli. Instead, our findings demonstrate that TNF-α, IL-1β, and chemical-hapten (DMBA) profoundly increases the surface expression of αvβ6. Notably, the increased expression of integrin αvβ6 on pKC resulted in an increased transactivation of LAP-TGFβ on pKC. As previously reported, UVB significantly suppressed the expression of integrins β6 and β8 on pKC. Notably, we were able to demonstrate that TNF-α also increased the expression of integrin αvβ6 in \textit{in vivo} KCs. Even though integrin αvβ6 is predominantly expressed in IFE KCs at steady state, we observed that IFE and IM KCs rapidly increase the mRNA and protein expression of integrin αvβ6 in response to I.D. TNF-α. This unique observation suggests that integrin αvβ6 may have a redundant function throughout the epidermis during inflammation.

Similar to our \textit{in vitro} studies, the increased in integrin αvβ6 directly resulted an increased in epidermal active TGFβ bioavailability. Although, it is unclear of the purpose for an increased in integrin αvβ6 in response to TNF-α, it is possible the excess active TGFβ may play a role in wound healing or leukocyte differentiation (Blanco-Mezquita, Hutcheon, Stepp, & Zieske, 2011; Mohammed et al., 2016; Y. Yang et al., 2019). Interestingly, we consistently saw a suppression in integrin β8 transcript on pKC and epidermal IM KCs, however the surface expression of integrin
αvβ8 heterodimer remain unchanged. It is difficult to speculate the purpose of integrin αvβ8 in inflammation, since our knowledge of integrin αvβ8 has been predominantly study in immune cells (Worthington et al., 2012). To the best of our knowledge, IM KC are the only non-immune cell where integrin αvβ8 is expressed. Thus, it appears that homoeostatic expression of integrin αvβ6 and αvβ8 is required for epidermal leukocyte retention, but they may play two distinct roles during inflammation. Altogether, our data demonstrates that inflammatory stimuli does not suppress TGFβ-activating integrins αvβ6 and αvβ8 on epidermal KCs.

Integrin αvβ6 is predominantly expressed in the epithelium lining of the skin, gut, lung, kidney, and uterus (Koivisto et al., 2018). We have previously shown that mice lacking integrin β6 are not able to establish CD8+T_{RM} in the gut (Mohammed et al., 2016). Thus, it appears that homoeostatic expression of integrin αvβ6 is key in maintaining leukocytes to epithelium barriers. Although TGFβ has been extensively described throughout the literature to participate in many biological processes. It is uncertain whether inflammatory stimuli can increase the expression of integrin αvβ6 that results a direct increase in TGFβ bioavailability in other epithelium cells remains to be determined.
4.0 Discussion

With this work we demonstrate that enforced TGFβR signaling in LC is sufficient to prevent TNF-α, IL-1β, epicutaneous C. albicans infection, and small chemical hapten (DMBA) induced LC migration. This extends our prior findings that homeostatic and UVB-induced LC migration can be inhibited by enforced TGFβRCA expression. Consistent with these observations, we demonstrated that loss of TGFβ signaling (decreased pSMAD2) in LC occurs in response to I.D administration of TNF-α. We also confirmed our prior observation that UVB suppresses the expression of TGFβ-activating integrins αvβ6 and αvβ8 in pKC that results in reduced TGFβ transactivation. However, contrary to our expectations, inflammatory stimuli such as TNF-α did not decrease but rather increased Itgb6 and αvβ6 expression *in vitro* and *in vivo* KCs. This was associated with an increase in KCs-mediated TGFβ transactivation. Thus, loss of TGFβ transactivation by KCs is associated with homeostatic and UVB-induced LC migration but not with inflammation-induced LC migration.

We had expected that loss of TGFβ signaling through the suppression of KCs-mediated transactivation would be a fundamental component in LC migration. The observation that forced expression of TGFβRCA can prevent LC migration indicates that high levels of TGFβR signaling can overcome a wide variety of migratory signals. The observation that pro-inflammatory stimuli increase KCs-mediated TGFβ transactivation indicates that these signals do not mediate LC migration through the extrinsic loss of TGFβ availability. Interestingly, it has been suggested that TNF-α -induced LC migration is inhibited by global ablation of TNFRII (p50), but not TNFRI
In the epidermis, TNFRII is believed to be preferentially expressed by LC (Eaton et al., 2015; Luo et al., 2006; S. Yang, Wang, Brand, & Zheng, 2018). Thus, TNF-α may induce migration by acting directly on LC to interrupt intrinsic TGFβ signaling. In contrast, LC migration in response to IL-1β is not affected by the loss of Myd88 which is a required component of canonical IL-1β receptor signaling (Didovic et al., 2016; Haley et al., 2012). Thus, IL-1β likely triggers LC migration through a LC-extrinsic mechanism that is independent of reduced TGFβ transactivation.

It has previously shown that systemic administration of either anti-IL-1β or anti-TNF-α, prevents TNF-α or IL-1β induced LC migration, respectively (Antonopoulos et al., 2001; Cumberbatch et al., 1997). Notably, TNF-α induced LC migration is inhibited in Caspase1−/− deficient mice. These observations suggest that LC migration requires both signals from TNF-α and IL1-β. Our findings suggest a model where IL-1β acts through a LC-extrinsic mechanism, but not through the suppression of TGFβ activating integrins, while TNF-α acts through an LC-intrinsic mechanism to elicit LC migration. Nonetheless, both cytokines appear to be essential for LC migration. At least in human fibroblasts, IL-1β has been shown to downregulate e-cadherin and β-catenin, thus it is possible that IL-1β maybe acting on KCs to suppress anchoring proteins, while engagement of TNF-α to the LC-TNFRII receptor programs LC for migration (Karmakar & Das, 2004).

Evidently, the loss of TGFβ signaling seems to be a requirement for DNFB and TNF-α induced LC migration (Bobr et al., 2012). Thus, it is possible that engagement of TNF-α to TNFRII results in a signaling cascade that intrinsically interrupts TGFβ signaling. Although, there is an
extensive amount of literature studying TNFR1 signaling, very little is known about how TNFRII signals. Thus, it is difficult to speculate the intrinsic inhibitory mechanism between TNFRII signaling and TGFβ signaling in LC. Nonetheless, since the loss of TGFβ signaling in response to TNF-α or DNFB is observed as decreased staining of pSMAD2 and pSMAD2/3, respectively. We could speculate that the interruption of TGFβ signaling by TNF-α or DNFB occurs upstream, prior to the phosphorylation of SMAD2 and SMAD2/3 intracellular proteins. Degradation of TGFβR it is an unlikely mechanism for the interruption of TGFβ signaling in LC, since expression of TGFβRCA is not compromised in response to migratory stimuli. Although not shown here, we did not observe significant changes in Tgfβr1 or Tgfβr2 transcripts in sorted LC from mice treated with I.D. TNF-α. In our LC^{TGFβRCA} mice, the TGFβR1 intracellular serine/threonine residues are replaced with a non-cleavable phosphomimetic amino acids (e.g., aspartic acid), which also prevents the binding of inhibitory molecules such as immunophilin FKBP12. This small immunophilin have been shown to dampen TGFβ signaling by binding to the intracellular serine/threonine residues of the TGFβR1 (Chen, Liu, & Massague, 1997; Wieser et al., 1995; Wrana et al., 1994). Hence, it is possible that TNFRII activation results in the suppression of TGFβ signaling through a FKBP12 dependent mechanism. Notably, these speculations are based under the assumption that induction of TGFβRCA does not impact LC responsiveness to migratory stimuli and that alternative pathways (e.g., non-canonical TGFβ pathways) are not actively participating in the induction of LC migration.

One interesting observation from our studies was that recently migrated LC in response to I.D TNF-α had regained sensitivity to TGFβ in the draining LNs. This is an unexpected finding,
since it has previously shown that TGFβ negatively regulates the antigen presentation function of classical human LCs in vitro. LC were activated with PAMPs, DAMPS, or co-stimulatory cytokines that resulted in the upregulation of MHCII and co-stimulatory molecules, which induced T-cell activation and differentiation. Addition of TGFβ to LPS-stimulated LC cultures suppressed the expression of MHCII and other co-stimulatory molecules which in turn dampened the ability of LC to efficiently present antigen (Geissmann et al., 1999). The suppressive effects of TGFβ could prevent LC from eliciting harmful immune responses in the LNs. Through several mouse transgenics it has been shown that ablation of TGFβ activating integrin αvβ8 on DC, mice developed an age-related wasting disorder associated with T-cell activation, aberrant T-cell associated antibody production, and early onset of colitis (Travis et al., 2007). Previously, we have demonstrated that epidermal LC expressed integrin αvβ8 which is not required for their epidermal retention (Mohammed et al., 2016). Thus, it is possible that LC-integrin αvβ8 could be a self-mechanism to prevent autoimmunity. Alternatively, regaining sensitivity to TGFβ in the draining LN could serve as a retention signal similarly to homeostatic epidermal LC. Either outcome presents an interesting contribution to our understanding of LC biology.

In these studies, we showcased that enforced TGFβR signaling in LC is sufficient to prevent epicutaneous C. albicans infection induced migration. Consistent with these results, we saw a dampened polarization of Th17 T-cell differentiation, which requires IL-6 and TGFβ. We have previously demonstrated that LC provide IL-6 for Th17 differentiation (Kashem et al., 2015). It is also possible that LC transactivates TGFβ through integrin αvβ8 to polarize a Th17 T-cell response. This is a possible role for integrin αvβ8 on LC, since it is now apparent that
transactivation of TGFβ by integrin αvβ8 on DC is essential for the development of Th17 cells. DCs that lack TGFβ activating integrin αvβ8 have reduced ability to promote Th17 cells in vitro. Moreover, mice lacking integrin αvβ8 on DCs have reduced numbers of Th17 cells in vivo (Melton et al., 2010). Although there is a more detailed understanding of the role of integrin αvβ8 in the immune system, the function of this TGFβ activating integrin expressed in non-immune cells is not entirely clear (Worthington et al., 2012). In our studies, we saw that integrin β8 on primary KCs was transcriptionally suppressed in response to inflammatory stimuli. However, surface expression of integrin αvβ8 remain relatively unchanged. Consistent with this finding, we also did not observe the specific depletion of LC in IM KCs, where integrin αvβ8 is predominantly expressed. Perhaps, transactivation of TGFβ by αvβ8 only serves as retention mechanism to position leukocytes in the epidermis that cannot be suppress by inflammatory stimuli.

The functional importance of increased αvβ6 expression in response to inflammatory stimuli remains unclear. TGFβ is required for differentiation of CD8+ T_{RM} in the epidermis and the gut epithelium (Hirai et al., 2019; Mohammed et al., 2016). We have previously demonstrated that in the absence of integrins αvβ6 and αvβ8 on KCs, CD8+ effector T-cells have dampened CD103 expression and failed to establish epidermal residency. Thus, it is possible that the increased expression of integrin αvβ6 in response to inflammatory stimuli is to support the differentiation of CD8+ T_{RM} (Hirai et al., 2019). Alternatively, the increase in integrin αvβ6 could participate in the recruitment of monocyte-derived LC to the skin epidermis during inflammation or their differentiation into short-lived LC (Ferrer et al., 2019; Mackay et al., 2013). As previously mentioned, during ontogeny the epidermis of mice deficient in the cytokine TGFβ1 (TGFβ1−/−) is
completely devoid from LC. However, entry of myeloid precursors into the epidermis during ontogeny is not affected (Borkowski et al., 1996; Kel et al., 2010). Moreover, CD8+ effector T-cells are recruited in normal numbers into the skin epidermis of mice lacking integrins αvβ6 and αvβ8 during *Vaccinia virus* infection (Mohammed et al., 2016). Thus, it is unlikely that the recruitment of monocyte-derived LC requires TGFβ during inflammation. However, the differentiation of monocyte-derived LC to short-lived LC requires the upregulation of langerin and EpCAM, which can be induced by TGFβ (Bigley et al., 2015). Thus, we speculate that the increased αvβ6 expression may be required for differentiation of short-lived LC and CD8+ TrM during inflammation.

Another explanation for the increased in αvβ6 expression in response to inflammatory stimuli is to aid in wound healing. Wound healing requires a finely regulated response from epithelial, inflammatory, and connective tissue. KCs have been described to modulate dermal fibroblast’s ability to synthesize collagen, a key factor in wound healing that is directly induced by TGFβ (B. S. Bauer, Tredget, Marcoux, Scott, & Ghahary, 2002; Lijnen & Petrov, 2002; Nowinski et al., 2002). *In vitro*, integrin αvβ6 mediates cell-adhesion and migration on fibronectin, vitronectin, and tenascin, all components of wound provisional matrix (Ljubimov, Saghizadeh, Pytela, Sheppard, & Kenney, 2001; Stepp & Zhu, 1997). In comparison to wild-type, integrin β6-/- deficient mice are not as efficient in promoting corneal wound healing (Blanco-Mezquita et al., 2011). Thus, these studies suggest an important role for integrin αvβ6 in modulating wound healing. Altogether, integrin αvβ6 may play multiple roles in skin biology. At steady-state, integrin
avβ6 could serve as a retention mechanism for epidermal leukocytes, while during inflammation it might support wound healing processes and the differentiation of recently recruited leukocytes.

These observations were possible through the development and optimization of several techniques. We developed a protocol for the quick isolation of primary KCs cultures from mouse neonates that were used for the high-throughput screening of several migratory stimuli with the purpose to detect changes in integrins avβ6 and avβ8 expression. These primary KCs cultures isolation protocols will aid in future studies that aim at gaining a better understanding of KCs biology. Although, we were not successful in creating a reporter mouse to study the kinetics of integrin β6 by in vivo microscopy, we did optimize antibodies that detect mouse TGFβ activating integrins on KCs. We validated that anti-αvβ6(6.3g9) and anti-αvβ8(C6D4)-PE antibodies specifically detect mouse integrins αvβ6 and αvβ8 via flow cytometry, respectively. As mentioned above, integrin αvβ8 has been described to play a significant role in controlling inflammation via different immune cells. To this date, detection of mouse integrin αvβ8 via flow cytometry has not been possible, thus we present an exciting new tool to study the expression and regulation of integrin αvβ8 in the immune system. The expression of integrin αvβ6 is restricted to epithelial cells. The optimization and validation of anti-αvβ6(6.3g9) has revealed that the homeostatic expression of integrin αvβ6 varies based on anatomical sites. For instance, we recently reported that KCs from the ear epidermis lack steady-state expression of integrin αvβ6 that could explain the inability of CD8+ T_{RM} to differentiate and establish residency (Hirai et al., 2021). Thus, the tools developed to study KCs TGFβ-activating integrins αvβ6 and αvβ8 can be utilized to expand our knowledge of their role in the immune system.
Although we did not observe the suppression of KCs TGFβ-activating integrins αvβ6 and αvβ8 in response to inflammatory stimuli. Depletion of potential pathogenic epidermal leukocytes through the suppression of TGFβ-activating integrins on KCs remains of therapeutic interest. We contribute to the field of LC biology by demonstrating that the loss of intrinsic TGFβ signaling in LC is key to mediate homeostatic, UVB, and inflammation induced migration. However, steady-state and UVB, but not inflammatory stimuli act through the suppression of KCs TGFβ-activating integrins. It is interesting to note that the two conditions where loss of KCs-mediated TGFβ transactivation triggers LC migration (i.e., homeostasis and UVB) are both associated with the induction of peripheral tolerance(Mutyambizi, Berger, & Edelson, 2009; Shklovskaya et al., 2011; Yoshiki et al., 2010). This is consistent with the observation that those LC that have migrated into regional LN in response to an artificial loss of autocrine TGFβ maintain an immature activation state(Bobr et al., 2012). Explorations of the genomic state of LC following different types of migratory stimuli represents an exciting future avenue to for exploration.
Bibliography


