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THE ROLE OF TGFβ SIGNALING IN ULTRAVIOLET RADIATION (UVB)-INDUCED CUTANEOUS INFLAMMATION AND CARCINOGENESIS

A Dissertation in

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by

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ABSTRACT

Inflammation is an integral component of carcinogenesis and chronic dysregulated inflammation is recognized as an important tumor promoter increasing cancer risk. Cytokines, chemokines and growth factors in the tissue microenvironment, play a critical role in regulating the immune response to foreign antigen or altered self-antigen. Ensuing tissue damage and tissue repair from inflammation contribute to tumor promotion in part by subversion of the protective immune response generated against the neoplastic antigens into a predominantly protumorigenic response. Environmental exposure to ultraviolet radiation (UVB) is the major cause of skin cancer and exposure at high doses can potently activate the immune system. UVB can act as a complete carcinogen in the skin by inducing mutations in the DNA and establishing a proinflammatory microenvironment. Transforming growth factor-β1 (TGFβ1) is a potent negative regulator of epithelial cell proliferation and a key immunoregulatory cytokine in the skin with a pivotal function in maintaining steady-state peripheral tolerance. Previous studies using the 2-stage skin carcinogenesis model with mice that have alterations in the TGFβ1 pathway showed that TGFβ1 can function as a tumor promoter as well as a tumor suppressor but its role in UVB-induced skin carcinogenesis is not known.

To study the role of TGFβ signaling in UVB-induced cutaneous inflammation and carcinogenesis, we used a pharmacological inhibition model with SB431542 (SB), a small molecule inhibitor of TGFβ type I receptor kinase and a genetic abrogation model, CD11c-dnTGFβRII in which mice express a dominant negative human transforming growth factor beta receptor II gene under the control of a CD11c promoter (CD11c-DNR), specific for dendritic cells. UVB-induced skin tumor formation in Skin Hairless-1 mice (SKH1) was suppressed by topical treatment with SB, as were CD4 and CD8 Tumor infiltrating lymphocytes (TIL), and

IFNγ^{+} TIL. However, there was no difference in the tumor size or tumor cell proliferation. To understand the mechanism of TGFβ modulation of tumoral immune response, we used acute and chronic models of UVB induced inflammation with SB. SB blocked acute and chronic UVB-induced skin inflammation, linked to reduced T cell activation and IFNγ secretion in the skin and skin draining lymph nodes suggesting that the difference in tumor inflammation could be critical to reduced tumor outgrowth when TGFβ1 signaling was blocked.

Dendritic cells are professional antigen presenting cells forming the bridge between the innate and the adaptive immune system and are key mediators of cutaneous inflammation. TGFβ signaling is important for the development of Langerhans cells (LCs), the epidermal DC subset in the skin. It is also implicated in the steady state epidermal maintenance of LCs. However, the role of TGFβ signaling for immunostimulatory function of LCs or other dermal DC subsets in response to an inflammatory stimulus such as UV irradiation is not clear. When we studied the effect of SB on DCs in an acute UVB model, we found that SB blocked p-Smad2 induction in dermal DCs, the migration of CD103^{+} CD207^{+} and CD207^{-} dermal DC subsets to the skin draining lymph nodes (SDLNs) and in an *ex vivo* ear explant assay. However, SB did not alter the p-Smad2^{+} percentage or the basal or UVB-induced migration of LCs implicating CD103^{+} CD207^{+} and CD207^{-} dermal DC subsets as key DC populations for UVB-induced skin inflammation.

To further determine the requirement of TGFβ signaling specifically in DCs, we used CD11c-DNR mice to study UVB-induced inflammation. Defective TGFβ signaling in DCs reduced the steady-state migration of all three DC subsets: LCs, CD103^{+} CD207^{+} and CD207^{-} dermal DC subsets. Acute UVB irradiation, however, reduced the migration of only CD103^{+} CD207^{+} and CD207^{-} dermal DC subsets to the SDLNs and CD207^{-} subset in a similar ear explant assay in

DNR mice compared to WT. This correlated with a reduction in p-Smad2^{+} and CD86^{high} subset of dermal DCs. There was no difference in UVB-induced migration of LCs between WT and DNR mice.

Finally, to test the physiological relevance of the UVB-induced migration of dermal DC subsets, we used a T cell driven pathological model of Contact Hypersensitivity. Defective TGFβ signaling in DCs in DNR mice led to an attenuated ear thickness response in CHS compared to WT. In addition, the synergistic increase in ear thickness, CD4, CD8 T cell activation and cytokine profile in the SDLNs in WTs with UVB plus CHS was significantly suppressed in DNR mice. Together, these results highlight the critical requirement of TGFβ signaling in DCs for an optimal cutaneous immune response to UV irradiation. In contrast to the well documented immunoinhibitory properties of TGFβ, these results suggest a model in which TGFβ signaling in DCs is important for UVB induced cutaneous inflammation as well as other cutaneous inflammatory responses mediated by dermal dendritic cells. Together, these studies highlight a novel tumor-promoting mechanism for TGFβ in UVB skin carcinogenesis.

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Abbreviations

SCC- Squamous cell carcinoma
BCC- Basal cell carcinoma
HNSCC- Head and neck squamous cell carcinoma
AK- Actinic keratosis
UVR- Ultraviolet radiation
UVB- Ultraviolet radiation subtype B
TGFβ1- Transforming growth factor β1
LAP- Latency-associated peptide
LTBP- Latent TGFβ binding protein
SB- SB431542
ALK5- Activin receptor-like kinase 5, TGFβ type I receptor
TβRI- TGFβ type I receptor
TβRII- TGFβ type II receptor
DNR- Dominant negative receptor
SMAD- Homolog of SMA and mothers against decapentaplegic
SBE- Smad binding element
EMT- Epithelial to mesenchymal transition
FACS- Fluorescently activated cell sorting
qRT-PCR- Quantitative reverse transcribed polymerase chain reaction
H&E- Hematoxylin and Eosin
TUNEL- Terminal deoxynucleotidyl transferase dUTP nick end labeling
BrdU- 5-bromo-2-deoxyuridine
DAB- 3,3'-diaminobenzidine
NFκb- Nuclear factor kappa B
STAT3- Signal transducer of activated T cells type 3
Ras- Protein subfamily of small GTPases
DMBA- 7,12-dimethylbenzathracene
TPA- 12-*O*-tetradecanoylphorbol-13-acetate
MAPK- Mitogen activated protein kinase
CDK- Cyclin-dependent kinase
JNK- Jun amino-terminal kinase
PI3K- Phosphatidylinositol 3-kinase
JAK- Janus kinase
ERK- MAP-kinase
MPO- Myeloperoxidase
GAPDH- Glyceraldehyde 3-phosphate dehydrogenase
LN- Lymph node
SDLN- Skin-draining lymph node
IL- Interleukin
CCL- Chemokines with consensus consecutive cysteine-cysteine sequence
ROS- Reactive oxygen species
iNOS- Inducible nitric oxide synthase
NO- Nitric oxide
RNS- Reactive nitrogen species
MMP- Matrix metalloprotease

VEGF- Vascular endothelial growth factor
CSF- Colony stimulating factor
GM-CSF- Granulocyte/monocyte colony stimulating factor
TNFα- Tumor necrosis factor alpha
IFNγ- Interferon gamma
MHC- Major histocompatibility complex
CD- Cluster of differentiation antigen
Ly6G- Surface marker for neutrophils
F4/80- Surface marker for macrophages
TCR- T cell receptor
NK- Natural killer cells
NKT- Natural killer T cells
DETC- Dendritic epidermal T cell
PMN- Polymorphonuclear cells
TAM- Tumor associated macrophage
M1- Classically activated macrophage
M2- Alternatively activated macrophage
TIL- Tumor infiltrating lymphocyte
CTL- Cytotoxic $CD8^+$ T lymphocyte
T_H- $CD4^+$ T helper lymphocyte
T_H1- T_H cells expressing IFNγ and Tbet
T_H2- T_H cells expressing IL-4 and GATA-3
T_H17- T_H cells expressing IL-17A/F and RORγT
Treg- CD4 T lymphocytes expressing TGF-β and FoxP3
FoxP3- Forkhead box P3
γδT cell- T lymphocyte expressing γδ chains of the T-cell receptor
DC- Dendritic cell
LC- Langerhans cell
pDC- Plasmacytoid DC
dDC- Dermal DC
CD207/Lang- Langerin
CHS- Contact hypersensitivity
DNFB- 2,4-dinitrofluorobenzene

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Chapter 1

1 INTRODUCTION

1.1 Chronic inflammation and Cancer

In 1863, Rudolf Virchow made a key observation that there was enhanced infiltration of leukocytes in neoplastic tissues implicating “lymphoreticular infiltrate” at sites of chronic inflammation as playing a crucial causal role in carcinogenesis [1]. Recent research has clearly shown that chronic inflammation increases cancer risk [13;14] 15 to 20 % of cancers are linked to inflammation from chronic infection [15], 30 % from inflammation due to physical agents like ultraviolet (UV) irradiation/chemical agents like smoking and inhaled pollutants [16] and 35 % due to autoimmune and metabolic disorder associated inflammation [17-19].

1.1.1 Acute inflammation versus chronic inflammation

Inflammation is a physiologic process in response to tissue damage resulting from pathogenic infection, chemical irritation and wounding [20;21]. Neutrophils are the first cells to respond to an inflammatory insult and migrate to the tissue in reponse to chemokines secreted by tissue resident macrophages and mast cells [4;22]. As the inflammation progresses, multiple other types of leukocytes and inflammatory cells are activated and attracted to the site of inflammation by a regulated network of cytokines, chemokines and growth factors [4;21;22]. All these mechanisms forge a protective line of defense against the infection or the source of insult with tissue damage as an unwanted byproduct of inflammation.

Acute inflammation is self-limiting and once the infectious or chemical/physical agent is cleared by the acute inflammatory cascade, there are mechanisms to regulate the inflammation from lasting too long [23]. A shift from tissue damage to tissue repair occurs involving both

proinflammatory and anti-inflammatory molecules [23]. Transforming growth factor β (TGFβ) [24] is an important immunomodulatory molecule for bringing the tissue back to homeostasis. The resolution of inflammation requires a rapid programmed clearance of infiltrating leukocytes as well as damaged cells; phagocytes play an important role in this event [25-27]. This event is tightly regulated by the anti-inflammatory role of TGFβ in the tissue microenvironment [28-30]

However, if the resolution of inflammation is dysregulated either due to non-clearance of the foreign antigen or the persistence of the environmental insult such as repeated UV exposure, the cellular response changes to a pattern of chronic inflammation where the inflammatory signature is dominated by components of the adaptive immune system such as lymphocytes, plasma cells and macrophages [20]. Macrophages and lymphocytes especially $T_{helpher}$ cells are rich sources of cytokines, chemokines, growth factors and reactive oxygen species that can directly cause DNA damage as well [4]. In carcinogenesis, where there is a persistence of the altered self-antigen from preneoplastic lesions, chronic inflammation acts synergistically with a tumor-initiating carcinogen exposure [31]. Such a proinflammatory microenvironment leads to continued tissue damage and acts as a mitogen for tumor-initiated mutated cells to proliferate uncontrollably ultimately leading to tumor formation[1].

1.1.2 The causal link between chronic inflammation and preneoplastic lesions

There are several reports of non-tumor associated inflammation leading to tumor formation from epidemiological and clinical studies [1;4;20] (Table 1.1). The link between increased colon cancer risk with inflammatory bowel disease [32-35] and increased lung cancer risk with prolonged *EBV* infection has been well-reported [36]. Gastric cancer, adenocarcinoma and mucosa-associated lymphoid tissue lymphoma caused by gastric *Helicobacter pylori* infection is

a well-documented model of inflammation driven carcinogenesis [37;38]. Hepatocellular carcinoma is associated with chronic hepatitis caused by hepatitis B and C [39]. Possible associations between inflammation from environmental factors such as asbestos and mesothelioma [36], silica and lung cancer [36], UVB irradiation and skin cancer have also been reported. Autoimmunity driven inflammation has been implicated as a strong cofactor for tumorigenesis [40;41]. Thus, there is strong evidence linking chronic and dysregulated inflammation to cancer development.

Table 1 **Chronic inflammatory conditions associated with neoplasms**

Pathologic condition	Associated neoplasm(s)	Aetiologic agent
Asbestosis, silicosis	Mesothelioma, lung carcinoma	Asbestos fibres, silica particles
Bronchitis	Lung carcinoma	Silica, asbestos, smoking (nitrosamines, peroxides)
Cystitis, bladder inflammation	Bladder carcinoma	Chronic indwelling, urinary catheters
Gingivitis, lichen planus	Oral squamous cell carcinoma	
Inflammatory bowel disease, Crohn's disease, chronic ulcerative colitis	Colorectal carcinoma	
Lichen sclerosus	Vulvar squamous cell carcinoma	
Chronic pancreatitis, hereditary pancreatitis	Pancreatic carcinoma	Alcholism, mutation in trypsinogen gene on Ch. 7
Reflux oesophagitis, Barrett's oesophagus	Oesophageal carcinoma	Gastric acids
Sialadenitis	Salivary gland carcinoma	
Sjögren syndrome, Hashimoto's thyroiditis	MALT lymphoma	
Skin inflammation	Melanoma	Ultraviolet light
Cancers associated with infectious agents		
Opisthorchis, Cholangitis	Cholangiosarcoma, colon carcnoma	Liver flukes (*Opisthorchis viverrini*), bile acids
Chronic cholecystitis	Gall bladder cancer	Bacteria, gall bladder stones
Gastritis/ulcers	Gastric adenocarcinoma, MALT	*Helicobacter pylori*
Hepatitis	Hepatocellular carcinoma	Hepatitis B and/or C virus
Mononucleosis	B-cell non-Hodgkin's lymphoma, Burkitts lymphoma,	Epstein-Barr Virus
AIDS	Non-Hodgkin's lymphoma, squamous cell carcinomas, Kaposi's sarcoma	Human immunodeficiency virus, human herpesvirus type 8
Osteomyelitis	Skin carcinoma in draining sinuses	Bacterial infection
Pelvic inflammatory disease, chronic cervicitis	Ovarian carcinoma, cervical/anal carcinoma	Gonnorrhoea, chlamydia, human papillomavirus
Chronic cystitis	Bladder, liver, rectal carcinoma, follicular lymphoma of the spleen	Schistosomiasis

Modified from refs 29, 67. MALT, mucosa-associated lymphoid tissue.

Table 1.1 **Chronic inflammation acts as a precursor for carcinogenesis:** The link between chronic inflammation and cancer is highlighted by the increased cancer susceptibility caused by chronic inflammation associated with different pathological conditions including infection. *Reprinted with permission from Macmillan publishers Ltd: Nature [4] © 2002.*

1.1.3 Tumor-associated chronic inflammation acting as a tumor promoter

Most of the solid tumors are infiltrated with immune cells [35]. This could represent either an anti-tumor or a pro-tumor response. The anti-tumor immune response is mainly dependent upon $CD8^+$ cytotoxic T cells (CTLs) and natural killer cells, which have potent anti-viral and anti-tumor functions [42]. The CTLs perform their cytotoxicity mainly through perforin/granzyme and induce target cell apoptosis by TRAIL, FasL-dependent mechanisms [43]. $T_{Helper}1$ and $T_{Helper}17$ cell lineages can both provide important help to boost cytotoxic immunity [44]. In addition, NKT cells can also be involved in surveillance of tumors [45;46]. Macrophages and Dendritic cells (DCs) can act as antigen-presenting cells and respond to danger /immunoregulatory signals such as type I IFN, IFNγ, FasL, TRAIL, GM-CSF, IL-12. [14;43;46;47].

However, tumors in most cases, through their ability to constantly mutate and evolve, subvert the immune response and exploit it for tumor promotion [35]. This phenomenon is described as “tumor escape” [48]. While the primary purpose of the host immune response is to recognize the tumor antigens as foreign and mount an anti-tumor response, once the immune infiltrates are within the tumor, they can be diverted by cytokines and growth factors in the tumor microenvironment towards a pro-tumorigenic response [49;50] (Figure 1.1).

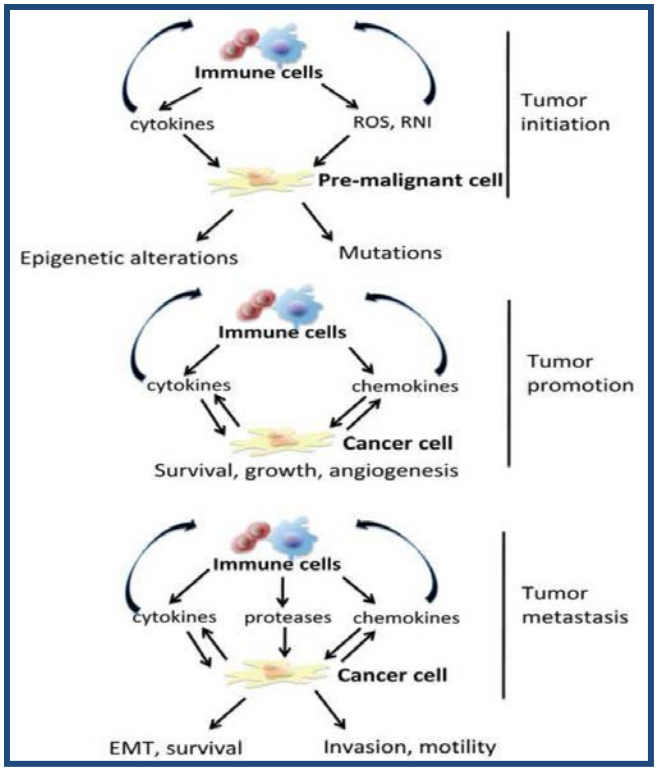

Figure 1.1 **Inflammation is an integral event of carcinogenesis and is both causal and resultant with respect to carcinogenesis:** Inflammation is associated with the different stages of tumorigenesis. In the initial stages, the immune system recognizes the altered self-antigen and mounts an immune response. The immune response can also induce mutations directly. Once an oncogenic mutation is sustained, the tumor-initiated clone can subvert the immune response and convert it into a protumorigenic response facilitating tumor promotion. In later stages, the growth factors and cytokines from the immune cells present in the tumor microenvironment allow the tumors to acquire invasive phenotype and metastasize. *Reprinted from [7] © 2010 with permission from Elsevier.*

Some of the key immune populations involved in immune responses to tumor are listed below:

Tumor-associated macrophages (TAMs) represent a major immune component of most tumor infiltrates [1;51]. TAMs can potently kill tumor cells and mount a potent protective response when they acquire the M1 phenotype secreting IL-12 and other anti-tumorigenic components. However, they can also produce growth and angiogenic factors including matrix metalloproteases which increase the invasiveness of the tumor and favor tumor growth [52]. In this case, they assume a M2 phenotype which is predominantly immunosuppressive and protumorigenic [53-55]. In response to TGFβ in the tumor microenvironment, TAMs spontaneously release copious amounts of IL-10, which is a potent immunosuppressor [56].

Dendritic cells (DCs) are professional antigen-presenting cells with a crucial role in the activation of antigen-specific immunity acting as a bridge between the innate and the adaptive immune responses. Another important function of DCs is the maintenance of tolerance in the periphery under homeostatic conditions [1]. Tumor-infiltrating DCs (TADCs) usually assume an immature phenotype defective in their ability to prime naïve T cells [57] in breast cancer and thyroid cancer, while mature DCs are restricted to the peritumoral or noninvasive regions of the tumor [57]. This distribution is distinct for TADC as opposed to TAM, which are evenly distributed throughout the tumor tissue [1]. Thus, TADCs are often poor inducers of downstream T cell activation to tumor antigens.

Tumor-infiltrating lymphocytes (TILs): The predominant T-cell population infiltrating tumors are the memory effector T cells and natural killer cells, while B cells are rarely present in the tumor microenvironment [58]. T_H1 and $CD8^+$ CTLs are important for anti-tumor responses. IFNγ plays a key role against cancers by enhancing the cytotoxic responses against tumors and also strengthening the innate immune system by activating Macrophages. The role of IFNγ in rejecting transplanted tumors is well established and has also been shown to be anti-angiogenic [59-63]. However, these $IFN\gamma^+$ T cells are also functionally disarmed within tumors in some cases where they are immunomodulated by the tumor microenvironment [31;64-66]. Signaling mediated by T cell receptor has also been reported to be defective in these TILs [67].The TILs in bronchial carcinoma, cervical carcinoma, Hodgkin's disease and Kaposi's sarcoma mainly produce T_H2 polarizing cytokines such as IL-4, IL-5 and are negative for interferonγ (IFNγ) [68]. There is also evidence showing IFNγ can act as a tumor promoter by enhancing monocyte/macrophage activation in hepatocarcinogenesis [69;70].

The correlation between T cell infiltration and prognosis is unclear [31]. While the presence of T cells in colon cancer represents better prognosis [71;72], a higher $CD4^+$ to $CD8^+$ ratio in breast cancer represents poor prognosis [73]. Many studies, however, show a direct correlation between increased infiltration of cytotoxic T lymphocytes and improved prognosis [74-76]. These different immune infiltrates, TAMs, TADCs and TILs constitute tumor-associated inflammation. Inflammation is associated with each stage of the carcinogenesis process (Figure 1.1). In the initial stages, the immune system recognizes the altered self-antigen and mounts an immune response. These immune cells infiltrating the preneoplastic lesions are potent stimulators of cellular proliferation, which can ultimately contribute to the carcinogenesis process. The most important function of a tumor promoter is to support the proliferation of initiated preneoplastic lesions.

Ultraviolet radiation (UVB) of the skin at high doses is a potent stimulator of cutaneous immune responses. Chronic UVB acting as a complete carcinogen can cause mutations in keratincoytes and melanocytes generating preneoplastic lesions ultimately leading to skin carcinogenesis. TGFβ is frequently overexpressed in disease conditions like cancer and fibrosis where overproduction of TGFβ drives disease progression by modulating the tumor immune microenvironment [77;78]. Our study focuses on the Dendritic cell (DC)-T cell crosstalk in a chronic UVB irradiation model and how that might act as a tumor promoter in a UVB skin carcinogenesis bioassay and explores the role of TGFβ signaling in this process.

1.2 The Protective Physiology of Skin

The skin forms the primary protective barrier against the external environment protecting the host from injury, infectious agents, water loss and ultraviolet radiation (UV) [79]. It is characterized by a sophisticated network of immune surveillance to combat invading pathogens as well as maintain peripheral tolerance. It also performs crucial functions like sensation of temperature, touch and pain [80]. The skin comprises two main layers- the epidermis and the dermis (Figure 1.2). The epidermis is the outermost compartment and consists of four strata [6]. The stratum basale consists of a single row of undifferentiated epidermal cells called the basal keratinocytes with keratins K5 and K14 [81] and is responsible for constantly renewing the cells of the epidermis [6]. As the basal keratinocytes differentiate and move to the next layer-the stratum spinosum, they also divide to replenish the basal layer. The keratinocytes in the stratum spinosum become columnar and synthesize keratins K1 and K10 [82]. The keratinocytes in the stratum granulosum synthesize additional proteins such as loricrin and filaggrin in addition to lipids. The stratum corneum is the outermost layer and is composed of dead keratinocyte-derived cells called corneocytes which are devoid of organelles [6]. This layer is mainly responsible for the barrier function of the skin. The epidermis also consists of additional specialized cells such as Langerhans cells (LCs), and melanocytes. LCs form an unique population of professional antigen-presenting cells in the epidermis that participate in cutaneous immune responses to environmental stimuli such as UV or foreign antigens. The functions of LCs are discussed in greater detail in the following chapters. Melanocytes synthesize the pigment, melanin and are important in protective pigmentation responses to UV exposure [83].

The dermis is separated from the epidermis by the basement membrane and contains hair follicles, sweat glands, sensory nerves, cutaneous vasculature, lymphatic vessels in addition to

fibroblasts and an elaborate network of immune cells. The dermal resident immune cells include a heterogenous population of dermal dendritic cells (which will be discussed in detail later) and plasmacytoid DCs (pDCs) and T cells subsets such as $T_{Helper}1$ (T_H1), $T_{Helper}2$(T_H2), T_H17, γδ T cells and natural killer T (NKT) cells. In addition, macrophages and mast cells are also present. Further, in the case of an inflammatory insult, there is a massive influx of neutrophils, monocytes/macrophages and B cells from the blood and the lymphatic system [84].

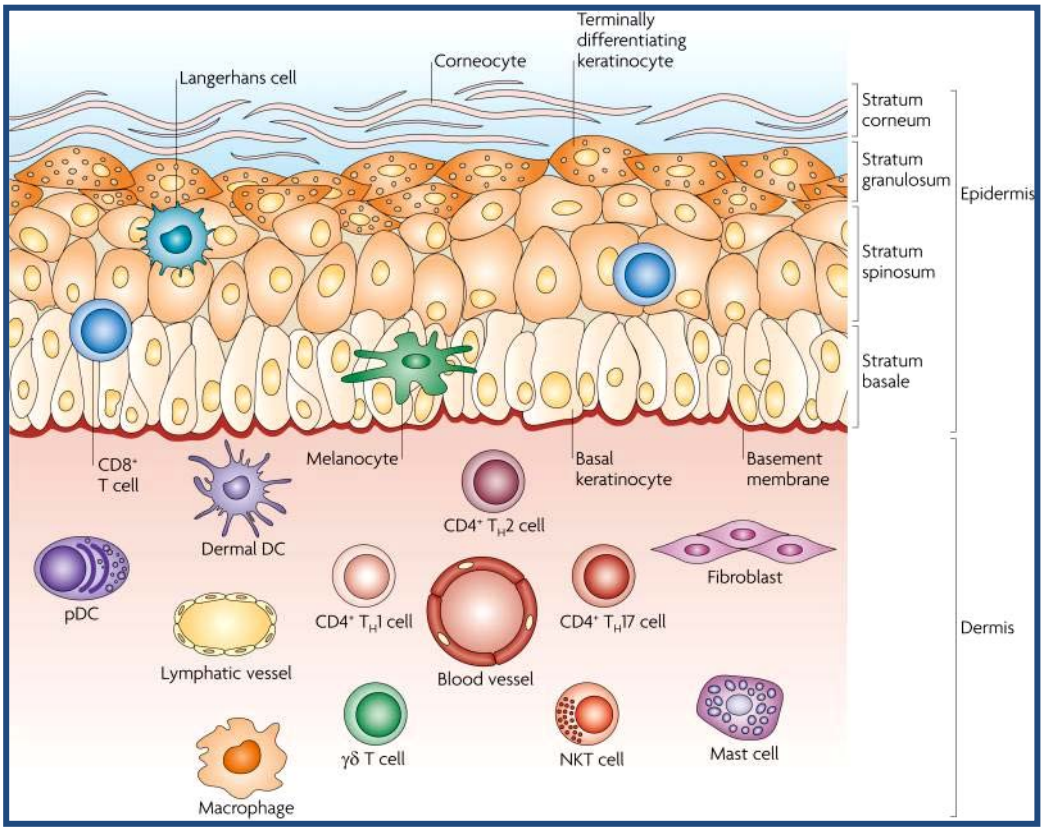

Figure 1.2 **Skin structure and the different immune components:** The complex structure of skin interspersed with different immune populations helps it to function as a key protective barrier. The epidermis contains four layers, stratum basale, stratum spinosum, stratum granulosum and stratum corneum. Keratinocytes, melanocytes, langerhans cells and rare population of CD8 T cells populate the epidermis. The dermis is composed of collagen, elastic tissue and many dendritic cell subsets including dermal cells and plasmacytoid (pDCs) and various T cell subsets, T_H1, T_H2, T_H17, γδ T cells and NK cells. In addition macrophages, mast cells and fibroblasts are also present in the dermis. *Reprinted with permission from Macmillan publishers Ltd: Nature Rev. Immun. [6] © 2009.*

1.2.1 Cutaneous antigen-presentation response mediated by Skin-resident Dendritic cell subsets

The first step in the cutaneous adaptive immune response is mediated by dendritic cells which act as antigen-presenting cells (APCs). They constitute 1-2% of the total cell numbers within the skin. Although, traditionally LCs were thought to be the primary antigen-presenting cells in the skin, numerous reports in the past decade have shown that both Langerhans cells and dermal dendritic cells can participate in this event depending on the type of inflammatory insult and the source of the foreign antigen [85;86]. The skin DCs respond to infection or the presence of an altered self-antigen within the tissue microenvironment by internalizing, processing the foreign antigen and migrating to the skin-draining lymph nodes (SDLNs) (Figure 1.3). Once in the lymph nodes, they increase the expression of co-stimulatory molecules such as CD80, CD86 and CD40 and present the processed antigen to antigen-specific naïve T cells. This causes the T cells to proliferate and differentiate producing a population of activated T cells [87] (Figure 1.4). Activated T cells then migrate to the site of insult where they act as effectors of inflammation through the release of several cytokines and chemokines for attracting other immune populations [88]. A population of antigen-specific memory T cells are also produced, which are retained in the circulation and are also resident in the skin for future recall responses to the antigen [6]. T-cell activation is critical in determining the type of immune response generated and there are several distinct subsets of T cells-CD4 T helper subsets:T_H1, T_H2, T_H17, $T_{regulatory}$ cells and CD8 T cells. The whole process is highly regulated by a network of cytokines and interleukins locally produced by dendritic cells, such as IL-1β, IL-6, IL-18, TNFα and by fibroblasts such as IL-6, TNFα [89;90].

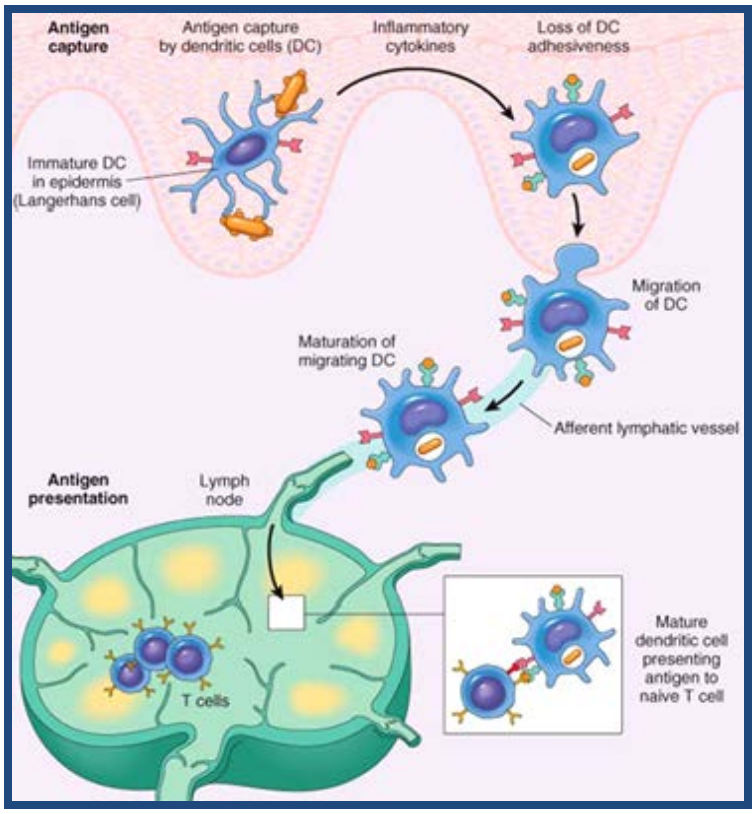

Figure 1.3 **Skin Antigen presentation: Adapted from (short communic;Symposcium;july2012)** When encountered by a foreign antigen, the peripheral dendritic cells (Langerhans cells shown) capture and process the antigen. Then they downregulate E-cadherin, become activated and migrate to the afferent skin draining lymph node (SDLN). They then upregulate the expression of costimulatory molecules such as CD40, CD80/86 and present the antigen to naïve T cells to activate them.

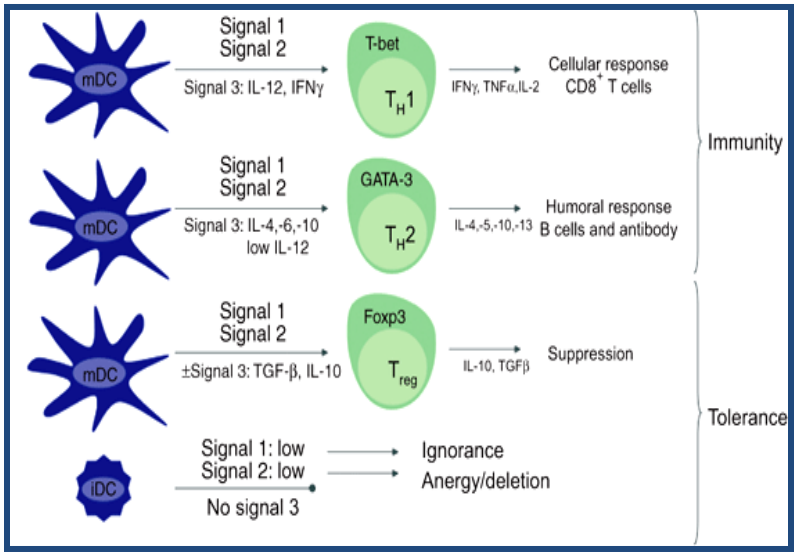

Figure 1.4 **DC-naïve T cell interaction in the SDLN:** The activation status of the DCs influences the immunogenic or tolerogenic nature of the adaptive immune response. The local cytokine profile of the DCs when interacting with the naïve T cells in the SDLNs dictates the lineage differentiation of naïve T cells: IL-12 and IFN-γ results in a T_H1 response; IL-4, IL-6, and IL-10 results in a T_H2 response; TGFβ and IL-10 lead to generation of $T_{regs.}$ Immature DCs also result in tolerance. *Reprinted with permission from Macmillan publishers Ltd: Mol. Therapy [11] © 2011.*

1.3 Overview of the TGFβ Signaling Pathway

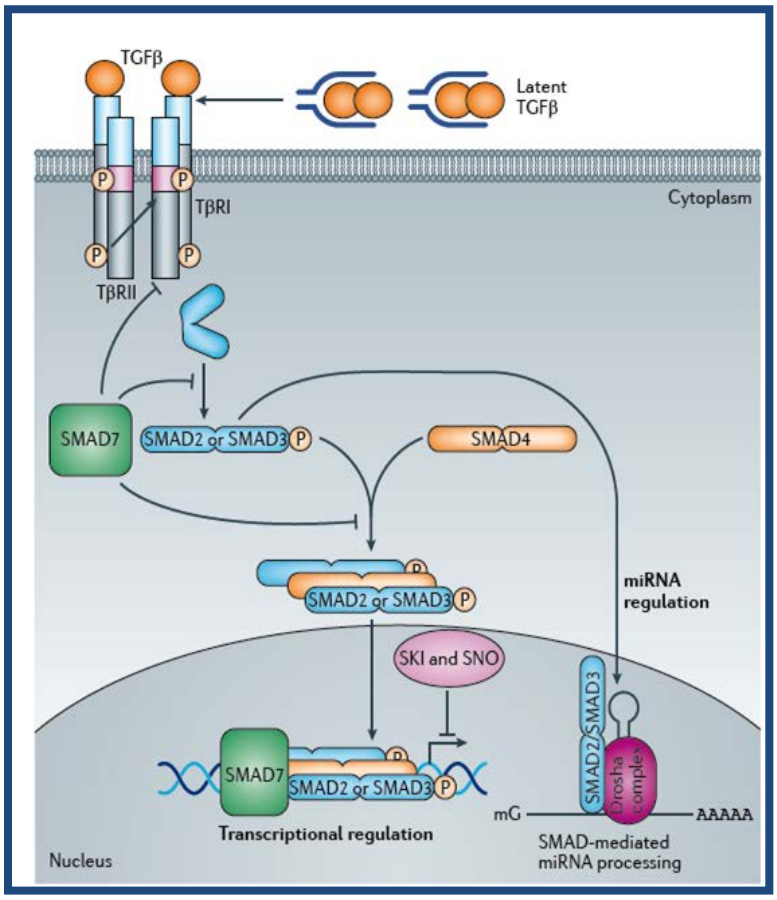

Figure 1.5
The TGFβ Signaling Pathway
TGFβ is synthesized as a large latent TGFβ complex consisting of dimeric TGFβ associated with its latency-associated peptide (LAP) and a latent TGFβ-binding protein (LTBP). TGFβ1 binds as a homodimer to the TGFβ type II receptor, which phosphorylates the type I receptor. The type I receptor is then able to phosphoylate receptor associated Smads2 and 3. Smad2/3 binds Smad4, which translocates as a complex into the nucleus, and bind to Smad binding elements (SBE) activating TGFβ target genes. Smad7 acts as a negative regulator of TGFβ pathway. *Reprinted with permission from Macmillan publishers Ltd: Nat.Rev.Drug Discov [10] © 2012.*

Transforming growth factorβ (TGFβ) is a secreted regulatory cytokine with pleiotropic effects on primary cellular functions such as proliferation, differentiation, migration and apoptosis. It is also known to play a critical role on other complex biological processes like development, fibrosis, wound healing, carcinogenesis and immune function [91-94]. There are 3 homologous TGFβ isoforms in mammals, TGFβ1, TGFβ2, and TGFβ3. They share a common receptor complex but they are expressed at different levels in different tissues [95]. TGFβ has a molecular size of 25 kDa and functions as a homodimer [96]. Among the three known isoforms of TGFβ, TGFβ1 is predominantly expressed in the immune system. TGFβ1 has been well established to be context-dependent; it can either stimulate or inhibit cell proliferation depending on the exact

concentration at which it is present and the target cell. While it is a growth inhibitor for epithelial, endothelial and hematopoietic cells it acts as a proliferator of mesenchymal cells and fibroblasts [92;94].

The active form of TGFβ can function in either a cell-surface bound form or soluble form. Activated TGFβ binds to its receptors, TGFβRI also known as ALK5-Activin-receptor like kinase5 and TGFβRII. The eight vertebrate smads are grouped into 3 categories: five receptor-associated Smads (R-Smad1, 2, 3, 4, 5 and 8), one common Smad-4 and two inhibitory Smads (I-Smad6 and 7). This is the canonical pathway.

The function of TGFβ is regulated at two levels: its level of expression and its activation state. It is secreted as a latent precursor form called Large Latent Complex (LLC) where the TGFβ propeptide complexes with Latent TGFβ binding protein (LTBP1,3 and 4) and its Latency Associated Peptide (LAP) and is sequestered in the extracellular matrix (ECM) [97-99]. This is then activated by the matrix proteases and transglutaminases in a 2-step process, first the release of LLC from the ECM followed by proteolytic cleavage of LAP to release active TGFβ, which is then available for interaction with the TGFβ receptor [100]. A number of additional mechanisms can activate the latent protein such as αvβ6 integrin, MMP9, thrombospondin-1 (TSP-1) expression as well as reactive oxygen species [101-104].

The TGFβ receptor consists of two transmembrane receptors, type I (also known as TβRI or ALK5) and type II (also known as TβRII) both of which possess serine/threonine kinase activity. There are also co-receptors called type III receptors whose main function is to regulate the accessibility of TGFβ family members to the receptors I and II. The TGFβ signaling pathway is a linear pathway [7] (Figure 1.7) wherein TGFβ1 binds to the type II receptor which then brings the type I receptor closer forming a heterotetrameric complex and trans-activates it by

phosphorylation [105;106]. This in turn, activates the serine-threonine kinase activity of TGFβR1 [106] and phosphorylates the C-terminal of receptor associated Smads, Smad2 and Smad3. Smad2 and Smad3 act as mediators of signaling downstream of TGFβ and activin signaling, while Smad1, Smad5 and Smad8 act as mediators of Bone morphogenetic protein (BMP) signaling. Once phosphorylated, these Smads form heterooligomeric complexes with Smad4, which then translocate to the nucleus and bind to the Smad binding elements (SBEs) along with other transcriptional cofactors to modulate the transcription of TGFβ target genes [107]. This is the canonical signaling pathway for TGFβ. But TGFβ can also activate mitogen-activated protein (MAP) kinase signaling molecules, including JNK, p38, ERK and PI3K/AKT. [108]. Unlike the Smad pathway, these alternative pathways can be activated by TGFβ in a cell type dependent manner. These pathways can also influence tumorigenesis independently of the Smad pathway.

There are a number of mechanisms to negatively modulate the TGFβ signaling pathway both extracellularly and intracellularly. Cell surface receptors such as betaglycan and endoglin modulate the accessibility of the TGFβ1 to the receptors [109;110]. Nuclear accumulation and transcriptional activity of the different Smads can also be modulated by the extent of phosphorylation of the linker region of Smads by multiple different kinases of the ERK, MAPK and cyclin-dependent kinase families [92]. In addition, phosphatases such as PPM1A modulating the levels of phosphorylated Smad2 and Smad3, inhibitory Smad6, 7 and ubiquitin ligases such as Smad ubiquitin regulatory factor1 (SMURF1), SMURF2 and HECT are also important in the regulation of the TGFβ signaling [111-116].

1.4 TGFβ1 pathway in Inflammation

TGFβ1 plays a key role in the immune system with its function as a potent immunoregulatory cytokine [117;118]. TGFβ is produced by every leukocyte lineage including lymphocytes, macrophages and dendritic cells. TGFβ1 is the predominant isoform of the TGFβ family expressed by the cells of the immune system [117]. In addition, it is an important cytokine involved in the cross-talk between keratincoytes, fibroblasts and the immune system in both steady state and inflammatory conditions [118]. In addition to its role as a chemotactic cytokine, it can also control the activation and survival of immune cells. However, the exact role of TGFβ is context-dependent, based on the the specific cell type or its differentiation state and/or the presence of other cytokines and costimulatory molecules [117]..

Monocytes, macrophages and langerhans cells can all secrete TGFβ and there are numerous reports, which show that TGFβ1 is required for the development and activation of monocytes into macrophages and langerhans cells. TGFβ is a known chemokine for recruiting monocytes from the blood into tissue [118]. The exact response of TGFβ depends on the tissue localization and the differentiation state of the monocytes. Resting monocytes express high levels of TGFβ type I and II receptors whereas the receptor levels decline as cells mature and become activated by agents such as LPS or IFNγ [119]. In general, TGFβ1 helps in the recruitment of monocytes to the site of inflammatory insult by multiple mechanisms but once they become differentiated, TGFβ acts to inhibit their response by inhibiting their antigen presenting function [120;121]. Described below is a brief introduction to the different cutaneous DC subsets before discussing the role of TGFβ signaling in the regulation of these different subsets.

1.4.1 The different skin-resident Dendritic Cell (DC) subsets

Dendritic cells form the primary component of the innate immune response that responds to an inflammatory insult. They are the first cells to encounter a foreign or non-self antigen and due to their constitutively high expression of MHC class II and costimulatory molecules they act as a bridge between the innate and the adaptive immune system potently activating an antigen-specific lymphocyte response [122]. They capture the foreign antigen, process it and migrate to the tissue draining lymph node where they efficiently present to naïve T lymphocytes to activate both $CD4^+$ and $CD8^+$ cells. Following this, depending on the local cytokine profile, either a T_H1 Response (IL-12 and TNFα) or T_H2 response (IL-4, IL-6) is elicited [123;124]. IFNγ is the characteristic cytokine of the T_H1 reponse.

Langerhans Cells

Langerhans cells (LCs) form an interdigitating network of professional antigen presenting cells specifically in the epidermis, which play an important role in maintaining peripheral tolerance and responding to foreign inflammatory stimuli. They act as phagocytic epidermal sentinels and constantly sample the skin milieu for altered self as well as foreign antigen. LCs represent the only $MHCII^+$ antigen-presenting cells in the epidermis and are the first described cutaneous DC subset. LCs are distinguished from DCs in other tissues by the presence of langerin (or CD207), a C-type lectin receptor associated with cytoplasmic organelles called Birbeck granules. It is a type II transmembrane Ca^{2+} dependent receptor, shared also by a small subset of dermal DCs-$CD207^+$ dermal DCs and a subset of DCs in the lungs [125]. TGFβ1 is important for the induction of Langerin expression and LC development [126].

LCs are derived from colony-stimulating factor-1 (CSF-1) dependent bone marrow precursors which migrate to the dermis right before birth [9;127;128]. In the first postnatal week, they

undergo a burst of proliferation and differentiate and populate the entire epidermis to constitute a dense network. In adults under steady state conditions, LCs assume a low level of proliferation to replace the LCs that exit the epidermis under homeostatic migration. Thus, LC numbers are maintained from locally proliferating precursors in steady state [129;130].

During inflammation, keratinocytes secrete proinflammatory cytokines such as IL-1β and TNFα and the LCs respond to these cytokines and become activated and acquire migratory capability. LCs then downregulate E-cadherin, allowing them to dissociate from neighbouring keratinocytes and upregulate α6-integrin to enable passage through the basal membrane to gain access to the afferent lymphatics in the dermis. LCs then enter the draining lymphatics finally reaching the paracortex region of the SDLNs in a CCR7 dependent manner where they efficiently present antigens to antigen-specific naïve T cells [131;132].

Dermal Dendritic cell subsets

LCs were long considered to be the prototypic DCs involved in skin immune responses. However, a number of DC subsets in the dermis are increasingly being characterized, which can perform both redundant and nonredundant functions with LCs. Since LCs and $CD207^+$ dermal DCs (dDCs) were both positive for CD207, until recently, $CD207^+$ dDCs were mistaken for migrating LCs in the dermis. A number of genetic ablation models have been used to selectively deplete LCs to characterize the dermal DC subsets and ascertain their specific contribution to immune surveillance and skin inflammatory function.

LangDTR-EGFP transgenic mice form one such model where *in vivo* diphtheria toxin (DT) treatment gets rid of both LCs and $CD207^+$ DCs. After DT treatment, LCs and $CD207^+$ dDCs repopulate the skin with different kinetics: the $CD207^+$ dDCs reappear in the dermis as early as 3-4 days after DT administration and 50% of the $CD207^+$ dDC compartment is reconstituted in

the dermis by 7 days. In contrast, LCs remain undetectable for up to 20 days and 50 % of the LCs are replaced slowly only by 3 weeks. The radioresistant property of LCs has also been exploited to study skin DCs where mice are lethally irradiated and reconstituted with donor bone marrow (BM) resulting in LCs of host origin and other DC subsets of donor origin.

Together these transgenic selective DC ablation models, bone marrow chimera models and the distinct repopulation kinetics of LCs and dermal DCs have demonstrated that the fraction of $CD207^+$ DCs in the dermis are not transmigrating LCs but represent a distinct population of dermal resident DCs. On further characterization, two markers have been identified- Epithelial Cell Adhesion molecule (EpCAM) and the integrin CD103, which are differentially expressed between the LCs and the $CD207^+$ dermal DCs.

The dermal DCs form a more heterogeneous population and are composed of two major subsets: $CD207^+$ and $CD207^-$ cells. Dermal DCs (dDCs) form networks in the form of dermal cords before they emigrate to the LNs. They are replaced every 10-15 days and mobilize quickly upon stimulation by foreign antigen and dDCs that have emigrated from the skin can be detected in the draining LNs 24-48 hours post stimulation. The migration of $Langerin^+$ dermal DCs is also CCR7 dependent [133]. $CD207^+$ dermal DCs are specialized in the cross-presentation of cell-associated self and viral antigens similar to LN resident $CD8\alpha^+$ DC [134;135]. In the lung, $CD207^+$ dermal DCs are required for the clearance of influenza virus infection [136].

Both LCs and dermal DCs can migrate to the SDLNs under steady state and in response to an inflammatory stimulus. After migration to the LNs, skin-derived DCs increase their MHCII expression.

The figure 1.8 shows the different DC subsets of the skin in steady state. The table below lists the composition of the different DC subsets in the dermis under steady state conditions where the heterogeneous Langerin$^-$ forms the majority subset:

Table 1.2 **The DC composition of steady state dermis (adapted from [9;12])**

DC subset	Steady State percentages in the Dermis
Langerin$^-$	80
Langerin$^+$CD103$^+$	2.5
Langerin$^+$CD103$^-$	2.5
Migratory Langerhans cells	6.5

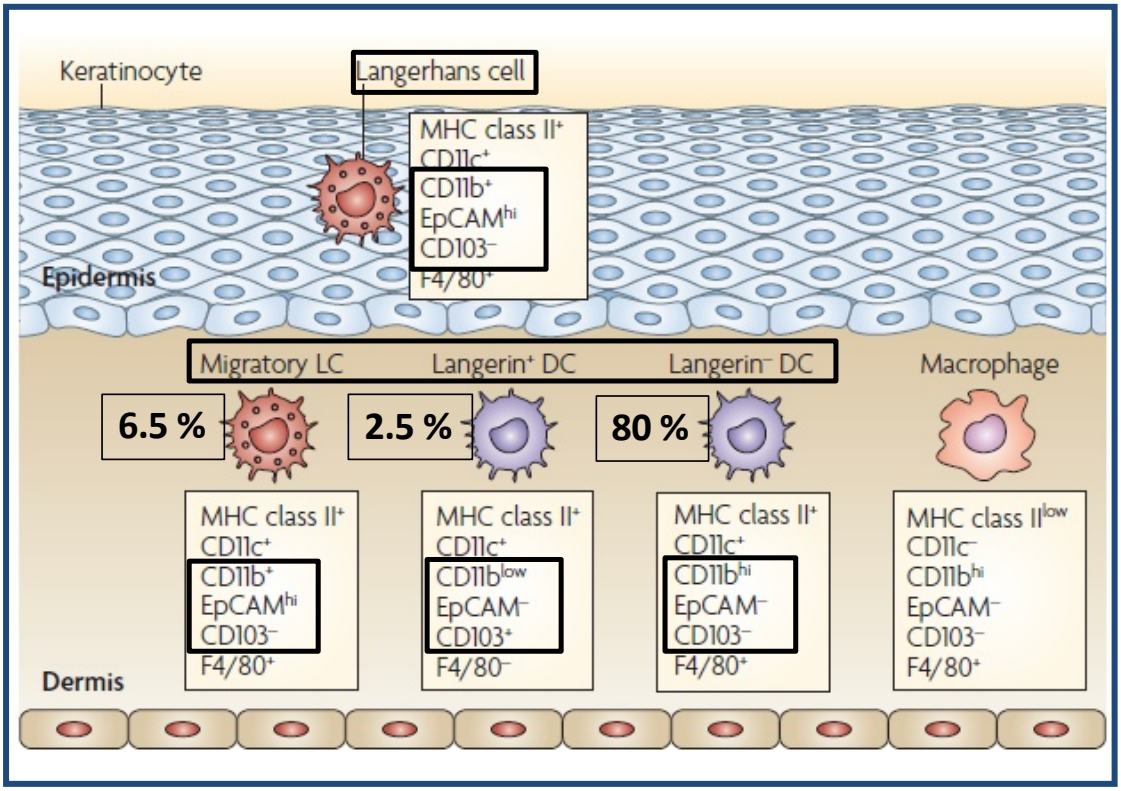

Figure 1.6 **The DC composition of steady state skin:** The skin contains two main populations of CD207$^+$ dendritic cells (DCs): Langerhans cells (LCs) in the epidermis and CD207$^+$ DCs in the dermis. Migrating LCs can also be present in the dermis on their way to the lymph nodes. LCs and dermal langerin$^+$ DCs can be distinguished based on their expression of CD11b, epithelial-cell adhesion molecule (EpCAM) and CD103. In addition to these, there are two other antigen presenting cells in the dermis: dermal CD207$^-$ DCs and dermal macrophages. *Reprinted with permission from Macmillan publishers Ltd: Nat.Rev.Immunol., [9] © 2008.*

A number of features distinguish dermal DC subsets from the LCs. The following table lists the differences between LCs and DCs of dermal origin.

Table 1.2 **The Epidermal Langerhans cells and the dermal DC subsets have distinct phenotypic markers and function: Adapted from [2]**

LCs-EPIDERMAL DCs	DERMAL DCs
Radioresistant	**Radiosensitive**
They express epithelial cell adhesion molecule (EpCam)	**$CD207^+$ dermal DCs do not express EpCam**
They require TGFβ signaling for development	**Their homeostasis is TGFβ independent**
LCs are long-lived	**Dermal DCs have higher turnover rate than LCs**
LCs are renewed from locally proliferating precursor cells in steady state.	**Continuous renewal of dermal DCs comes from bone marrow precursors**
Host origin in bone marrow (BM) chimeras	**Donor origin in BM chimeras**
Birbeck granules present	**Birbeck granules absent**

TGFβ1 has distinct roles in **1) DC development 2) DC homeostasis and 3) DC response to immunostimulation**

1.4.2 TGFβ and DC development:

LCs formed the first hematopoietic cell type whose development was shown to be absolutely dependent upon TGFβ. This discovery was initially made in TGFβ1-/- mice where LCs were completely absent. Similar results were obtained in mice deficient in two other components of the TGFβ signaling pathway Id2 and Runx3 [137]. Additionally, Id2-/- mice had reduced CD8α DCs. Not surprisingly, Id2 was upregulated during DC differentiation both *in vitro* and *in vivo* [138]. Further, there was a 50% reduction in the number of LCs in heterozygous TGFβR1-deficient mice suggesting that LC development is dose dependent on the availability of TGFβ [139]. When TGFβ1+/- SCID BM cells were injected into irradiated TGFβ1-/- SCID mice, the

recipient mice were able to produce LCs suggesting that autocrine TGFβ1 produced by LC precursors is sufficient for LC development [140]. Another study also reported that autocrine production of TGFβ1 is responsible for LC development in the epidermis [129]. However, BM cells from TGFβ1-/- mice were also able to generate donor derived LCs when injected into irradiated WT recipients, suggesting that a paracrine source of TGFβ1 is sufficient to support LC development [128;129]. Nevertheless TGFβ is clearly required for the development of LCs irrespective of the source.

In contrast, the development of dermal DC subsets does not require TGFβ. Dermal DC frequencies and maturation represented by the surface expression of MHCII and CD86 and steady state migration were not affected with CD11c-specific deletion of TGFβ type I receptor [141]. Similarly, Langerin^{+} dDC development is unaffected in TGFβ1 deficient mice and is therefore TGFβ independent [142].

1.4.3 TGFβ and DC homeostatic function: TGFβ mediates the maintenance of peripheral tolerance facilitated by DCs

Steady state immunotolerance is achieved by a continuous flow of immature DCs from the skin to the draining lymph nodes. Non-activated or immature DCs can still present antigen in the absence of sufficient co-stimulation resulting in T cell tolerance [143]. The immunostimulatory role of TGFβ in DC function is context specific and the mechanism is largely unclear. In contrast, the role of TGFβ in maintaining tissue homeostasis and self-tolerance is well-demonstrated at the level of T cells by maintaining T cell anergy [144] and also at the level of dendritic cells by inducing tolerogenic DCs [129] in the periphery. Tolerogenic DCs refer to the lineage of DCs, which can induce antigen-specific unresponsiveness or tolerance in central lymphoid organs and in the periphery [145]. Tolerogenic DCs can either be naturally occurring

DCs or induced DCs. It has been reported that addition of TGFβ *in vitro* to monocyte-derived DCs led to the differentiation of tolerogenic DCs and favored T cell anergy and differentiation of regulatory T cells [144]. TGFβ secreted by splenic stromal cells are also responsible for the differentiation of regulatory DCs [146] and TGFβ secreted by gut epithelial cells favors differentiation of regulatory T cells by interstitial DCs [147]. Thus, TGFβ from paracrine cell types in the DC microenvironment can also modulate the DC differentiation state.

The role of TGFβ signaling in DCs for maintenance of tissue homeostasis: Knowledge from mouse models

In mice with a deletion of the TGFβ receptor 1 (TβR1) in dendritic cells ($CD11c^+$), LCs initially seeded the epidermis but disappeared 1 week after birth [139]. TβR1-deficient LCs also demonstrated spontaneous maturation leading to increased migratory potential based on increased expression of MHC class II, CCR7, CD40, CD86 and downregulation of E-cadherin. LCs in TGFβ and TGFβRII deficient mice in the epidermis prematurely adopt the phenotype of homeostatically migrating LCs while still in the epidermis [148]. Therefore, migrating LCs were reduced in the dermis and SDLNs of adult CD11c-TβR1 mice whereas the number of $Langerin^+$ dermal DCs was similar to the WT mice. This shows that TGFβ signaling is largely dispensable for $Langerin^+$ dermal DCs under steady state conditions.

In CD11c-dnTGFβRII transgenic mice expressing a dominant negative human transforming growth factor beta receptor II gene under the control of a CD11c promoter, specific for DCs, TGFβ signaling was blocked in DCs and NK cells. In response to Leishmania infection, the blockade of TGFβ signaling in NK cells led to uncontrolled secretion of IFNγ, promoting T_H1 differentiation. However, DC homeostasis or IL-12 secretion from DCs remained largely unaffected. In general, TGFβ acts as an important immunomodulator for maintaining tissue

homeostasis with DCs acting as the mediators and its absence polarizes DCs to a more inflammatory phenotype leading to uncontrolled downstream activation of T cells creating a proinflammatory pathological state.

1.4.4 TGFβ signaling in immunostimulatory DC function

TGFβ1 is a critical immune regulator highly expressed by LCs. In addition, the homeostatic migration of LCs has been largely linked to the differentiation of regulatory T cells. TGFβ treated monocyte derived DCs are resistant to maturation in response to treatment with hapten or UV leading to decreased T cell stimulatory function [149]. In addition, TGFβ can immobilize DCs interfering with the migration and transport of antigen to the draining lymph nodes for generating an adaptive immune response. TGFβ can also directly induce DC apoptosis in tumors [129]. CCR7 gene activity in DCs can also be inhibited by TGFβ signaling *in vitro* [150]. The inhibitory effect of TGFβ on DC-mediated lymphocyte function is well-known [151;152]. However, TGFβ regulation of DCs, especially in immunostimulatory conditions is largely unclear and preliminary reports suggest that different cutaneous DC subsets may perform distinct functions.

Contact Hypersensitivity (CHS) Model for studying immunostimulatory function of cutaneous DCs

CHS is a relevant mouse model for allergic contact dermatitis where painting of a contact sensitizer onto the skin leads to mobilization of skin DC and priming of hapten-specific T cells in SDLNs during the sensitization phase. Re-exposure to the same hapten after a time-period of 5-7 days on a distant location such as the ear induces a transient ear swelling reaction mediated by IFNγ^+ CD8 T cells and regulated by IL-10$^+$ CD4 T cells. For a long time, LCs were considered to be the primary mediators of initiation of CHS. But recent studies showing that the ear swelling

was similar or reduced but not abolished upon inducible ablation of LCs suggest a redundant role for LCs with dermal DCs in CHS response. In an inflammatory setting induced by DNFB or *C. albicans* infection, p-Smad2/3 expression characteristic of TGFβ pathway activation was limited to LCs that were not activated with phenotype of low CD86/MHCII [148]. There are also reports that suggest that epidermal LCs are responsible for UVR induced immunosuppression and are negative regulators of the anti-Leishmania reponse. However, there is another study which claims LCs are not required for UVR-induced suppression of contact hypersensitivity [153;154] CHS response is under intense investigation and there are many contradictory reports to the specific roles of LCs vs dermal DCs. Thus, the role of different DC subsets and their dependence on TGFβ signaling, in response to an inflammatory stimulus such as UVB irradiation or in an inflammatory setting such as CHS [155] remains controversial and deems extensive investigation.

1.4.5 TGFβ signaling and T cells

TGFβ plays a critical role in determining the response of T lymphocytes by acting 1) directly on them and also by 2) modulating the function of antigen presenting cells within the skin. TGFβ1 is also a well-established modulator of T cell development and effector function. TGFβ was first observed to inhibit the proliferation of human B and T cells [151]. TGFβ also potently suppressed T cell proliferation *in vitro* [152].The data from TGFβ1 deficient mice supported this result. However, there have been conflicting reports on the exact role of TGFβ1; while some studies highlight its ability to inhibit IL-2 dependent T cell proliferation, others show that TGFβ1 enhances T cell expression of specific cytokines and their capacity to respond to stimulation. TGFβ1 has also been shown to modulate the survival of T lymphocytes [3;117]. With respect to differentiation and lineage specification, there is again conflicting reports of whether TGFβ1

supports the development of T_H1 or T_H2 response. While presence of TGFβ1 during the priming of naïve T cells drives them toward a T_H1 phenotype, [156] exogenous addition of TGFβ inhibited the T_H1 phenotype [157;158].

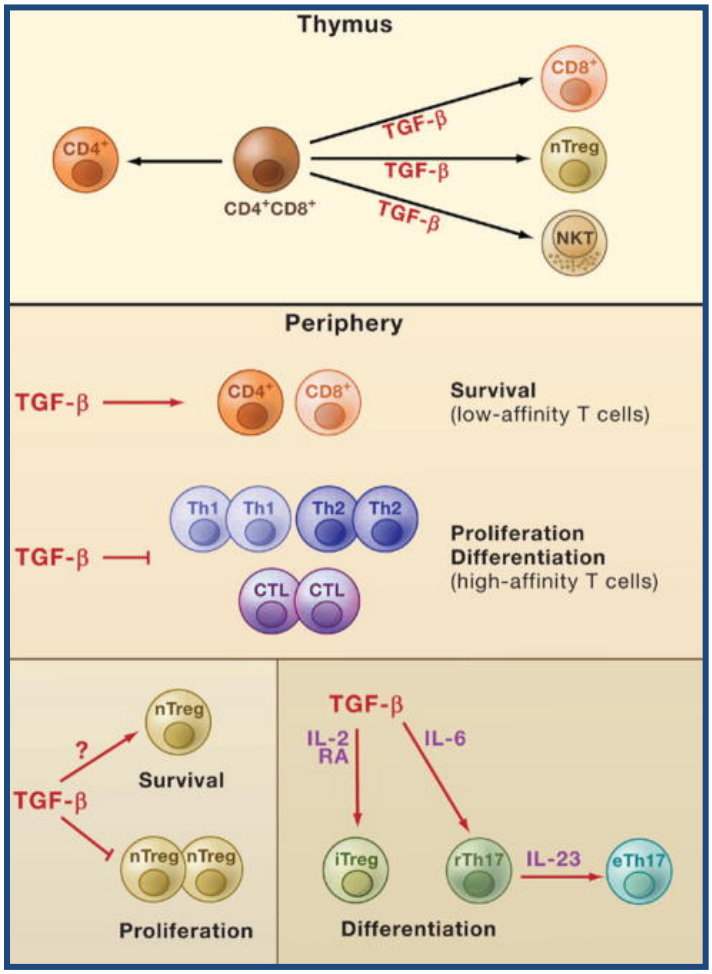

Figure 1.7 **TGFβ is required for the development, survival, homeostasis, tolerance, and immunity.** TGFβ regulates T cell development, homeostasis, tolerance, and immunity. TGFβ signaling in T cells promotes development of natural killer T (NKT) cells, natural regulatory T (nTreg) cells, and CD8+ T cells in the thymus. TGF-β signaling in T cells in the periphery is essential for the survival of low-affinity $CD4^+$ and $CD8^+$ T cells. It also modulates immune tolerance by inhibiting high-affinity $CD4^+$ and $CD8^+$ T cell proliferation. It is important in the differentiation of T helper 1 (T_H1), T_H2, and cytotoxic T lymphocytes (CTL). In addition, TGFβ signaling in nTreg cells inhibits their proliferation while supporting their maintenance in peripheral lymphoid organs. TGF-β also positively regulates differentiation of induced Treg (iTreg) cells and Th17 cells with help from other factors. *Reprinted from [3] © 2008 with permission from Elsevier.*

1.4.6 The role of TGFβ signaling in T cells for maintenance of tissue homeostasis: Knowledge from various mouse models

TGFβ signaling supports the survival of naïve CD4 and CD8 T cells that interact with low affinity to self-antigens to maintain a diverse repertoire of naïve T cells (Figure 1.9). In TGFβ deficient mice, there was a hyperactivation of T cells and progressive infiltration of leukocytes into multiple organs leading to an inflammatory disorder developing into a rapid wasting

syndrome reducing the animal lifespan to 3-4 weeks after birth resembling an autoimmune disorder [159-161]. In these mice, there was enhanced expression of MHC class I and class II molecules and was associated with enhanced infiltration of macrophages and CD8 T cells [162]. $CD4^+$ or $CD8^+$ depletion rescued the inflammatory phenotypes suggesting that T cells are the main mediators of the inflammatory disorder [163]. In addition, bone marrow chimera recipients with deletion of TGFβRII in the bone marrow cells mimicked the lethal immune pathology of TGFβ-/- mice [164].

A transgenic mouse model that specifically overexpresses dominant negative TGFβ II receptor (DNRII) on T cells highlights the importance of TGFβ signaling in maintaining CD8 homeostasis in the periphery. CD2-DNR mice exhibited deregulated growth of $CD8^+$ memory T cell population resulting in a hyperproliferative disorder which acts as a tumor-initiating event with associated chromosomal aberrations that eventually transforms into lymphoma [165;166].
A transgenic mouse model that overexpresses a dominant negative TGFβ II receptor (DNRII) specifically in CD4 T cells highlights the importance of TGFβ signaling in maintaining CD4 T cells in an immature state. CD4-dnTβRII mice displayed less severe autoimmune pathology when compared to TGFβ-/- mice. In the absence of TGFβ mediated T cell regulation, T cells undergo spontaneous activation resulting in increased, T_H1, T_H2 differentiation ultimately leading to autoimmune inflammatory bowel disease and mice lethality within 6-8 months. [167]

T-cell specific TGFβRII deletion similar to $TGF\beta^{-/-}$ mice leads to a more aggressive disease where there is a generalized infiltration of activated proinflammatory T_H1 T lymphocytes that results in a lethal autoimmune disease [168]. Thus, TGFβ inhibits immune function by two distinct mechanisms acting through T cells, by 1) directly acting on the lymphocytes [151] and 2) indirectly by promoting the function of T_{reg} cells [168].

1.4.7 TGFβ and $T_{regulatory}$ cells

There are two distinct subsets-nT_{regs} and iT_{regs}. While nT_{regs} develop in the thymus, express IL-2 receptor α chain (CD25) and maintain self-tolerance in an antigen-independent manner, iT_{reg} cells develop in the periphery in response to self or tumor antigens and express variable levels of CD25. The absence of TGFβ-dependent regulation of Tregulatory cells has been implicated in the early fatal mutifocal inflammatory disorder in TGFβ-/- mice and the TGFβ-/- T_{reg} cells also proliferate at a higher rate than the WT T_{regs} [168]. However, bone marrow chimera experiments with repletion of WT T_{regs} to neonatal TGFβRII deficient mice did not rescue the phenotype of autoimmune disease entirely, suggesting that T cell independent factors such as antigen presenting cells might also play a role in the development of the inflammatory condition [168]. In addition, in a bone marrow transfer model of WT T_{regs} into a model with T cell-specific deletion of TGFβ-RII, the spontaneously activated T cells were resistant to suppression by WT T_{regs} [168-171].

Active immune suppression by TGFβ1 and $Foxp3^+$ T_{regs} is an important mechanism for maintaining peripheral T cell tolerance [117]. In the presence of anti-inflammatory signals such as TGFβ, IL-10 and corticosteroids, DCs can induce tolerance by T cell deletion or the induction of T_{reg} cells [172]. In the absence of IL-2, TGFβ enhances the conversion of naïve $CD4^+$ T cells to $Foxp3^+$ T_{reg} cells by targeting peptide-agonist ligands to DCs [173;174] [145]. TGFβ is also expressed at high levels in T_{regs} as cell-surface bound form [175;176]. The tissue maintenance of T_{regs} is dependent on TGFβ signaling [168]. In addition, naïve T cells in the presence of excess of TGFβ can differentiate into induced T_{regs} (iT_{regs}) in peripheral tissues, which is associated with Smad3 driven Foxp3 expression [177-180]. A role for TGFβ signaling has also been established in the development of natural regulatory T cells (nT_{reg}). However, T cell responsiveness to TGFβ

is not required for the development or peripheral function of thymic-derived T_{reg} cells. Further, the function of TGFβ1-/- T_{reg} cells was abrogated by anti-TGFβ monoclonal antibody, indicating that functional TGFβ can be provided by a non-T_{reg} paracrine cell source implicating the importance of antigen-presenting cells[171]. Indeed, the induction of iT_{regs} in the gut is facilitated by $CD103^{+}$ dendritic cells in a TGFβ and retinoic acid (RA) dependent manner [181-183]. Dendritic cells in the gut associated lymphoid tissue (GALT) play a major role in inducing iT_{regs} to avoid unwanted activation of the immune system in response to frequent DC sampling of of innocuous gut microflora.

1.4.8 The inhibitory properties of TGFβ on T cell subsets

Naïve CD4 T cells differentiate into three major types of effector T cells upon activation by antigen-presenting cells [123]. Based on the cytokine profile, CD4 effector T cells-T helper cells can be categorized as T_H1-IFNγ, T_H2-(IL-4, IL-13 and IL-5), and T_H17-(IL-17 and IL-22) cells. There is considerable information available on T cell-specific target genes of TGFβ. TGFβ regulates the effector function of T cells at multiple levels. TGFβ can directly inhibit expression of T-bet and Gata-3, the transcription factors involved in T_H1 and T_H2 polarization respectively [184-188]. Although TGFβ can directly inhibit T-bet expression *in vitro* [185], a cross of mice with TGFβRII deletion with T-bet encoding *Tbx21* gene deleted mice, did not rescue the inflammatory condition suggesting that TGFβ regulation of T cell activation can be T-bet independent [189]. The expression of genes important for T cell differentiation and function such as STAT4, IFNγ, granzyme-B in addition to GATA3, T-bet, are suppressed by TGFβ. TGFβ also functions by suppressing T cell proliferation by inhibiting the secretion of IL-2 through direct binding of Smad3 to the IL-2 promoter [152;190;191]. TGFβ also promotes the synthesis of anti-inflammatory IL-10 in a Smad dependent manner [192].

TGFβ mediated inhibition of CTL function during antitumor immunity can occur through several mechanisms: by direct suppression of gene expression of cytolytic genes such as granzyme-A, granzyme-B and FAS ligand [193]. TGFβ can also inhibit CD8 T cells by suppressing the expression of effector molecules like IFNγ and perforin [184]. $CD4^+$ T cells from CD4-DNRII TGFβ transgenic mice spontaneously differentiate into T_H1 and T_H2 cells [167] suggesting that TGFβ suppresses the activation of naïve $CD4^+$ T cells in steady state conditions.

1.4.9 The immune-promoting properties of TGFβ

TGFβ signaling in T cells has been implicated in the thymic development of T cells from $CD4^+CD8^+$ to $CD8^+$ cell stage. TGFβ signaling is also necessary for NKT cell development as their numbers in the thymus and the periphery are greatly reduced in T cell-specific TGFβRII-deficient mice [168;194]. The reduced numbers of NKT cells and $CD8^+$ T cells in these mice suggests that TGFβ signaling is required for the development, maintenance and survival of specific T cell subsets [168].

Under stimulation with anti-CD3 in the presence of TGFβ, $CD8^+$ T cells displayed enhanced proliferative capabilities while retaining their cytotoxic function [195]. TGFβ also enhanced the expression of CD154 (CD40L), TNFR2 and the production of TNFalpha on human T lymphocytes [196]. TGFβ1 can enhance the survival of T cells in the periphery by inhibiting apoptotic machinery [197] and also support the clonal expansion of activated T cells and generation of memory T cell response [198] (Figure 1.10). Whereas TGFβ1 can also cause activation-induced cell death in T cell populations [199;200]. In addition to T_{reg} lineage differentiation, TGFβ is critical for the T_H17 cell lineage differentiation under inflammatory conditions, [201-203] which has been implicated in autoimmune pathologies [204-206].

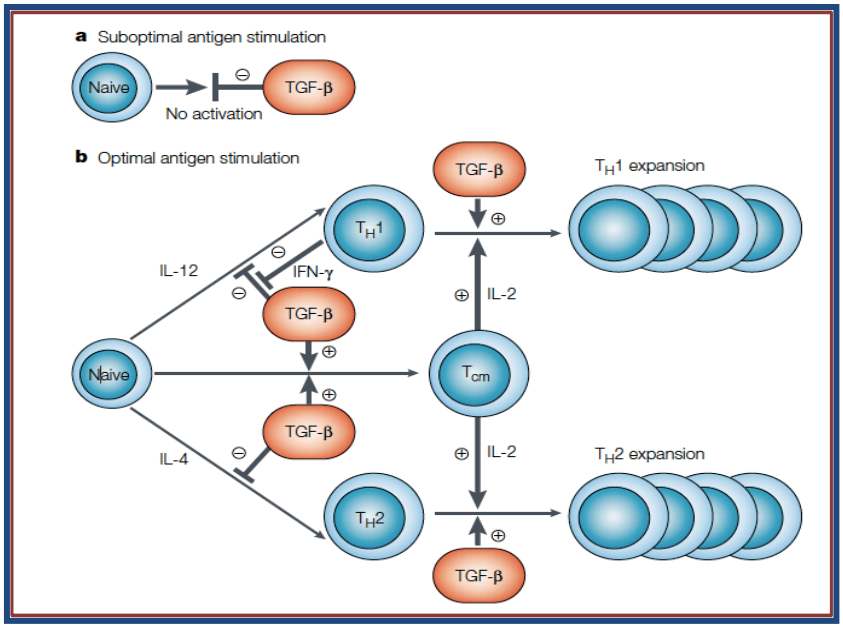

Figure 1.8 **The context dictates whether TGFβ inhibits or promotes T cell proliferation** Suboptimal stimulation of a naive T cell in the presence of TGFβ results in inhibition of IL-2 production and subsequent inhibition of T-cell proliferation and activation. Whereas optimal costimulation or IL-2 presence results in T-cell activation and proliferation from naïve T cells. TGFβ can inhibit this process, however, IFNγ secretion by previously differentiated T_H1 cells can block the inhibitory effect of TGFβ, thereby permitting the generation of T_H1 cells. Secondary signals such as presence of IL-2 along withTGFβ can support the enhanced clonal expansion of these cells. *Reprinted with permission from Macmillan publishers Ltd: Nat.Rev.Immunol. [5] © 2002.*

1.5 Ultraviolet Irradiation of the skin

Sunlight is composed of a continuous spectrum of electromagnetic radiation that is divided into three main bands of wavelength: Ultraviolet (UV), visible and infrared. Ultraviolet radiation (UV) present in sunlight is a key environmental carcinogen. UV light can be further subdivided into three major sections: UVA (320-400 nm), UVB (280-320 nm) and UVC (200-280 nm). While UVC is effectively blocked from reaching the earth's surface by the stratosphere, UVA and UVB reach the earth's surface in quantities sufficient to have biological impact on human skin. Of these two, UVB is widely accepted to be the major contributor of UV induced erythema,

sunburns eventually leading to skin tumors. Melanoma and non-melanoma skin cancer (NMSC) are the most common types of cancer. Nonmelanoma skin cancer encompassing basal cell carcinoma and squamous cell carcinoma is the most frequent cancer among Caucasian populations [207]. There were over 1,000,000 new cases of Squamous cell carcinoma (SCC) reported in the US in 2010. It is a malignant tumor of the squamous cells, which form the top layers of the epidermis and it frequently results due to chronic sun-exposure. The tumors develop most frequently on the sun-exposed face, neck, bald scalp, hands, shoulders, arms and back. SCCs can be classified into two major types depending on the specific stage, actinic keratosis, the earliest form of SCC and keratoacanthoma, a rapidly growing form of SCC. Exposure to UV in conjunction with an inability to tan is the most important risk factor to NMSC and the frequency of NMSC is highest in individuals who tan poorly and sunburn easily [208].

Acute UV irradiation usually refers to a single exposure of UV. DNA has been identified to be the major chromophore for UV irradiation [8]. UV irradiation induces lesions in the DNA among adjacent pyrimidines in the form of two types of dimers: 1) cyclobutane pyrimidine dimers (CPDs) between adjacent thymine (T) or cytosine (C) residues and 2) pyrimidine (6-4) photoproducts between adjacent pyrimidine residues [8]. Both lesions occur most frequently in foci of tandem pyrimidine residues and are recognized as ‘hot-spots’ of UV-induced mutations [8;209]. The action spectrum for CPD formation is very similar to that for erythema with a maximum between 290-320 nm [210]. Moreover, 6-4 photoproducts are repaired much more rapidly than CPDs in mammalian cells [211] and so CPDs are believed to be the major contributor of mutations in mammals. These UV-induced DNA lesions lead to mutations in the DNA if not repaired. The mutations in the form of C to T and CC to TT transitions are “signature” UV mutations [212].

1.5.1 UV and molecular events:

UV radiation is also known to alter cellular homeostasis via DNA damage, the generation of reactive oxygen species, activation of damage response pathways such as p53 and activation of nuclear transcription factors involved in inflammation, importantly, NFκB.

1.5.2 DNA Repair mechanisms:

All mammalian cells are equipped with different DNA repair systems to protect the genome from any DNA lesions caused by mutagenic agents such as UV. Among the different repair pathways such as direct repair, base excision repair, mismatch repair, double-stranded repair, and nucleotide excision repair (NER), NER is the predominant repair machinery employed by UV irradiated cells to repair CPD and (6-4) photo adducts. NER removes bulky DNA in two distinct subpathways, wherein damages incurred in actively transcribed genes are removed by a quick mechanism called Transcription-coupled repair and the damages sustained in other parts of the genome by a slower process called global genome repair (GPR) [213].

1.5.3 p53-dependent DNA damage response pathway

Despite the inherent capacity of the mammalian cells to repair damaged DNA, some of the UV-induced lesions escape the damage repair machinery. One of the key damage response mechanisms the cells possess to prevent such DNA damage to be sustained in the cells is mediated by p53. These damaged cells rapidly undergo p53 mediated growth arrest [8]. The p53 pathway is activated by both upregulation at the message level and also by phosphorylation at primarily Serine 15 and Serine 20 residues [214]. Upon activation, p53 induces cell-cycle arrest at the G1 phase, which provides the cells with a window wherein the DNA damage could be repaired in a p53-dependent manner before they proceed to the S phase [215]. p21/WAF1/CIP1 is an important mediator of this response as it inactivates the cyclin dependent kinase (CDK)-

cyclin complex by competing for the CDK, pushing the cell into growth arrest [216]. GADD45 is an important molecule involved in DNA damage repair, which is activated by p53 [217].

The cells that are DNA-damaged beyond repair are shunted to an apoptotic pathway controlled by p53. As a transactivator of transcription, p53 upregulates the expression of pro-apoptotic genes such as Bax, Fas/Apo-1 or downreglates the expression of anti-apoptotic genes such as Bcl2 [218;219]. The Fas/Fas-L interactions have been implicated in the formation of sunburn cells with UV irradiation [220].

Growth arrest and DNA damage response are activated sequentially and are both mediated by p53 transactivation to prevent the mutated DNA to be transmitted to daughter cells, which could result in dysregulation of key growth control genes ultimately leading to carcinogenesis.

Apoptosis peaks at 24 hours post UVB following which the dermis and the epidermis undergo a proliferative cycle associated with increased protein, DNA and RNA synthesis and increased mitotic activity between 48 and 72 hours to replace the apoptotic cells and return the skin to homeostasis. This increased proliferation in turn, results in hyperplastic epidermis and dermis [221].

1.5.4 NFκB transcription program

NFκB represents another important transcription program induced by UV, which is key for inflammatory genes, cell proliferation genes and anti-apoptotic genes [222]. NFκB is important for the maturation of dendritic cells [223] and the development of lymphocytes [224-227]. NFκB plays a critical role in immune mediation by modulating the expression of cytokines and adhesion molecules in inflammatory cells [228;229]. TNFα is the canonical target of the NFκB pathway. The lack of IKKβ in keratinocytes enhances TNFα dependent inflammation [230].

UV induced secretion of IL-1, IL-6, TNFα and Vascular endothelial growth factor (VEGF) are key target genes of the UV stimulated NFκB transcription program [224;231]. UVB also induces several NFκB-mediated genes in HeLa cells [232] and HaCaT cells including IL-1, TNFα and MMP-1 [233]. This pathway is also important in UVB induced generation of reactive oxygen species [234;235].

1.5.5 Skin pigmentation

The tanning response following UV irradiation is biphasic-an immediate pigment darkening (IPD) and a delayed formation of new melanin. IPD results from redistribution of melanin already present, which form nuclear caps to protect epidermal basal cell nuclei. Delayed tanning response is associated with an increase in activity as well as number of melanocytes and is associated with an increase in melanocyte tyrosinase activity. As a result, there is an accumulation of melanin granules in the epidermis.

As a result of tanning responses, hyperkeratosis of the stratum corneum and hyperplasia of the skin post UVB, there is usually an increase in the UV dose required to cause visible erythema under chronic UV irradiation. This phenomenon has been termed “photoadaptation”- a diminished future response to equivalent doses of irradiation [236].

Most of the histological changes with acute UV exposure disappear in the absence of further UV, and the skin returns to normal within 1-2 weeks time. Whereas, repeated exposure to UVR leads to the development of photoadaptation and photoprotection [237-239] associated with hyperplastic skin, hyperkeratosis of the stratum corneum, increased melanocyte function and a general disruption of normal skin architecture, which pose key risk factors for tumorigenesis.

1.5.6 The cutaneous immune response to UV exposure:

The physiological changes caused by UV exposure of skin in the 280-320 nm UVB range can be categorized into acute and chronic effects. The acute response is primarily characterized by rapid erythema, edema and tanning whereas the chronic responses encompass photoaging and photocarcinogenesis [240;241].

The pathophysiology of acute erythema can be further divided into three overlapping phases: 1) early vasodilatory phase, 2) the inflammatory phase followed by 3) the late regressive phase. The exact time-course of these events is a function of the UVB dosage regimen. The **early vasodilatory phase** is primarily mediated by prostaglandins and nitric oxide [242-244]. Nitric oxide (NO) is a potent endogenous vasodilator and UVB causes a dose dependent release of NO from keratinocytes, which is associated with increased cGMP [243]. Nitric oxide synthase (NOS) acting along with cyclooxygenase has been shown to contribute to UVB induced visible erythemal response of the skin [244].

The second phase, **the inflammatory phase**, is characterized by an influx of immune cells as a result of increased vasodilation and chemokine secretion. The polymorphonuclear neutrophils are the first immune cells to respond and they start accumulating as early as 3-6 hours post irradiation [245]. This is followed by activation of skin resident dendritic cell populations, the epidermal Langerhans cells and the heterogenous dermal dendritic cell subsets. The DCs, keratinocytes along with lymph-node resident naïve T cells constitute the Skin associated lymphoid tissue (SALT) [246;247]. While UVR has been shown to induce the migration of Langerhans cells to the SDLNs [248-251], there is little information about the effect of UV on dermal dendritic cell subsets as the different dermal DC subsets have been characterized only in the recent years.

The DCs exit the cutaneous compartment with the dermal DCs forming dermal cords before they reach the afferent lymphatics, leading them into the skin draining lymph nodes (SDLNs). The dermal DCs reach the SDLNs 48 hours post UVB while the LCs take 48-72 hours because of the additional time required to penetrate through the basement membrane before they reach the afferent lymphatics (Figure 1.5).

The T cells, mostly $CD4^+$ T cells begin to arrive in the dermis between 2 and 4 days after a single UVB dosage, however the intraepidermal T cell compartment follow delayed kinetics [252]. The T cells are T_H1 polarized, possess activation markers and act as key effectors of UVB induced erythema along with neutrophils [253]. Keratinocytes exposed to UVB are well known to produce various proinflammatory cytokines such as TNFα, IL-8 and IL-6 [254]. IFNγ produced by T_H1 cells synergize with these proinflammatory cytokines resulting in a pronounced UVB-induced edema response [240;253]. TNFα and IL-8 act as key mediators and are associated with the upregulation of adhesion molecules such as ICAM-1 and ELAM-1 on the vascular endothelial cells contributing to the infiltration of immune cells from the vasculature [255].

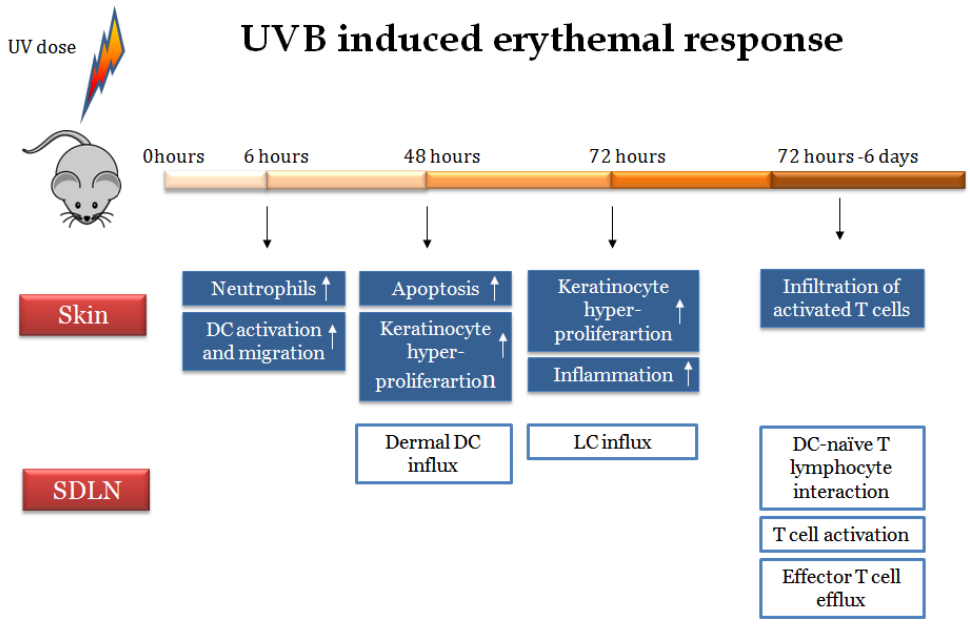

Figure 1.9 **UVB induced erythemal response:** A time-course of the different UVB induced cutaneous immune events in the skin and the skin draining lymph nodes

Regressive phase

The **regressive phase** precedes the regression of UVB-induced inflammatory responses acting to suppress UVB-induced inflammation. Some of the immune cells are common between the inflammatory phase and regressive phase. The $CD4^+$ T cells assume a $T_{regulatory}$ phenotype as opposed to a T_H1 phenotype and cytokines such as TGFβ produced by T_{regs} may play a role in establishing an immunosuppressive microenvironment post UVB treatment. UVB irradiated keratinocytes can also produce a host of cytokines such as IL-10, TGFβ and IL-1 receptor antagonist [256-258] that also have immunosuppressive activity. Neutrophils can also contribute to the regression by secreting IL-10 and IL-1 receptor antagonist [259;260].

The histological changes following UV include thickening of the stratum corneum, epidermis and dermis as well as intercellular and perivascular swelling in the dermis and perivascular infiltration.

CD4 and CD8 lymphocytes form an integral part of the response to UVB radiation as they modulate the immune infiltrates both in acute as well as chronic settings. However, the exact role of sustained increase/activation of CD4, CD8 cells has not been well understood especially because most of the studies that report the activation of $IL\text{-}10^+$ CD4 T cells and T_{reg} cells involve low doses of UVB [261;262]. In addition, low doses of UVB decrease the magnitude of T_H1 response in contact hypersensitivity [263] compared to high UVB doses. In studies that use UVB doses close to the MED, (Minimum Erythema Dose), there was an overall increase in the dermal $CD3^+$ T cells [264]. In another study, there was an immediate (6 hr) increase in the number of $CD4^+$ T cells and was sustained till 48 hr [253] along with an increase in $CD8^+$ cells after 24 hr. The T_H1 polarizing cytokines, IFNγ and IL-2 in addition to TNFα have also been shown to increase by 6 hr after UVB irradiation.

1.5.7 Long-term effects of UV on skin

Skin photoaging

Skin photoaging is the result of chronic sun exposure. The clinical symptoms include dryness of skin, irregular pigmentation characterized by flat patches of increased pigmentation and persistent hyperpigmentation, wrinkling of skin, and telangiectasia- dilation of preexisting blood vessels creating small focal red lesions. Most of the chronic effects of UV have a genetic basis and are prevalent in fair-skinned caucasians with a history of chronic sun exposure and frequently manifested in the sun exposed head, neck and upper extremities.

Non-melanoma skin cancer (NMSC)

Carcinogenesis is a multistep process involving initiation, promotion and progression. Chronic exposure to UV leads to a gradual degradation in the integrity of skin structure and function. It occurs as a result of accumulation of lesions from repetitive DNA damage from UV and the effects of chronic inflammation. These cumulative injuries to the cutaneous tissue ultimately lead to the development of skin cancers. This is a sequential process with the induction of mutations with UV, followed by enrichment for cells with mutations, which offer them a selective growth advantage with a simultaneous escape from immune surveillance (Figure 1.6). UV irradiation by itself can cause mutations, increase cutaneous proliferation and can ultimately lead to skin tumor formation; hence, UV is a “complete carcinogen”.

UV carcinogenesis often involves the inactivation of one or more tumor suppressor genes and/or the overactivation of growth-stimulatory proto-oncogenes. Some of the pathways commonly mutated with UV carcinogenesis are p53, patched, p19 and ras. This in turn leads to defective apoptosis and clonal expansion of keratinocytes selected for mutations in key pathways, which offer them preferential growth advantage. These lesions eventually develop into epidermal

dysplasias and actinic keratosis. Some of these can progress further to the malignant form of the tumor, Squamous Cell Carcinoma (SCC). p53 is the most frequently mutated gene in human cancers with mutations identified in 50 % of all human cancers, with a even higher frequency 50-90 % in NMSCs and are associated with advanced malignancies [265-268]. Mutations in the CDK inhibitor 2A (CDKN2A) encoding $p16^{INK4a}$ and $p19^{ARF}$ are also associated with the formation of SCCs and BCCs [269;270].

Among the oncogenes, ras oncogene has been frequently involved in initiated papillomas from the skin [271] and mutations in ras gene have been detected in 20-40 % of UV-induced mouse skin cancers [272;273;273].

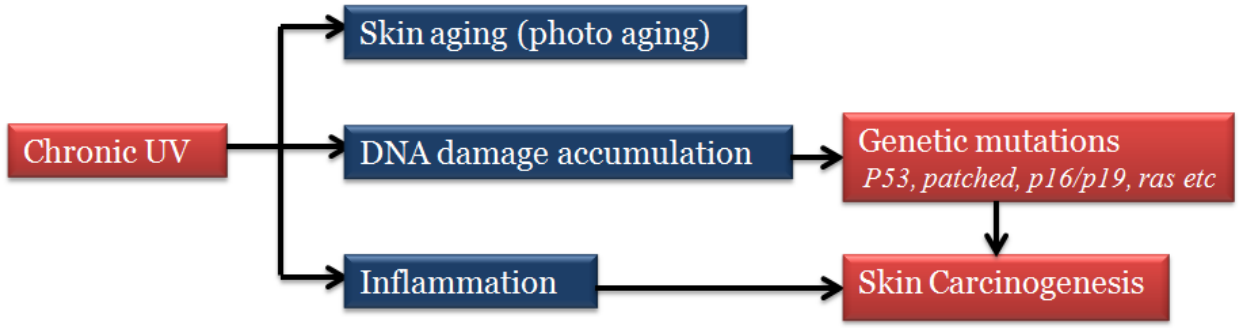

Figure 1.10 **The long term effects of chronic UVB irradiation** *Reprinted from [8] © 2004 with permission from Elsevier.*

1.6 TGFβ Signaling and UVB responses in skin

The role of TGFβ1 signaling pathway in UVB has not been clearly established. There are a few reports, which show the downregulation of the TGFβ receptor II and an induction of inhibitory Smad7. TGFβ1 expression has also been shown to be downregulated with UVB treatment [274-279]. However, there are a few studies, which have looked at the activation of TGFβ1 [279] pathway directly in terms of phosphorylation of Smad2. Furthermore, the kinetics of

phosphorylation of Smad2 in response to doses close to MED (Minimum Erythema Dose) and the exact downstream targets of this phosphorylation and their role in UVB responsiveness of the skin remain to be established. This study focuses on understanding the role of TGFβ1 signaling in UVB induced skin inflammation and UVB carcinogenesis using pharmacological and genetic models of TGFβ pathway ablation.

1.7 TGFβ Signaling in carcinogenesis

TGFβ was initially discovered as a negative growth regulator of epithelial cells and fibroblasts [280]. TGFβ also strongly inhibits the growth of multiple cell types including endothelial, hematopoietic and immune cell lineages [281]. The role of TGFβ in the regulation of epithelial cell development and maintenance has been studied extensively in normal tissue homeostasis as well as in the context of carcinogenesis. TGFβ activates p21-CIP1 and p15-INK4B transcription to mediate cell cycle arrest at the G1 phase in epithelial cells [282]. In addition, TGFβ also antagonizes the expression of Myc, which is important for cellular proliferation [283]. Both in cancer and immune system, TGFβ1 is more abundant than TGFβ2 or TGFβ3 [284;285]. Importantly, TGFβ1 expression is frequently upregulated in human cancers [77;78].

Although the primary and most direct properties of TGFβ in tissue regulation are growth-inhibitory for the epithelial cells and immunosuppressive for the immune system, the specific role of TGFβ in cancer progression has been established to be biphasic. Numerous reports have documented the dual role of TGFβ in skin carcinogenesis, which is both context-dependent and stage dependent [286;287]. TGFβ can inhibit cell cycle progression in epithelial cells and promote apoptosis, which often relates to a tumor-suppressive function of TGFβ [288]. In non-transformed and premalignant epithelial cells, TGFβ is well known for its ability to inhibit cell proliferation and promote an epithelial to mesenchymal transition (EMT) [94;288]. Transgenic

mice that expressed the TGFβ1 transgene [289] or TGFβ superfamily members [290] in the epidermis had reduced papilloma formation in the 2 stage chemical carcinogenesis model. On the other hand, carcinomas are often reported to secrete excess TGFβ1, in which case, it acts as an oncogene by increasing invasion and metastasis [286]. Most of the tumor-promoting properties of TGFβ have been linked to loss in responsiveness of tumor cells to TGFβ and dysregulation in the negative feedback loop for TGFβ.

TGFβ can also enhance tumor invasiveness and metastatic properties by enhancing EMT by acquisition of fibroblast-like properties and promoting an immunosuppressive microenvironment in an established tumor. In breast and skin cancer, EMT contributes to cancer progression as the tumor cells become increasingly invasive as they acquire myofibroblastic phenotype. [291] TGFβ transgene overexpression has been shown to increase tumor latency in a mouse model of mammary carcinoma while at the same time, it has also been shown to be necessary for inducing tumor invasiveness [292-295]. In chemical carcinogenesis studies with TGFβ1 transgenic mice, though there was a decrease in the number of papillomas, the papillomas that formed had enhanced malignant conversion with increased incidence of spindle cell carcinoma [296]. Hence, the exact role of TGFβ in tumorigenesis is a function of the specific tumor stage. In addition to the direct roles on tumor cells of epithelial origin, TGFβ is also important for the different heterotypic interactions between the tumor cells and the immune populations as well as endothelial cells in the stromal microenvironment. The diverse repertoire of immune subsets in the tumor microenvironment can promote or suppress tumor progression depending on the specific context of interaction and there exists a balance between protumorigenic and antitumorigenic functions and TGFβ plays an important node of regulation with the potential to switch the balance.

1.7.1 Link between TGFβ signaling and Human cancer

Studies on the specific contribution of TGFβ to tumorigenesis have yielded conflicting results and both increases as well as losses in TGFβ pathway components have been reported. Mutations and loss of TGFβR1 and TGFβR2 have been detected in many common human cancers such as breast, colon, pancreas, prostrate, stomach, esophagus, liver, lung and brain [78;297]. Mutations in a single allele of TGFβR1 have been linked to increased cancer risk [298;299].

Smad signaling has also been shown to be frequently lost in human cancers. Smad4 mutation, deletion and loss of expression have been reported in a number of cancers including breast, ovary, colon, intestine, pancreas, esophagus, liver, lung and cervical cancers [78;297]. Smad2 loss has also been detected in cervical, colon, lung and liver cancers [300]. In addition, TGFβ pathway silencing can also occur through epigenetic alterations of the TGFβR1 and TGFβR2 genes in addition to Smad4 [301-303]. Moreover, loss of TGFβ signaling is correlated with poor patient prognosis with an increased propensity for relapse of breast cancer [304;305]. Thus, TGFβ suppresses primary tumor growth but can promote metastasis through EMT of the responding carcinoma cells.

1.7.2 Link with Skin cancer

A loss of TGFβ1 expression is associated with Human Head and Neck SCC (HNSCC) and cervical SCC [306-309] and increased risk of malignant progression in the 2-stage chemical carcinogenesis model [310;311].

Mutations in TGFβ type I and type II receptors have been identified in HNSCC and SCC while mutations in TGFβ signaling components such as Smad2, Smad4 and Smad3 are less frequent. Loss of TGFβ signaling occurs more often through downregulation of TGFβ type I or type II receptors in upto 60 % of tumors. Disruption of Smad function occurs through loss of

heterozygosity for Smad4 or reduced message levels or protein levels for Smad4 in HNSCC and esophageal SCC [312-315]. In addition, loss of Smad4 and Smad2 is strongly correlated with poorly differentiated tumors [316].

1.7.3 The effect of TGFβ on immune cells in the tumor microenvironment

The overproduction of TGFβ in the tumor microenvironment enhances EMT of initiated tumor cells and also establishes an immunosuppressive environment for the tumor cells to thrive [93;317]. Overproduction of TGFβ is associated with metastasis and poor patient prognosis. A successful immune response requires the proper activation and maturation of antigen-presenting cells of the innate immune system that present antigen to adaptive immune cells. TGFβ presence can modulate the activation, maturation and differentiation of both innate and adaptive immune cells including natural killer cells, dendritic cells, macrophages, neutrophils and CD4, CD8 T cells. TGFβ also contributes to the immunosuppressive environment indirectly through supporting the differentiation of regulatory T cells, which serve to downregulate the overstimulation of the immune system by the persistence of tumors.

1.7.4 Dendritic cells in tumors

DCs are not only important for the initial activation of the adaptive immune responses but are also important for subsequent regulation of immune responses under chronic immune stimulation [318]. DCs can control the course of the immune response to tumorigenesis by virtue of their polarization toward immunogenic vs tolerogenic DCs. TGFβ can immobilize DCs interfering with their migration and transport of antigen to the draining lymph nodes responsible for generating an adaptive immune response and also directly induce DC apoptosis in tumors and tumor-draining lymph nodes [319]. Increased production of TGFβ has also been shown to reduce

the tumor infiltration of DCs within skin tumors as well the migration of DCs to the tumor draining lymph nodes resulting in decreased T cell response and is frequently linked to a progressive tumor phenotype [320;321].

Tumor derived TGFβ also modulates the activation status of tumor-infiltrating DCs by down-regulating their expression of MHC class II as well as co-stimulatory molecules such as CD40, CD80 and CD86 and also by downregulating their proinflammatory signature: TNFα, IFNα, IL-12, and CCL5 [117;322]. The increased TGFβ presence in the tumor microenvironment polarizes immature DCs towards tolerogenic DCs that secrete copious TGFβ in the lymph nodes promoting the development of tumor-specific T_{regs} in a TGFβ and IL-10 dependent manner [323-326] [327-329].

1.8 TGFβ pathway inhibition in Disease

TGFβ is frequently overexpressed in disease conditions like cancer and fibrosis where the overproduction of TGFβ drives disease progression through its immunomodulatory properties [10]. The generally accepted paradigm for TGFβ in carcinogenesis is its biphasic action by suppressing carcinogenesis at early stages but promoting tumor progression once they are in advanced stages [296;304;330]. As the tumors progress, they become unresponsive to the TGFβ1 ligand, however, due to dysregulation of the negative feedback loop, there is excessive secretion of tumor-derived TGFβ in the microenvironment, which in turn supports EMT and tumor metastasis leading to poor prognosis [304;330]. However, there are also reports which suggest TGFβ signaling promotes skin tumor development and inhibition of TGFβ signaling in a pharmacological as well as genetic model enhances malignant progression [331;332]. Hence, the specific context of disease and the temporally distinct host-tumor interactions dictate the pro vs anti-tumorigenic function of TGFβ. While designing therapeutic strategies for cancer by

inhibiting TGFβ pathway components, it is of utmost importance to selectively retain the anti-tumorigenic properties of TGFβ signaling. In addition, the mode of drug delivery has to be designed in such a way that it is restricted to the tumor microenvironment, since all cell types have receptors for TGFβ and inhibiting TGFβ signaling can lead to a variety of side-effects exemplified in TGFβ knockout models [161;333-335]. However, multiple self-healing squamous epithelioma (MSSE) patients who carry a rare germline-null mutation in the TβRI gene develop only self-limiting non-malignant skin lesions and are largely free of inflammatory complications [10;336]. The function of TGFβ is highly context dependent and in addition, it also varies with cell type as well as interactions with other cellular populations in the milieu as well as cross talk with other pathways intracellularly. This is one of the major issues with targeting the TGFβ pathway for treating disease conditions [10].

TGFβ-induced EMT has been linked to acquisition of stem-cell like properties by tumors reminiscent of cancer stem cells with enhanced tumor-initiating, invasive and malignant phenotype and increased resistance to chemo and radiotherapy [337-339]. TGFβ inhibitors have been shown to be successful [10] to different extents in preclinical studies and mouse models to deplete the stem cell compartment and downregulate stem cell markers such as CD44, Id1 in different cancers including breast cancer [339], glioblastoma [340-342] and chronic myeloid leukemia [343]. In multiple myeloma, TGFβ receptor I kinase inhibitor SD208 decreased IL-6 and vascular endothelial growth factor (VEGF) secretion from bone marrow derived stem cells, which in turn decreased tumor growth [344]. However, increased metastases have also been reported from disseminated tumor cells in the case of systemic TGFβ inhibition. [345]

Fibrosis is a disease condition associated with dysregulated fibroblast function, which is linked directly to excessive TGFβ activity [346;347]. With tissue injury, there is excessive secretion of

TGFβ followed by induction of αVβ6 integrin, which binds and activates latent TGFβ [348]. Active TGFβ then promotes the differentiation of fibroblasts into myofibroblasts, which cause excessive extracellular matrix (ECM) deposition leading to scarring [349]. As TGFβ plays such a central role in fibrosis, TGFβ receptor inhibitors were primarily designed as prospective treatment for fibrotic diseases. A number of TGFβ pathway inhibitors have since been used in mouse models and preclinical trials against pulmonary fibrosis [350-353], renal fibrosis [347;354] and cardiac fibrosis [355;356] and have shown tremendous promise.

1.8.1 Inhibition of TGFβ1 by small molecule inhibitors – SB431542

The different drug design strategies include small molecule inhibitors specific for TGFβ pathway receptors, as well as signaling components, monoclonal antibodies and gene therapy [10]. Small molecule inhibitors were developed with the potential of selectively retaining the tumor suppressive properties of TGFβ1 while inhibiting its tumor promoting functions [357]. SB431542 (Figure 1.11) is one of these inhibitors with potential therapeutic applications. SB431542 (SB) (4-[4-(3,4-Methylenedioxyphenyl)-5-(2-pyridyl)-1H-imidazol-2-yl]-benzamide) is a commercially available small molecule inhibitor for the TGFβ1 pathway.

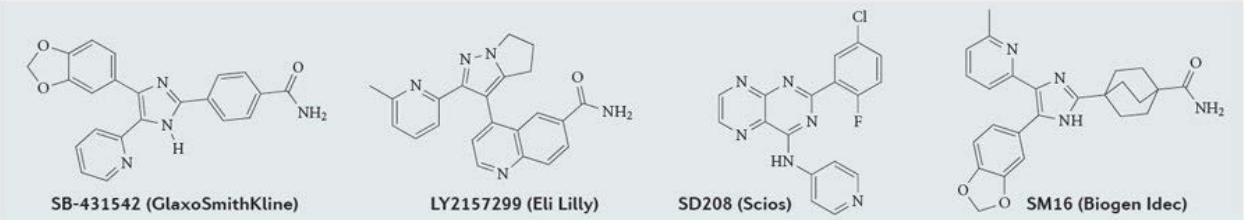

Figure 1.11 **Small molecule inhibitors to target the transforming growth factor-β (TGFβ) signalling pathway.** Depicted are the molecular structures of small-molecule inhibitors identified to target the transforming growth factor-β (TGFβ) signalling pathway. SB-431542, LY2157299, SD208 and SM16 are all ATP mimetics that inhibit TGFβ receptor type I (TβRI) kinase activity. *Reprinted with permission from Macmillan publishers Ltd: Nat.Rev.Drug Discov [10] © 2012.*

It has been reported as an inhibitor of the TGFβ type I receptor with specificity towards ALK4, ALK5 and ALK7 receptors [331;358;359]. It acts as a competitive inhibitor for the kinsae domain of the receptor where ATP binds; it has been shown to inhibit the *in vitro* phosphorylation of both Smad2 and Smad3. It has no direct effect on BMP signaling pathway or the ERK, JNK, p38 MAP kinase pathways [358].

1.9 Hypothesis and specific aims

TGFβ1 plays a dual role in cancer acting as both a tumor suppressor and a tumor promoter. Studies in literature have also reported conflicting results for the role of TGFβ1 signaling. Transgenic TGFβ expression inhibits formation of benign tumors while promoting malignant conversion [290;296] while blockade of TGFβ signaling has also been reported to accelerate two-stage chemical skin carcinogenesis [360-362]. Studies from our lab have shown that TGFβ is required for tumor promotion in chemical carcinogenesis [331;363], associated with increased inflammation. TGFβ is also a key immunomodulatory cytokine for different lineages of the hematopoietic stem cells and is important for maintaining tissue homeostasis and peripheral self-tolerance. However, the role of TGFβ in DC function during an immune response has been shown to be context specific and the mechanism is largely unclear. Based on literature and our preliminary results, we hypothesized that TGFβ signaling in DCs is essential for UVB-induced inflammation in the skin and UVB skin carcinogenesis. The overall goal of this study was to understand the role of TGFβ1 signaling in DCs in the elicitation of cutaneous immune responses and its relevance in cutaneous pathology. To addess this hypothesis, we devised the following specific aims:

Specific Aim1: To test the role of TGFβ signaling in UVB skin carcinogenesis bioassay using a pharmacological model of inhibition for the TGFβ type I receptor.

Specific Aim2: To test the contribution of TGFβ signaling in an acute and short-term model of UVB induced skin inflammation using a pharmacological and (DC-specific) genetic model of deficient TGFβ signaling.

Specific Aim3: To test the role of defective TGFβ signaling in DCs in responses to hapten sensitization in a Contact Hypersensitivity (CHS) experimental model.

Chapter 2 gives evidence for the importance of TGFβ signaling in the UVB irradiation responses of skin by showing activation of the TGFβ pathway at the level of phosphorylated Smad2 in total skin and in dermal Dendritic cells. Further, we show that the pharmacological inhibitor SB blocks this UVB-induced pSmad2 activation displaying the validity of our model. We further show that SB suppresses tumor formation in UVB skin carcinogenesis assay which is associated with reduced infiltration of Tumor-infiltrating lymphocytes (TILs), $CD4^+$ and $CD8^+$ and $CD4^+IFN\gamma^+$ and $CD8^+IFN\gamma^+$ T cells compared to the vehicle treated tumors. A short-term UVB irradiation model with SB phenocopied this reduction in T cell activation both in the skin-draining lymph nodes (SDLNs) and the skin. Further, we show that activation of TGFβ signaling in dermal DC subsets with UVB but not Langerhans cells is important for their reduced migration to the SDLNs, which in turn is linked to reduced inflammation with UVB.

Chapter 3 uses a genetic model of DC-specific TGFβ signaling defect to validate that the reduced inflammation and migration of dermal DC subsets to the SDLNs with UVB relative to WT mice is due to defective TGFβ signaling specifically in DCs. Further, Contact Hypersensitivity (CHS) responses of CD11c-DNR transgenic mice revealed a crucial role for TGFβ signaling in DCs for skin sensitization to hapten exposure resulting in reduced ear thickness. In addition, defective TGFβ signaling in DCs also abrogated the synergistic increase in CHS response with UV observed in WT mice. Consistent with this, there was reduced T cell activation and cytokine profile in the SDLNs of CD11c-DNR mice with UVB after hapten sensitization compared to WT mice. These results provide evidence for the critical role TGFβ signaling in DCs plays in eliciting an immune response to an inflammatory stimulus.

These studies are not only important for understanding the mechanism of TGFβ signaling in UVB induced carcinogenesis but is also important for understanding the basic mechanism of

immune activation within the skin. Our studies provide new insight into how cutaneous TGFβ signaling in DCs may represent a common mode of regulation for different T cell mediated cutaneous pathologies.

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Chapter 2

Suppression of Ultraviolet B-Induced Skin Tumors by the ALK5 Inhibitor SB431542 is Associated with Reduced T cell Activation and Lymph Node Migration of Skin Dendritic Cell Subsets

2.1 ABSTRACT

Transforming Growth Factor-β1 (TGFβ1) is a pleiotropic cytokine in the skin that can function both as a tumor promoter and suppressor in chemically induced skin carcinogenesis, but the function in UVB carcinogenesis is not well understood. Treatment of SKH1 mice with the activin-like kinase 5 inhibitor SB431542 (SB) to block UVB-induced activation of cutaneous TGFβ1 signaling suppressed skin tumor formation but did not alter tumor size or tumor cell proliferation. 30 week tumors that arose in SB treated mice had significantly reduced percentage of $IFN\gamma^+$ tumor infiltrating lymphocytes compared to control mice. SB blocked acute and chronic UVB-induced skin inflammation and T cell activation in the skin draining lymph node and skin, but did not alter UVB-induced epidermal proliferation. We tested the effect of SB on migration of skin dendritic cell populations since dendritic cells are critical mediators of T cell activation and cutaneous inflammation. SB blocked UVB-induced Smad2 phosphorylation in dermal DC, lymph node and ear explant migration of $CD103^+$ $CD207^+$ and $CD207^-$ skin DC subsets but did not affect basal or UV-induced migration of Langerhans cells. Together these results suggest that TGFβ1 signaling has a tumor promoting role in UVB-induced skin carcinogenesis and this is mediated in part through its role in UVB-induced migration of dermal DC and cutaneous inflammation.

2.2 INTRODUCTION

Ultraviolet radiation (UVB) is a key environmental mutagen, acting as both an initiator and promoter of skin cancer [1]. Chronic inflammation is a hallmark of carcinogenesis and has been widely implicated to be a potent tumor promoter [2]. High doses of UVB radiation leads to vasodilation, erythema and inflammation [3] while suberythemal doses cause local and systemic immunosuppression [4]. Langerhans cells (LCs) in the epidermis, and $CD103^{+}CD207^{+}$ and $CD103^{-}CD207^{-}$ dendritic cell (DC) subsets in the dermis are key mediators of the cutaneous inflammatory response [5;6]. LCs and dermal DC subsets can be differentially activated by inflammatory stimuli [7-9] including UV irradiation [10], and LCs are thought to mediate the tolerogenic response to suberythemal doses of UV. However, the mechanism of UV-induced DC activation and inflammation in the skin in response to erythemal doses of UV is not clear.

Transforming growth factor beta 1 (TGFβ1) is a pleiotropic cytokine that acts on multiple immune cell types including DC to either promote or suppress inflammation. *In vitro*, TGFβ1 can suppress dendritic cell function [11-14], and transgenic models with DC specific inactivation of TGFβ signaling exhibit exacerbated severity of several inflammatory diseases [15;16]. However, under some conditions TGFβ treated dendritic cells can promote Th17 polarization [17;18]. In the skin TGFβ1 signaling in LC is essential for epidermal residency and maintenance of an immature phenotype [19-22], but the role of TGFβ1 signaling in other DC subsets is poorly understood.

We previously reported that TGFβ had a proinflammatory and tumor promoting role in chemically-induced skin carcinogenesis [23;24] and that elevated TGFβ1 expression in the epidermis caused dermal DC migration to the SDLN and enhanced contact hypersensitivity (CHS) responses [25]. Here, we investigate the effects of ALK5 inhibition during UV

carcinogenesis. Here we show that inhibition of TGFβ1 signaling suppresses UVB-induced tumor formation but enhances malignant progression. Both responses are linked to reduced lymph node activation of T cells and IFNγ^+ CD4 and CD8 T cells in UVB irradiated skin and skin tumors that in turn is linked to reduced migration of dermal dendritic cells in response to UVB. Our results implicate TGFβ signaling in dermal dendritic as a primary response to UVB that regulates cutaneous inflammation and UVB induced skin tumor formation.

2.3 MATERIALS and METHODS

2.3.1 Mice: Age-matched (6–9 week) and sex-matched male Skin Hairless mice (SKH1) were used for the pharmacological inhibition studies of TGFβ1 signaling with SB. All animals were treated according to approved Institutional Animal Care and Use (IACUC) protocols.

2.3.2 UVB Irradiation: SKH1 mice were irradiated with 2.4 kJ/m2 (MED) from UV bulbs (American Ultraviolet Light Co.) covered with cellulose triacetate (KODAK) to filter out UVC radiation, and produce UV wavelengths between 280-320 nm as described (Melnikova, 2005 12582 /id). Irradiance was measured using a UVX radiometer (UVP, Upland Ca). Mice were pretreated with 200µl of 10 µM SB431542 (SB) (Sigma) or 200µl acetone 1 hour prior to the UV treatment. Primary keratinocytes were exposed to UVB with a Cl-1000 ultraviolet crosslinker (UVP, Upland CA) fitted with bulbs emitting predominantly UVB wavelength. Culture media was removed from cells and after a PBS wash, cells were exposed to UVB in PBS after which original media was replaced.

2.3.3 UVB Carcinogenesis: 7 week-old SKH1 mice (10-13 per group) were treated with UVB to induce tumors at the MED dose (described above) three times a week with pretreatment of 200µl of acetone or 200µl of 10 µM SB (Sigma) for 25 weeks and the tumors were harvested at the end of 30 weeks. The number of papillomas per mouse (>1 mm in diameter) was counted, and measured using a digital vernier calipers and a tumor profile was constructed. The harvested tumors were digested into a single cell suspension and immunophenotyped for tumor infiltrating lymphocytes and IFNγ secretion as described above.

2.3.4 Antibodies: The following antibodies were purchased from Ebioscience, San Diego, CA: anti-CD16/32 (93), and APC eFluor 750-anti-CD45 (30-F11), FITC- and eFluor 450-anti-MHCII (M5/114.15.2), PE- and Alexaflour 700-anti-CD11c (N418), FITC-anti-CD4 (GK1.5), PECy5-

anti-CD8α (53-6.7), PE-anti-CD103 (2E7), PECy7-anti-B220 (RA3-6B2), PercpCy5.5-anti-CD11b (M1/70), PE-anti-CD62L (MEL-14), and PECy5-anti-CD44 (IM7). The following antibodies were purchased from BD Pharmingen, San Diego, CA: PE-anti-CD45 (30-F11), and PECy7-anti-IFNγ (XMG1.2). The following antibodies were purchased from BioLegend: PercpCy5.5-anti-CD40 (3-23), PECy5-anti-CD197 CCR7 (4B12). Alexa 568-anti-Epcam (G8.8) and Alexa 647-anti-CD207 (L31) antibody conjugates were generated as previously described (Gaiser, 2012 14003 /id).The following antibodies were purchased from Cell Signaling technology: p-Smad2 (#3101 for western blotting; #9510 for FACs analysis), Smad2/3-3102, p53-2524, GAPDH-2118, p21-6246 (Santa Cruz), Actin-1501 (Millipore). Antibodies used for immunohistochemistry: CD45 (BD Pharmingen#550539), CD3-ε (M-20) (Santa Cruz-1127)

2.3.5 Flow Cytometry: Dendritic cells were isolated from the inguinal lymph nodes, the epidermis and dermis as described (Mohammed, 2013 14087 /id). Single cell suspensions of DCs were incubated with CD16/32 followed by staining for extracellular surface antigens. For anti-CD207 staining, cells were fixed and permeabilized using fixation/permeabilization buffer (Ebioscience) and incubated with anti-CD207 antibody in 0.2% saponin buffer. For phospho-Smad2 staining, the cells were fixed with 2% paraformaldehyde for 10 minutes followed by staining for surface antigens. Cells were then permeabilized by 90% methanol for 30 minutes and then stained for p-Smad2. Cells were analyzed using a Fortessa LSRII (BD Biosciences, San Jose, CA). Single-cell suspensions were prepared from the inguinal lymph nodes and the UV-exposed dorsal skin as described (Mohammed, 2010 13018 /id). Following incubation with PMA/ionomycin and Brefeldin A (eBioscience, San Diego, CA) for 4.5 hours at 37 °C, the cells were stained for surface antigens and then fixed with 4% paraformaldehyde, and permeabilized with 0.2% saponin buffer and stained for intracellular IFNγ. Cells were acquired on FC500

(Beckman Coulter, Indianapolis, IN) and analyzed using the FlowJo software (Tree Star, Ashland, OR).

2.3.6 Ear Explant Cultures: Ears of SKH1 mice with one ear treated with the vehicle and the other ear with SB, were irradiated with UVB at 1 MED. They were excised immediately after a single MED dose of UVB. They were then rinsed in 70% ethanol, and then in a solution containing 200 IU ml^{-1} penicillin and 200 µg ml^{-1} streptomycin for 5 minutes to make them sterile. Only the dorsal halves of the ears were presumed to be exposed to UVB. The dorsal and ventral halves were split from the attached cartilage and the dorsal halves were cultured in complete RPMI media (10% fetal calf serum) for 72 hours at 37 °C. The dendritic cell populations migrate out of the ear skin and into the media mimicking migration to the SDLN from the skin *in vivo*. The culture media was then harvested to analyze the cells that migrated from the explant, enumerated, stained and analyzed by flow cytometry similar to 2.3.5.

2.3.7 Epidermal sheet immunofluorescence: Epidermal sheets were prepared as described (Nagao et al., 2009). For staining, epidermal sheets were rehydrated in phosphate-buffered saline and incubated overnight with FITC-anti-MHCII antibody at 4' C. Slides were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and analyzed on an Olympus BX61 microscope (Olympus America, Center Valley, PA).

2.3.8 Analysis of Protein and RNA: RNA and protein were isolated and analyzed by quantitative RT-PCR and immunoblotting as described [23].

2.3.9 Statistical Analysis: All statistical analyses were performed using the GraphPad Prism software and the values are expressed as Mean±SEM (GraphPad Software, La Jolla, CA). A two-tailed Student's *t*-test was performed to compare the groups. P values of significance were represented as: * $p<0.05$, ** $p<0.01$.

2.4 RESULTS

2.4.1 ALK5 inhibition suppresses UVB-induced Smad phosphorylation in skin and reduces outgrowth of UVB-induced skin tumors

To test the effect of UV irradiation on the TGFβ1 pathway, we treated the skin of 7 week-old SKH1 mice with UVB at the minimum erythema dose (MED) of 2400 J/m^2 [26]. There was a rapid activation of the pathway measured in terms of phosphorylated Smad2 and phosphorylated Smad3, direct targets of ALK5 kinase, as early as 2 hours and was maintained through 48 hours (Figure 2.1 A). This increase at 2 hours and 6 hours was blocked with a 1 hour pretreatment with 10 μM SB431542 (SB) (Figure 2.1 B). In contrast, the characteristic UVB DNA damage response induction of p53 and p21 was unaffected with SB pretreatment suggesting that the effects of SB inhibition are specific to the TGFβ signaling pathway, and that SB pretreatment was not acting as a non-specific sunblock. Consistent with this, there was significant decrease in p-Smad2 levels between UV-treated TGFβ+/+ mice and TGFβ-/- mice (Figure 2.1 C). The increase in p-Smad2 and p-Smad3 in the skin was not associated with an increase in TGFβ1 message (Figure 2.1 D). Further, there was a similar induction of p-Smad2 *in vitro,* with UVD-irradiated SKH1 primary keratinocytes, (Figure 2.1 E) as well as a UVB dose-dependent increase in p-Smad2 levels at 50 mJ/cm^2 and 75 mJ/cm^2 (Figure 2.1 F). This increase was blocked entirely with 1 hour pretreatment with 1 μM SB (Figure 2.1 G).

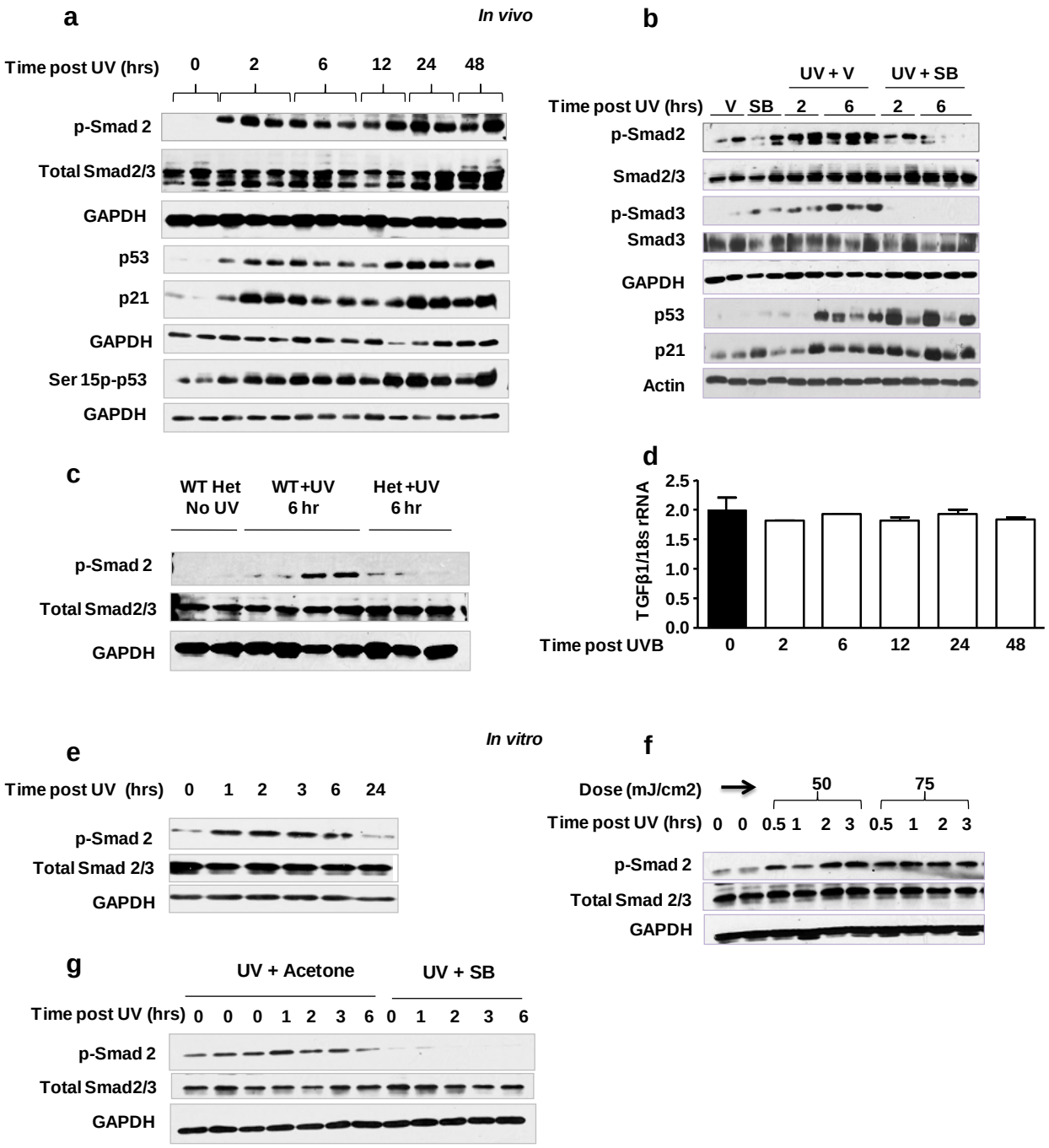

Figure 2.1 SB431542 blocks UVB-induced Smad phosphorylation in skin. Skin hairless (SKH1) mice were exposed to 2.4kJ/m^2 Ultraviolet irradiation UVB IR (1 MED) and skin was harvested at the indicated time points. (A) Immunoblot of time-course of skin tissue post-UVB. (B) Immunoblot of skin tissue at indicated times post UVB from SKH1 mice pretreated with 10 μM SB43152 (SB) or acetone (V) for 1 hour before UVB. (C) TGFβ+/+ and TGFβ+/- mice on SKH1 background were exposed to 2.4kJ/m^2 UVB IR (1 MED) and immunoblot of skin tissue was prepared at indicated times post-UVB. (D) mRNA was prepared from total skin and the relative expression of TGFβ1 transcript was measured by quantitative RT-PCR. (E) Immunoblot of time-course of SKH1 primary keratinocytes exposed to 75 mJ/cm^2 (F) Immunoblot of dose-response of SKH1 primary keratinocytes exposed to UVB IR. (G) Immunoblot of primary keratinocytes at indicated times post UVB from SKH1 mice pretreated with 1 μM SB43152 (SB) or acetone (V) for 1 hour before UVB.

To determine if inhibition of TGFβ1 signaling with topical SB could block UVB-induced skin tumor formation similar to its effects in the 2-stage chemical carcinogenesis model [23], we treated 7 week-old SKH1 mice in groups of 10-13 mice with 1 MED UVB 3X per week with or without SB. Mice were treated with this protocol for 25 weeks and tumors harvested after an additional 5 weeks. Tumor development (lesions greater than $1mm^2$) in both acetone and SB treated mice was apparent at week 18 but the tumor number per mouse was reduced by 50 percent in the SB treated mice at all subsequent time points (Figure 2.2 A). However, there was no difference in overall tumor size or distribution at any time point, or difference in tumor cell proliferation at study end (Figure 2.2 B, E). Histopathology of tumors taken after 30 weeks showed that there were equivalent percentages of benign lesions (hyperplasias and papillomas) in both groups, but there was a trend towards less progressed malignancies in the vehicle treated mice compared to SB treated mice (Figure 2.2 C, D).

Since T cell infiltration has been linked to both tumor suppression and progression [27] we isolated leukocytes from control and SB treated tumors and analyzed frequencies of myeloid cells and TIL by flow cytometry. There was no significant difference in myeloid cells measured by flow cytometry ($CD11b^+$ $Ly6G^+$; $CD11b^+$ $Ly6G^-$ or $CD11b^+$ $F480^+$ $Ly6G^-$) between tumors from vehicle or SB treated tumors (Figure 2.3 C). In contrast, the frequency of $CD4^+$ as well as $CD8^+$ T lymphocytes within the tumors that developed in SB treated mice was reduced 50% relative to vehicle control (Figure 2.3 A). Additionally, compared to vehicle control tumors the percentage of $IFN\gamma^+$ $CD4^+$ TIL was reduced from 6% to 1.5% and $IFN\gamma^+$ $CD8^+$ TIL reduced from 7% to 4% in SB treated mice (Figure 2.3 B).

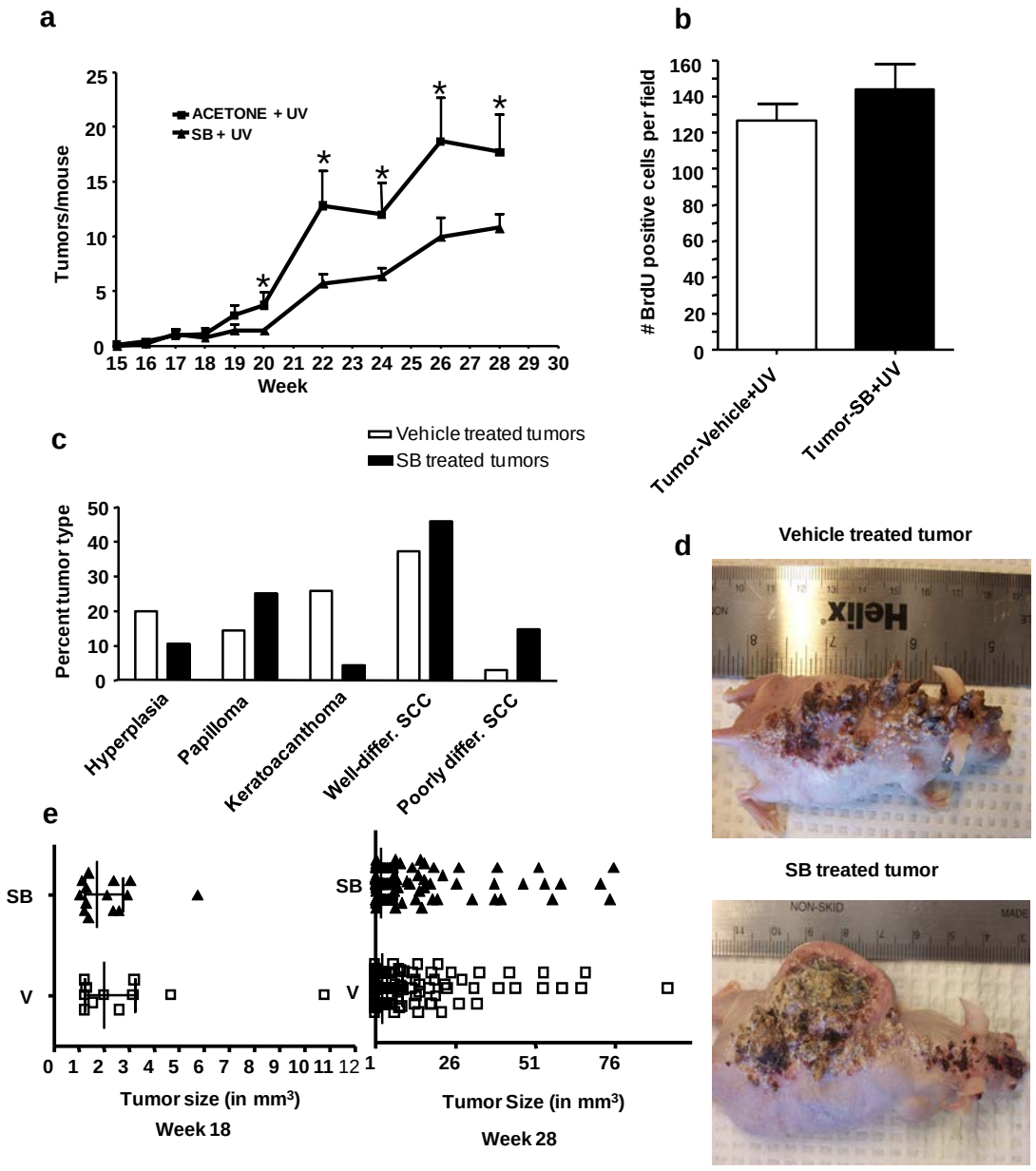

Figure2.2: SB431542 blocks development of skin tumors. (A)Skin tumor number per week in SKH1 mice treated with 2.4 kJ/m2 ultraviolet irradiation (UVB IR) 3X per week for 25 weeks with 1 hr pretreatment with SB431542 (n=13) or acetone vehicle (n=10). Lesions > 1 mm in diameter were counted. (B) BrdU positive tumor cells per field. Tumor sections were stained with anti-BrdU by immunohistochemistry and the number of positive cells per 40X field determined, averaged from 3-5 fields per tumor, n=7-10 tumors for each group. (C) Tumor grade determined blindly from H&E stained sections, n=35 and 48 tumors in control and SB treated groups. (D) Representative tumor images from vehicle and SB treated groups. (E) Tumor size distribution profile for an early time point-18 weeks and a later time-point-28 weeks. * indicates significantly different from acetone treated group at indicated time points $p<0.05$. V= vehicle.

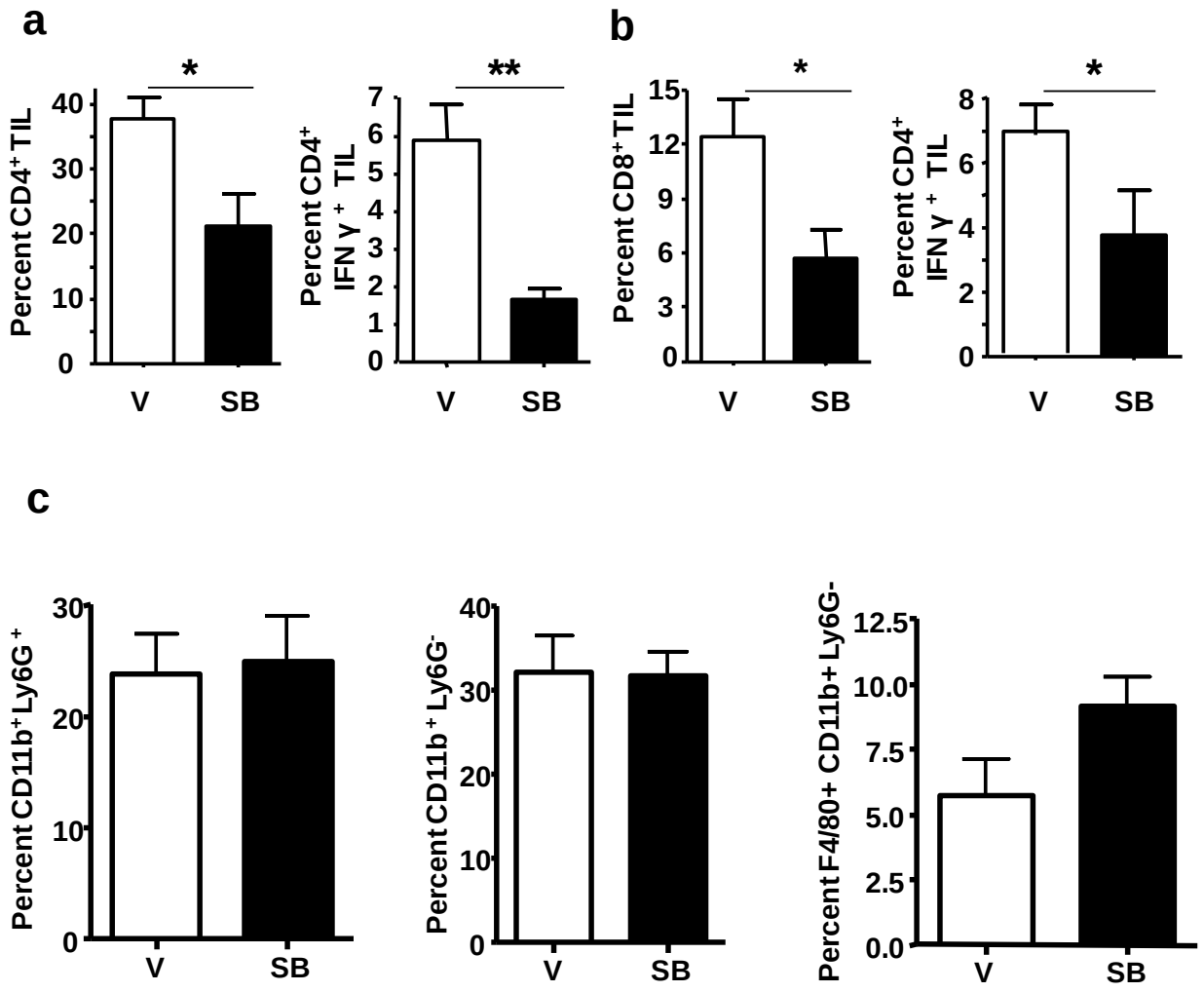

Figure 2.3: Skin tumors arising in SB431542 treated mice have reduced frequency of IFNγ$^+$ tumor infiltrating lymphocytes. Leukocytes were isolated from tumors arising in UVB irradiated mice treated with vehicle or SB431542 at 30 weeks. (A) The percentage of CD4$^+$ and CD8$^+$ T cells in total viable CD45$^+$ leukocytes. (B) The percentage of IFNγ$^+$ CD4$^+$ and CD8$^+$ T cells of viable CD45$^+$ leukocytes. (C) The percentage of CD11b$^+$ Ly6G $^+$ (representing neutrophils) and CD11b $^+$ Ly6G$^-$ and F4/80$^+$ CD11b$^+$ Ly6G$^-$ (representing macrophages) of viable CD45$^+$ leukocytes. N= at least 7 tumors per group. Error bars = ±SEM. **P<0.05 relative to indicated group; **P<0.01 relative to indicated group.*

2.4.2 ALK5 inhibition suppresses UVB-induced skin inflammation and UVB-induced T cell activation in lymph node and skin.

To understand how ALK5 inhibition could suppress tumor outgrowth we examined the effects of SB on UVB induced inflammation as this is critical for promotion of skin tumors. 7 week-old SKH1 mice were irradiated with UVB 3X on alternate days at 2.4 kJ/m^2 with or without a daily topical treatment of SB and after 1 week skin was analyzed by immunohistochemistry (IHC) and flow cytometry for changes in cutaneous responses to UVB. UVB caused a 5-fold increase in total skin leukocytes ($CD45^+$) and T ($CD3^+$) cells and this was significantly suppressed by topical SB treatment, by 35% and 50% respectively (Figure 2.4 A, B). Flow analysis showed that SB treatment significantly suppressed the UVB-induced increase in $IFN\gamma^+$ $CD4^+$ T helper lymphocytes in the skin (Figure 2.5 A). Although UVB did not significantly increase $IFN\gamma^+$ $CD8^+$ T lymphocytes at this time point, SB by itself reduced the frequency of these cells in the skin (Figure 2.5 A).

Topical SB also suppressed UVB-induced T lymphocyte activation within the skin draining lymph nodes (SDLN). With UVB there was a significant increase in $CD44^+CD62L^{low}$ central memory $CD4^+$ and $CD8^+$ T cells from 3 to 10 million and 2.5 to 6 million per lymph node respectively. SB suppressed this increase in memory cells by 3 fold and 2 fold respectively (Figure 2.5 B). UVB also caused a 4-fold increase in $IFN\gamma^+CD4^+$ T cells and 2 fold increase in $IFN\gamma^+CD8^+$ T cells, which was reduced to control levels by SB (Figure 2.5 C).

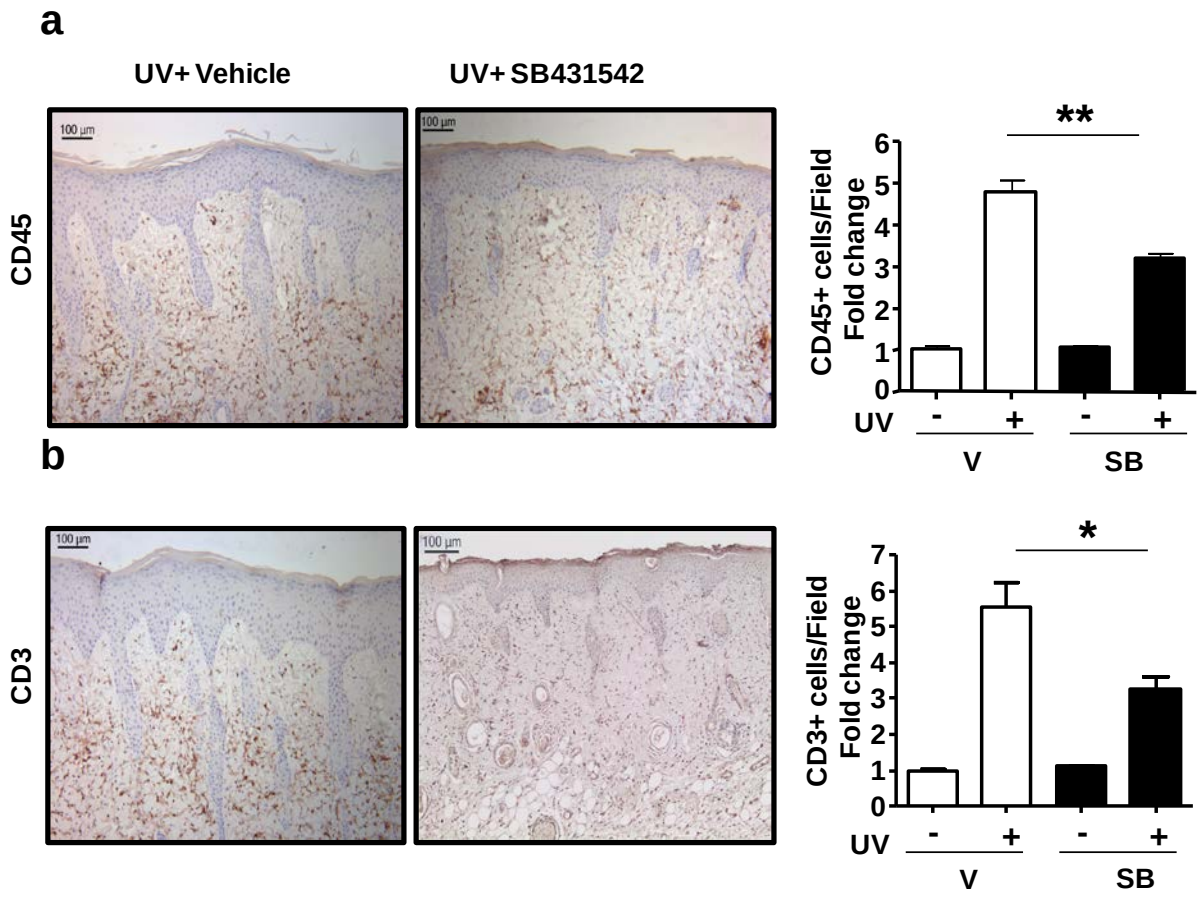

Figure 2.4: SB431542 suppresses UVB-induced skin inflammation. SKH1 mice were irradiated 3X over 1 week with 1 MED ultraviolet radiation and treated daily with either acetone vehicle (V) or SB431542 (SB). Quantitation of (A) $CD45^+$ and (B) $CD3^+$ cells in 7-10 random fields of view (FOV) from at least 3 stained sections per group. The average of the vehicle treated control was 29.52 $CD45^+$ cells/FOV, and 29.62 $CD3^+$ cells/FOV. n=2 for controls and n=3-4 for UVB IR groups. Error bars = ± SEM. * $p < 0.05$; ** $p < 0.01$. Scale bar = 100µm. n=2 for controls and n=3-4 for UVB irradiated groups.

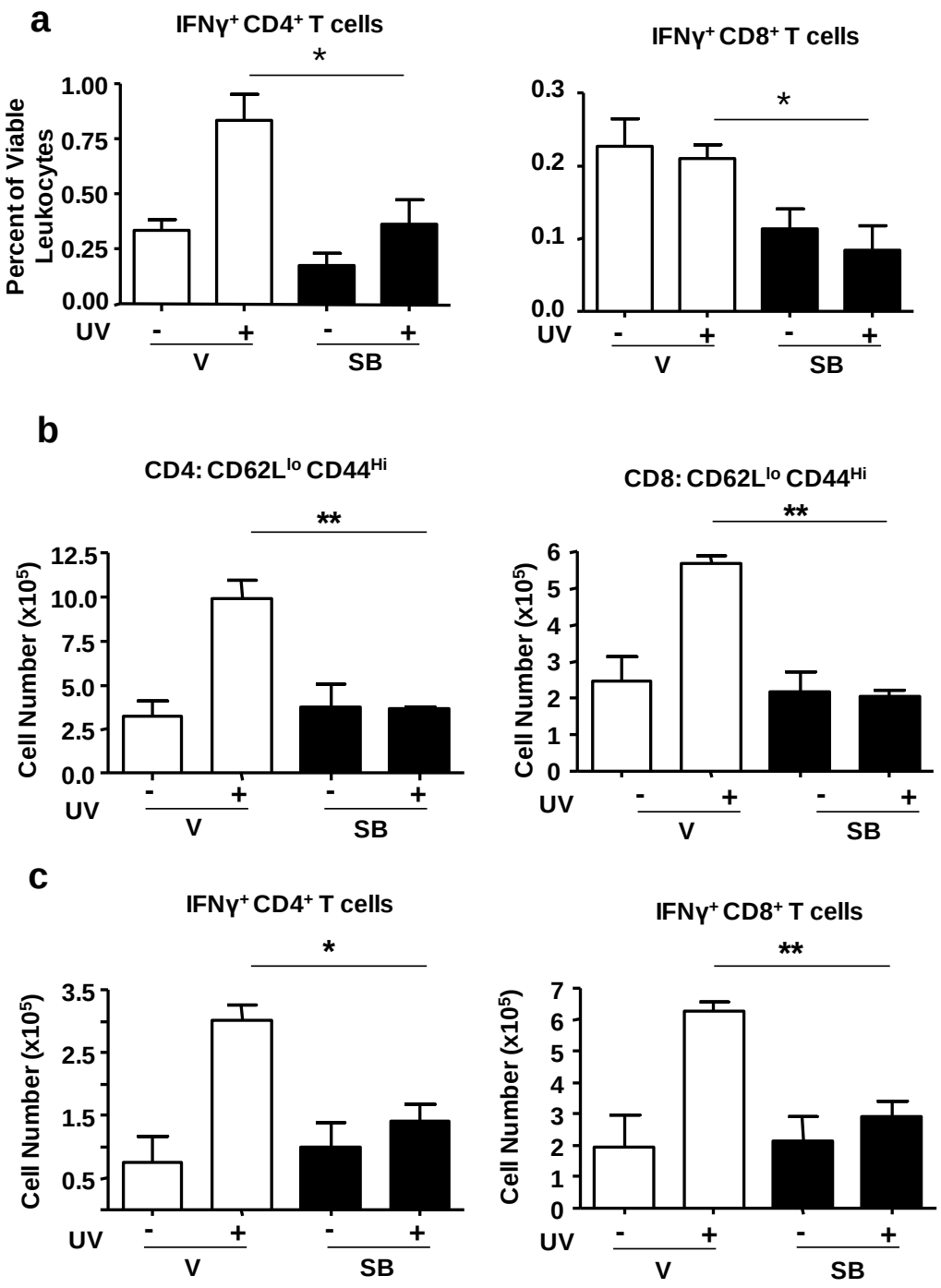

Figure 2.5: SB431542 suppresses UVB-induced skin Th1 and T cell activation in lymph node. SKH1 mice were irradiated 3X over 1 week with 1 MED ultraviolet radiation and treated daily with either acetone vehicle (V) or SB431542 (SB). (A) Percentage of $CD4^+$ $IFN\gamma^+$ T cells and $CD8^+$ $IFN\gamma^+$ T cells in viable CD45+ leukocytes isolated from SKH1 skin (B) Percentage of effector $CD4^+$ and $CD8^+$ T cells (CD44Hi CD62LLo) from SDLN of mice. (C) Percentage of $IFN\gamma^+$ $CD4^+$ and $CD8^+$ T cells in SDLN of mice treated as in a. n=2 for controls and n=3-4 for UVB irradiated groups. Error bars = ±SEM. **P<0.05 relative to indicated group; **P<0.01 relative to indicated group.*

Figure 2.6A shows that SB had the same effect on T cell activation in the SDLN and skin with a 2 week chronic UVB irradiation protocol (6X on alternate days, with or without dailytreatment). There was no significant increase in Th17 cells in UVB irradiated skin, or effect of SB on IL17^{+} T cells in the skin (Figure 2.6 B). There was no significant difference in myeloid cells measured by flow cytometry (CD11b^{+} Ly6G^{+}; CD11b^{+} Ly6G^{-} or CD11b^{+} F480^{+} Ly6G^{-}) or the number of Myeloperoxidase^{+} (MPO^{+}) cells infiltrating the UV-treated skin between SB and vehicle treatment (Figure 2.7 A, B, C and D). There was no significant difference between the groups in UVB-induced epidermal proliferation, as measured by anti-BrdU IHC (Figure 2.7 E). These results show that suppression of UVB-induced T cell activation by topical SB occurs early and is maintained in tumors even after 5 weeks removal from SB treatment.

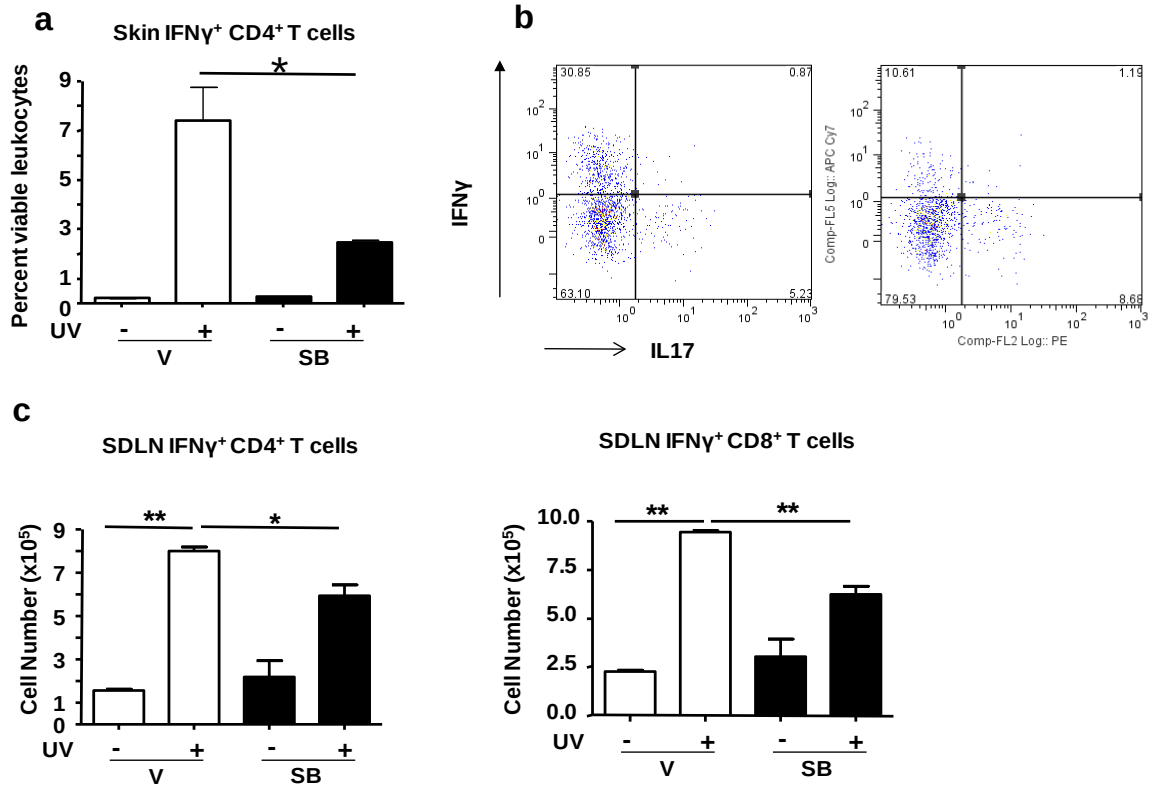

Figure 2.6: SB431542 suppresses UVB-induced Skin Th1 cells and T cell activation in lymph node after chronic (2 weeks) UVB IR. SKH1 mice were irradiated 3X over 2 weeks with 1 MED ultraviolet radiation and treated daily with either acetone vehicle (V) or SB431542 (SB). (A) Percentage of CD4^{+} IFNγ^{+} T cells in viable CD45^{+} leukocytes isolated from SKH1 skin (B) Representative flow profile of IFNγ^{+} and IL-17^{+} quadrants within the CD4^{+} T cell subset (C) Percentage of IFNγ^{+} CD4^{+} and CD8^{+} T cells in SDLN of mice treated as in a. n=2 for controls and n=3-4 for UVB irradiated groups. Error bars = ±SEM. **P<0.05 relative to indicated group; **P<0.01 relative to indicated group.*

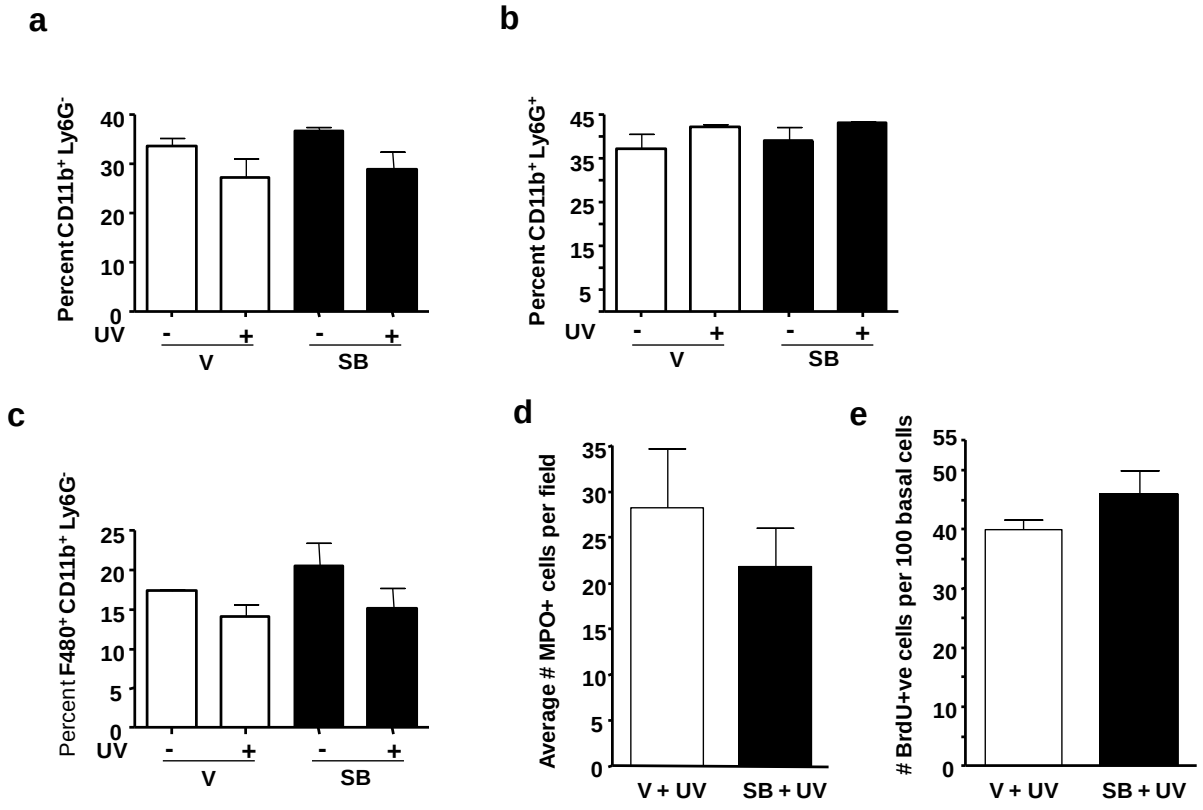

Figure 2.7: SB431542 does not change the skin neutrophil/macrophage profile or keratinocyte proliferation after chronic (2 weeks) UVB IR. SKH1 mice were irradiated 3X over 2 weeks with 1 MED ultraviolet radiation and treated daily with either acetone vehicle (V) or SB431542 (SB). (A) Percentage of $CD11b^+$ $Ly6G^-$, (B) $CD11b^+$ $Ly6G^+$ and (C) $F480^+$ $CD11b^+$ $Ly6G^-$ in viable $CD45^+$ leukocytes isolated from SKH1 skin. n=2 for controls and n=3-4 for UVB irradiated groups . Quantitation of (D) MPO^+ (representing neutrophils and (E) $BrdU^+$ cells in 7-10 random fields of view (FOV) from at least 3 stained sections per group. n=3-4 for UVB irradiated groups. Error bars = ±SEM. **P<0.05 relative to indicated group; **P<0.01 relative to indicated group.*

2.4.3 SB blocks UVB induced migration of dermal DC subsets to the SDLNs and in ear explant culture

Since dendritic cells are potential mediators of UVB-induced inflammatory responses, we next tested whether UVB increased Smad2 phosphorylation in cutaneous DC subsets, and if this could be inhibited by SB. At 2 hours post UVB irradiation we analyzed the p-Smad2 levels in different epidermal and dermal DC subsets by flow cytometry, gating dendritic cells as $CD45^{+}$ $MHCII^{high}$ $CD11c^{+}$ cells. UVB caused a small but statistically significant increase in percentage of p-$Smad2^{+}$ dermal dendritic cells from 7 percent to 9.5 percent and this increase was blocked by topical SB treatment (Figure 2.8). Importantly, there was no difference in the percentages of phospho-$Smad2^{+}$ Langerhans cells from the epidermis with UVB and SB did not decrease the p-Smad2 levels below the baseline (Figure 2.11 A, B).

To test the role of TGFβ1 pathway in UVB IR induced activation and migration of skin resident DCs, we analyzed migratory DC subsets in SDLN 72 hours post-UVB irradiation with or without SB pretreatment. After gating on $MHCII^{high}$ $CD11c^{+}$ cells for DCs migrating from the skin and excluding LN resident $MHCII^{high}$ $CD8^{+}$ DCs and $MHCII^{high}$ $B220^{+}$ DCs, we defined three DC subsets in the LN for analysis that responded to UVB: $CD207^{+}$ $CD103^{+}$ ($CD207^{+}$ dDCs), $CD207^{+}CD103^{-}$ and $CD207^{-}$ (Figure 2.9). At steady state, $CD207^{-}$ dDCs constituted the highest percentage of DCs in the LN.

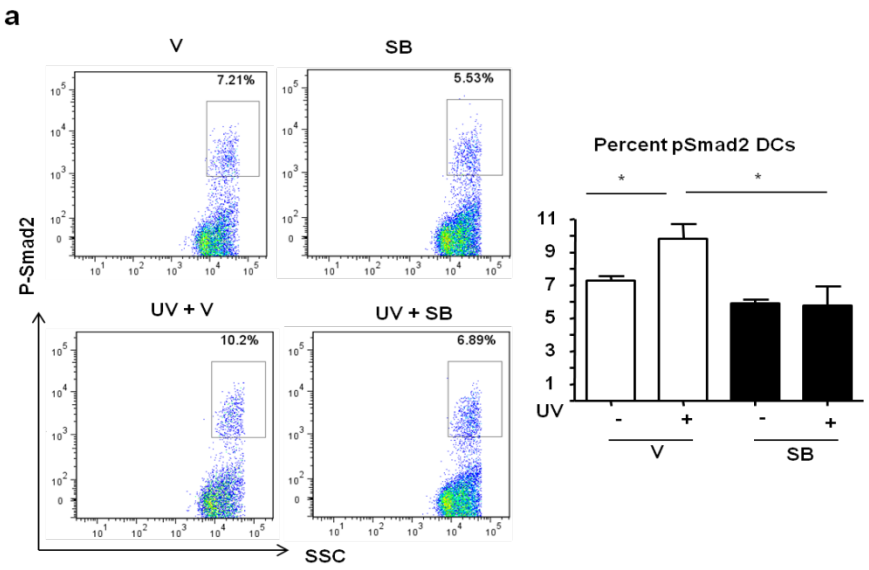

Figure 2.8: SB431542 blocks UVB-induced p-Smad2 induction in dermal DCs. (A) Representative FACS profile and quantitation of p-Smad2^{+} cells in MHCII^{Hi} CD11c^{+} dendritic cells (DCs) isolated from dermis of SKH1 mice 2 hours after UVB IR pretreated 1 hour with SB431542 (SB) or acetone (V). Error bars = ± SEM. *$P<0.05$ relative to indicated group*

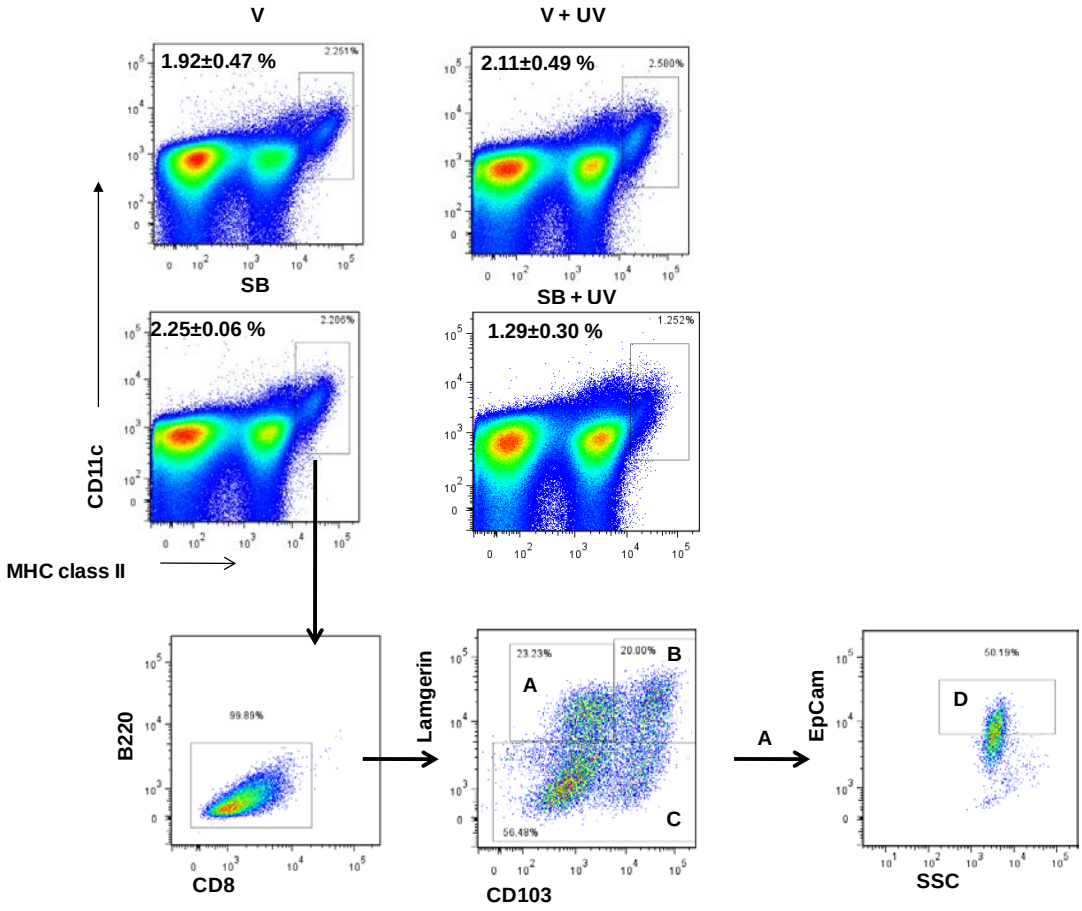

Figure 2.9: SB431542 reduces the DC percentages in the SDLNs after UVB compared to vehicle+UV group: Gating strategy to identigy (B) CD207^{+} CD103^{-} dermal DC subset, (C) CD207^{-} dermal DC subset and (D) Langerhans cells on inguinal skin draining lymph node cells gated on CD45^{+}.

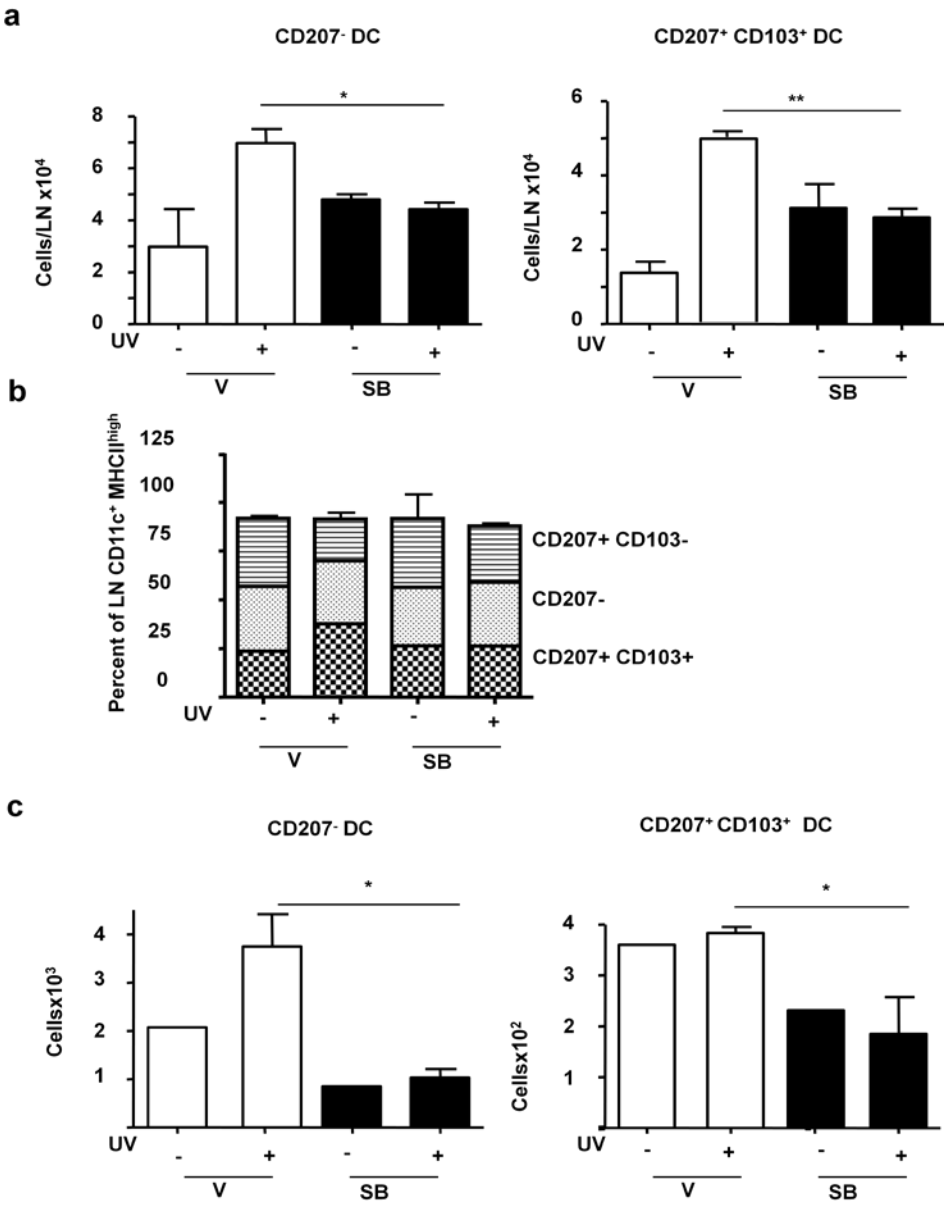

Figure 2.10: SB431542 blocks UVB-induced migration of dermal DC. (A) Numbers of CD207- and CD207+ CD103+ DC subsets in skin draining lymph nodes (SDLN) of SKH1 mice 72 hours post-UVB IR with 1 hr pretreatment with SB431542 or vehicle. (B) Percentages of indicated DC subsets in SDLN 72 hours after UVB IR with 1 hr pretreatment with SB431542 or acetone (V). (C) Effect of SB432541 on UVB IR-induced migration of dendritic cells in ear explant culture 72 hours post UVB. n=2-3, controls; n=3-4, UVB IR groups. Error bars = ± SEM. **P<0.05 relative to indicated group; **P<0.01 relative to control mice or indicated group.*

UVB IR significantly increased the total numbers of MHCII^{high} CD11c^{+} cells in the SDLNs from 0.21 to 0.4 million but in the presence of SB, MHCII^{high} CD11c^{+} cells only increased to 0.27 million following UVB IR. UVB increased the CD207^{-} subset in the SDLN 2.3 fold from $3x10^4$ to $7x10^4$ cells per LN and CD207^{+} dDCs subset 3.5 fold from $1.4x10^4$ to $5x10^4$cells per LN (Figure 2.10 A), and this increase was suppressed by SB. UVB also caused a selective increase in the proportion of the CD207^{+} dDC subset in the SDLN, which did not occur in SB treated mice (Figure 2.10 B). Similar results were obtained with chronic UVB, where mice were irradiated 3 times with UVB over 1 week, and daily with SB or acetone. Again, SB suppressed UVB-induced migration of dDC to the SDLN, but in this case chronic SB treatment also reduced the steady state levels of dDC in the SDLN (Figure 2.12 A, B).

To further analyze the dependence of UVB induced DC migration on TGFβ signaling, we prepared paired ear explant cultures from SKH1 mice treated with vehicle or SB 1 hour prior to UVB irradiation and measured migration of dendritic cell subsets into the culture media after 72 hours. Both baseline migration and UVB-induced migration of the CD207^{-} DC subset was suppressed by SB and baseline migration of the CD207^{+} dDC subset was suppressed by 50% (Figure 2.10 C). There was no effect of a single SB treatment on LC numbers in the SDLN with or without UVB, or on migration in an ear explant culture assay (Figure 2.13 A, B) but multiple SB treatments reduced the steady state numbers of LC in the SDLN (Figure 2.13 C). In addition, there was no significant difference in the LC numbers within the epidermis with SB treatment measured by MHCII immunoflourescence staining 48 hours after UVB (Figure 2.13 D, E).

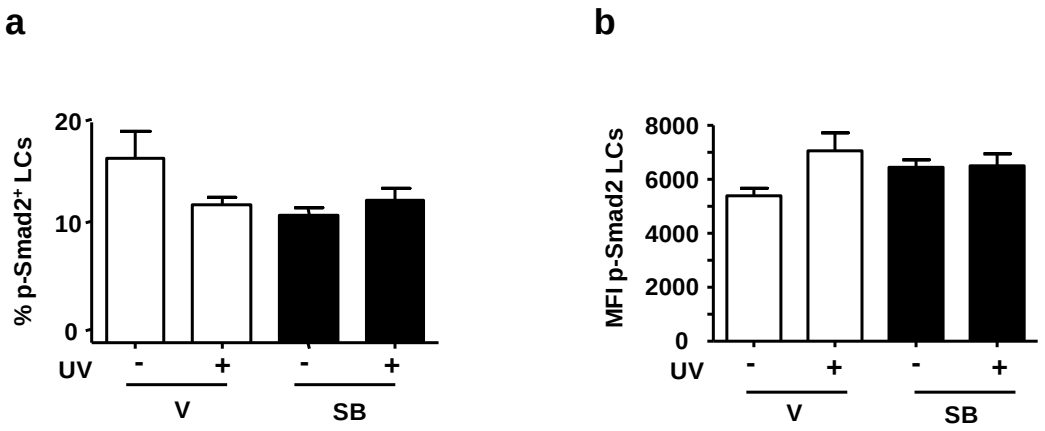

Figure 2.11: UVB and SB treatment do not change the phospho-Smad2 status of Langerhans cells. SKH-1 mice were pretreated with 10 μM SB43152 or acetone (V) for 1 hour followed by UVB treatment at MED dose. LCs were isolated from the epidermis 2 hours after UVB and stained for p-Smad2 and analyzed by FACs. (a)Percentages of phospho-Smad2^{+} LCs from UV treated or untreated epidermis gated on MHCII^{high} CD11c^{+} cells. (b) Mean Fluorescence Intensities (MFI) of phospho-Smad2 staining in LCs from UV treated or untreated epidermis

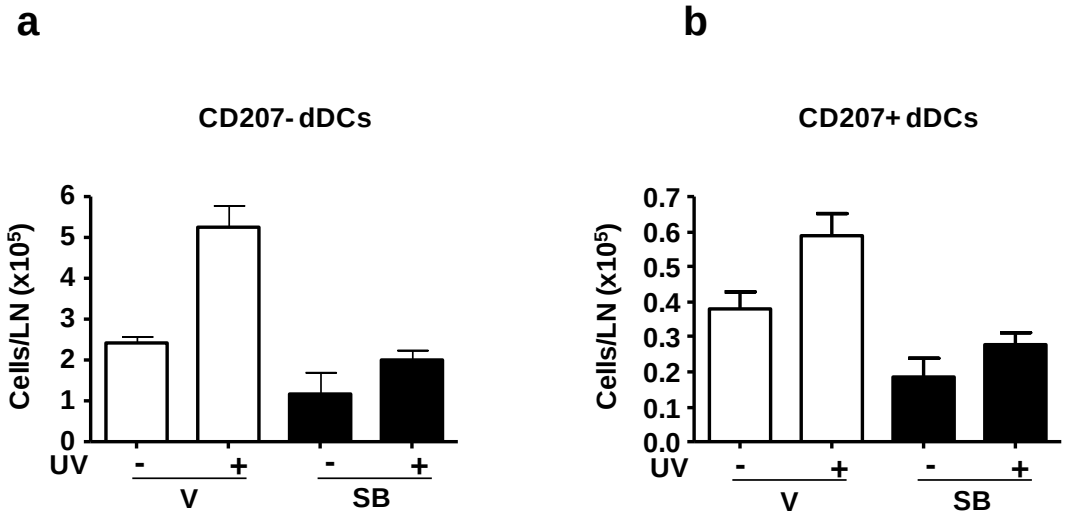

Figure 2.12: Topical ALK5 inhibition suppresses chronic UVB-induced migration of dermal DC subsets. SKH-1 mice were treated with 10 μM SB43152 or acetone (V) everyday with UVB treatment on alternative days for 1 week. Lymph nodes were harvested 40 hr post last UVB treatment and multicolor flow cytometry was used to immunophenotype dendritic cells by gating for MHCIIhigh CD11c+ cells, B220- and CD8-. (A) Cell numbers of the CD207- dDC subset in SDLN. (B) Cell numbers of the CD207+ CD103+ dDC subset in the SDLN

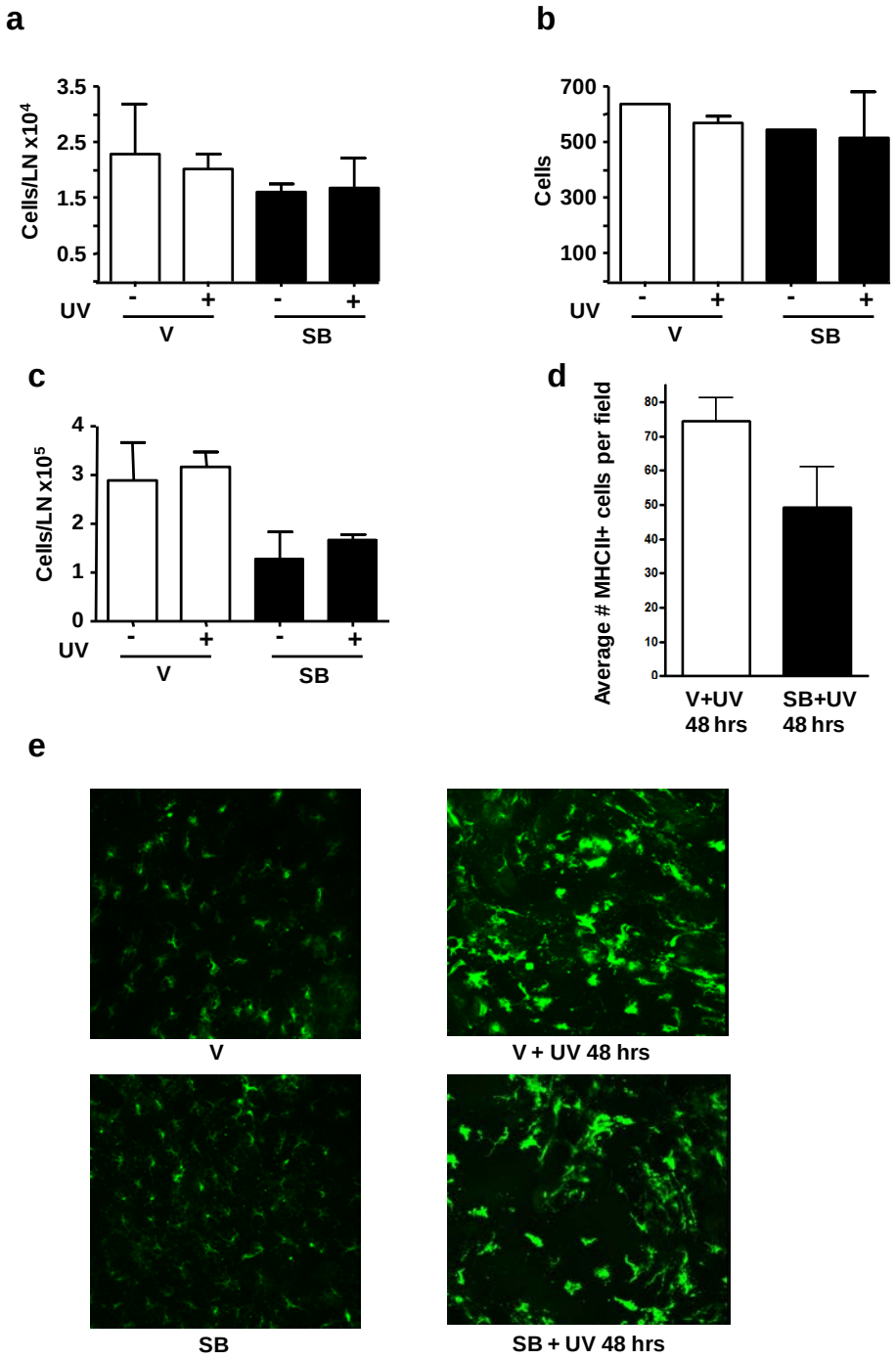

Figure 2.13: Effect of SB431542 on UVB induced migration of Langerhans cells(a) Numbers of $CD207^{+}$ $CD103^{-}$ DC subset in the SDLN of SKH-1 mice 72 hours after 1 MED UVB IR dose with 10 μM SB43152 or acetone (V) pretreatment. (b) Numbers of $CD207^{+}$ $CD103^{-}$ DC subset in media after 72 hours of ear explant culture. Ears were harvested immediately after a single MED UVB with 10 μM SB43152 or acetone (V) pretreatment split and floated in complete RPMI media and cultured for 72 hours. (c) LC numbers in SDLN of mice exposed to UVB IR for 1 week. SKH-1 mice were treated with 10 μM SB43152 or acetone (V) everyday with UVB treatment on alternative days for 1 week. Lymph nodes were harvested 40 hours post UVB IR and multicolor flow cytometry was used to immunophenotype the dendritic cells. (d and e) Epidermal sheets prepared 48 hours after UVB were stained for MHCII+ cells representing Langerhans cells. Quantification of the cells in 7-10 random fields of view (FOV) from 4 stained sections for controls and 6 stained sections for UV treated groups.

2.5 DISCUSSION

Acute UVB irradiation is a potent activator of cutaneous immune responses. Low dose UV is immunosuppressive, while UVB IR doses equal to or greater than the MED cause an inflammatory response [3;28;29], that is linked to accelerated tumor development [26;30;31]. Our previous studies showed that TGFβ1 has proinflammatory activities in skin carcinogenesis and immune responses [23-25]. Similar to effects on chemical skin carcinogenesis, the ALK5 inhibitor SB also suppressed UV-induced skin carcinogenesis, but enhanced the number of SCC and poorly differentiated SCC. We found no difference in initial p53 responses to UVB irradiation, UVB-induced epidermal proliferation, tumor volume at any time point or tumor cell proliferation at study end, but rather a significant decrease in T cell activation and $IFN\gamma^+$ CD4 and $CD8^+$ T cells, that was evident in skin and SDLN 72 hours after 1 UV exposure and maintained with 1 and 2 weeks of chronic UVB and in tumors 5 weeks after SB treatment ceased, suggesting that this difference is linked to suppression of tumor formation and potentially to enhanced progression. While it remains to be demonstrated directly, our results are consistent with a previous study demonstrating a tumor promoting role for IFNγ in the two-stage skin carcinogenesis model [32], but also for the well established role of IFNγ in tumor surveillance [33].

While it is possible that SB could be acting directly on T cells in skin or the SDLN, genetic blockade of TGFβ1 signaling in $CD8^+$ T cells enhances Tc17 cells [34], but we did not observe any significant change in $IL17^+$ T cells in SB treated mice. Instead our data support the idea that TGFβ1 signaling in dendritic cells is critical for this T cell response to UVB irradiation. There are multiple dendritic cell subsets in the skin but their role in responses to UV and effects of TGFβ1 are only partially understood. Langerhans cells have been implicated in UV induced

tolerance [35-37] possibly through UV inhibition of LC migration [37-39], although cyclopyrimidine dimer positive $CD207^+$ LCs have been detected in the SDLNs after chronic UV exposure [40]. The effect of UVB on dermal dendritic cell subsets is not clear as these earlier studies did not differentiate between migratory LCs and $CD207^+$ dDCs, and the effects on $CD207^-$ dDC have not been reported [41-43]. While *in vitro* studies have largely implicated TGFβ1 as an immunosuppressive cytokine for dendritic cells [12-14] and mouse models have shown an absolute requirement for TGFβ1 signaling in LC for epidermal residency and maintenance of an immature phenotype, [19-22], we recently showed that overexpression of TGFβ1 by keratinocytes causes rapid migration of dermal DC subsets and provoke an immunostimulatory response in a CHS assay [25]. In contrast to some studies implying UVB-induced inactivation of TGFβ1 signaling in skin [44-46], our results show that UVB irradiation at a MED, inflammatory dose activates TGFβ signaling as measured by increased pSmad2 and pSmad3 by immunoblotting of whole skin and pSmad2 levels by flow cytometry in dermal dendritic cells. Since we did not observe an increase in TGFβ1 transcript levels this suggests, at least at the level of whole skin, that pathway activation results from activation of latent TGFβ1, but this remains to be demonstrated conclusively.

We consistently observed a UV-induced increase in SDLN migration of $CD207^-$ and $CD207^+$ dDC with $CD207^-$ forming the major subset. Similarly, in ear explant cultures of UV irradiated mice there was an increase in the migration of $CD207^+$ dDC and $CD207^-$ subsets although as expected with the inflammatory setting of these cultures [47] the controls by themselves showed enhanced migration. Within the total DC subset migrating to the SDLNs, there was a selective increase in $CD207^+$ dDCs migration relative to the other DC subsets and this migration was significantly suppressed when TGFβ1 signaling was inhibited. $CD207^+$ dDCs

are important in skin immunity with roles in antigen presentation [48], generating adaptive immune responses, [8;49] and contact hypersensitivity [7]. Previous studies have reported TGFβ signaling to be nonessential for the steady state signaling and function of $CD207^+$ dDCs [8;21]. However, our results indicate that the UVB induced migration of $CD207^+$ dDCs as well as $CD207^-$ DCs was reduced with pharmacological inhibition of TGFβ signaling. Coupled with the significant suppression of UV-induced T cell activation in the SDLN and skin and suppression of cutaneous inflammation these data strongly implicate TGFβ1 signaling in $CD207^+$ dDC in UV-induced cutaneous inflammatory immune response.

Given the importance of TGFβ1 signaling in LC biology and epidermal residency as well as effects of low dose UV on LC, it is surprising that UVB at 1 MED dose or short term SB treatment had minimal effect on LC migration although this is consistent with the lack of change in pSmad2. As with the other DC subsets repeated SB dosing did impact LC numbers in the SDLN in the absence of UVB. It is possible the high UVB doses that we have used causes apoptosis in LCs [38;50] which masks any increase in migration over steady state. However, we did not observe significant differences in LC numbers in epidermal sheets at 24 hr or 48 hr post UVB between vehicle and SB treated skins.

A critical question is whether TGFβ1 signaling is important only for steady state migration of dDC, and the effect on UVB induced migration is superimposed on that migration defect, or if activation of TGFβ1 signaling is essential for UVB induced migration. The observation that approximately 7% of dermal DC were $pSmad2^+$ in the steady state is consistent with the former hypothesis. In addition, multiple applications of SB altered steady state DC numbers while a single application of SB did not cause significant reduction in steady state DC numbers in the SDLN even though UVB induced migration was blocked. These results suggest

that long term inhibition of TGFβ1 signaling in dermal DC affects steady state migration while acute inhibition with SB does not. It is also possible that the difference in dermal DC migration is due to an indirect effect through synergy with other inflammatory pathways or the migrated dermal DCs with blocked TGFβ1 signaling are deficient in T cell activation and this contributes to the reduced inflammatory response.

Taken together these results suggest a model in which TGFβ1 rather than acting as an immunosuppressive cytokine, promotes dermal dendritic cell migration in response to UVB irradiation and in conjunction with subsequent T cell activation and cutaneous inflammation promotes UV induced skin tumor formation.

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Chapter 3
Abrogation of TGFβ signaling in dendritic cells leads to their reduced lymph node migration and contact hypersensitivity responses with Ultraviolet B irradiation

3.1 ABSTRACT

Transforming Growth Factor-β1 (TGFβ1) is an important immunoregulatory cytokine in the skin with key roles in development and function of immune subsets such as dendritic cells (DCs) and T cells. Importantly, TGFβ has been shown to be required for the development of epidermal DCs-Langerhans cells (LCs) but not for dermal DC subsets- $CD207^-$ dDCs and $CD103^+$ $CD207^+$ dDCs. However, the role of TGFβ signaling in the immune activation for these subsets is not clear. Mice expressing a dominant negative TGFβ Type II receptor (DNR) specifically in $CD11c^+$ cells displayed reduced steady-state migration of all three DC subsets compared to WT C57Bl/6 mice. Acute Ultraviolet B (UVB)-induced migration of dermal DC subsets, $CD207^-$ and $CD207^+$ dDCs in DNR to skin-draining lymph nodes (SDLNs) was reduced in DNR compared to WT mice, which correlated with a reduced percentage of p-$Smad2^+$ and $CD86^{high}$ dermal DCs in DNR mice following UVB. There was no significant difference in UVB-induced migration of LC subset between DNR and WT mice. Consistent with this, the UVB-induced migration of $CD207^-$ and $CD207^+$ dDCs subsets in a chronic UVB model as well as migration of $CD207^-$ subset in an ear explant assay was suppressed in DNR when compared to WT mice with no difference in LCs. In addition, ear thickness response measured in a contact hypersensitivity model (CHS) with and without UVB, was reduced in DNR mice compared to WT with an associated reduction in activation status and IFNγ, IL17 and IL2 cytokine profile of $CD4^+$ and $CD8^+$ T cells. Together, these results suggest that TGFβ signaling in DCs is important for UVB-induced skin inflammation and for optimal response to hapten sensitization in a CHS model, both mediated through the DC-T cell arm of the immune response.

3.2 INTRODUCTION

Ultraviolet radiation (UVB) is a cutaneous immunomodulator that can activate dendritic cell (DC) subsets in the skin and is important in skin pathologies such as skin cancer and allergic contact dermatitis [1-3]. There are multiple subsets of DCs that are in steady-state migration between the skin and skin draining lymph nodes (SDLNs). The function of steady-state DC migration is to perform constant immunosurveillance of the skin for altered or foreign antigens and establish peripheral tolerance in the absence of any foreign antigen by maintaining T cells in naïve state in the SDLNs. Langerhans cells represent the only $MHCII^+$ DC subset in the epidermis and efficiently acquire antigens in the periphery and migrate to the lymph nodes where they present antigens to naïve T cells [4;5]. In the dermis, the two distinct DC (dDC) subsets, $CD207^-$ dDCs and $CD103^+$ $CD207^+$ dDCs also respond to UVB irradiation and can participate in immunosurveillance and antigen presentation in the skin [6]. $CD103^+$ dermal DCs representing 3 % of total dermal DCs, have been implicated as the sole DC subset capable of cross-presentation and $CD8^+$ T cell activation in the draining lymph nodes [6-14]. Cross-presentation is essential for $CD8^+$ T cell priming and involves the presentation of exogenous as opposed to endogenous antigens on MHC class I molecule. The $CD207^-$ dDC constitute the majority of dermal DCs and are a heterogeneous subset whose role in cutaneous antigen presentation has not been established [6;11].

Transforming growth factor β1 (TGFβ1) is an important cytokine with effects on several key cells of the immune system and its role in cutaneous immune responses as well as maintenance of steady state immunotolerance is not completely understood. TGFβ1 can be proinflammatory under specific conditions by promoting Th17 polarization as well as immunosuppressive by supporting T regulatory cell development or directly inhibiting dendritic cell function [15-18]

[19;20]. TGFβ1 has been shown to be important for LC ontogeny whereas it is dispensable for other $CD207^+$ dermal DC subsets in steady state, however, its role in immune activation is largely unknown [21-23]. To study the significance of TGFβ signaling for DC immunoresponsiveness, we used a mouse model in which a dominant negative TGFβ Type II receptor (DNR) is expressed specifically in $CD11c^+$ cells, representing DCs. [24]. DC or T cell specific knockdown of TGFβ1 signaling increases the susceptibitily to autoimmune disease with spontaneous wasting disease in T cell specific DNR mice and DC specific ablation of TGFβ type II receptor acting through dysregulation of $T_{regulatory}$ cells in both cases [25;26]. However, CD11c DNR mice do not display overt pathology. Here, we investigate the effect of DC-specific TGFβ1 pathway inactivation on the activation and migration of the different skin resident DC subsets to the SDLNs in response to UVB IR. In order to test the effect of inactivation of DC TGFβ1 signaling on downstream T cell function, we took advantage of experimental Contact hypersensitivity (CHS) model, which is a T cell driven pathology.

CHS is a type IV delayed-type hypersentivity response and is an experimental model for clinical human allergic contact dermatitis [27;28]. Allergic contact dermatitis is caused by an inappropriate cutaneous immune response to chemicals called contact allergens. When these chemicals enter the skin they bind to cutaneous proteins to generate potently immunogenic antigens. This process is called haptenization. A number of inorganic chemicals, jewellery and some cosmetics can act as contact allergens in select individuals.

The CHS model consists of two temporally and mechanistically distinct immune phases to achieve the optimal CHS response: the sensitization phase and the elicitation phase. [29]. In the sensitization phase, a topically administered chemical such as 2,4-Dinitro-1-Flouro-Benzene (DNFB) acts as the hapten. The hapten or haptenated self-proteins act as “danger signals” and

are recognized by pattern recognition receptors primarily Toll-like receptors TLR2, TL4[30] and NOD-like receptors [31] in innate immune cells and lead to a potent inflammatory reaction characterized by the secretion of several pro-inflammatory cytokines including interleukin-1β (IL-1β). Keratinocytes also express most functional TLRs [32] and are known to secrete IL-1β, IL-18 and TNFα which play a role in DC maturation in response to haptens [33-35]. Haptenated self-proteins are then taken up by skin-resident DCs, which become activated and migrate out through the afferent lymphatics to the skin-draining lymph nodes where they interact with naïve T cells. IL-12 has been shown to be important although is not required for this event. [30] All these events comprise the sensitization phase and are mediated primarily by skin-resident dendritic cells.

The main advantage of the CHS model is the ability to dissect out the role of dendritic cells versus activated T cells in an immune response. The hapten-specific activated CD4 and CD8 T cells are the main effectors of the CHS response, but the dendritic cells function as key mediators of the initial sensitization event, by trafficking the DNFB to the draining lymph nodes and priming the hapten-specific naïve CD4 and CD8 T cells. Once activated, these hapten-specific T cells emigrate from the lymph nodes and enter the bloodstream and recirculate in the blood reaching secondary lymphoid organs [29].

The final event in the process is the resensitization/elicitation phase after 5-7 days following the initial exposure to the hapten, when the T cells in the lymph node acquire potent proliferative properties [36;37]. There is a recall response of the memory T cells to the site of resensitization/challenge where the T cells interact with hapten-primed skin DCs. [29]. The activated effector CD8 T cells secrete copious amounts of proinflammatory cytokines such as interferonγ (IFNγ) [36;38] and elicit the adaptive immune response with cytolysis of haptenized

cells mediated by perforin/granzyme and keratinocyte apoptosis mediated by Fas/FasL binding, which manifests as a visible rash with erythema, edema, papules and thickening of skin in clinical cases of contact dermatitis. The elicitation phase takes 24-48 hours to develop in mice after reexposure to hapten. [29;39-42]. During the elicitation phase, keratinocytes in addition to antigen-presenting DCs and other endothelial cells in the dermis play an important role in presenting MHC-class I associated hapten complexes to activated memory CD8 CTLs, which are recruited into the skin at the site of challenge. In turn, keratinocytes act as important targets of CTL induced apoptosis. [43]

Key features of the CHS response are 1) the inflammatory response is systemic and the skin is challenged on a site (either footpad or ear) distant from the site of initial sensitization 2) the inflammatory response is antigen-specific and is dependent on antigen-specific cytolytic $CD8^+$ and $CD4^+$ T helper cells. [27].

In order to test whether the UVB induced reduction in the migratory subsets of $CD207^-$ dDCs and $CD207^+$ dDCs in DNR mice leads to any functional differences in a $INF\gamma^+$ $CD8^+/CD4^+$ T cell driven pathology such as CHS, we performed a CHS assay in the presence and absence of UVB. Here, we show that TGFβ signaling in DCs is important for the UVB mediated activation and migration of skin resident DC subsets. We further show that defective TGFβ signaling in DCs is linked to reduced lymph node effector differentiation of T cells and $IFN\gamma^+$ CD4 and CD8 T cells in response to UVB and reduced ear thickness in a CHS bioassay. Our results implicate a common mechanism of immunomodulation to foreign inflammatory stimuli such as UVB and hapten stimulation in CHS mediated through TGFβ signaling in dermal DCs .

3.3 MATERIALS and METHODS

3.3.1 Mice: CD11c-dnTGFβRII transgenic mice expressing a dominant negative human transforming growth factor beta receptor II gene under the control of a CD11c (*Itgax*) promoter were obtained from Jackson Laboratories and were genotyped for the transgene as described [24] and age matched (6-9 weeks) nontransgenic (WT) and transgenic littermates were used for experiments. All animals were treated according to approved Institutional Animal Care and Use (IACUC) protocols.

3.3.2 UVB Irradiation: CD11c-DNR transgenic mice on C57BL/6 background were exposed to MED of 5.4 kJ/m2 from UV bulbs (American Ultraviolet Light Co.) covered with cellulose triacetate (KODAK) to filter out UVC radiation, and produce UV wavelengths between 280-320 nm as described [44]. Irradiance was measured using a UVX radiometer (UVP, Upland Ca). Mice were shaved 48 hours prior to UVB irradiation.

3.3.3 Antibodies: The following antibodies were purchased from Ebioscience, San Diego, CA: anti-CD16/32 (93), and APC eFluor 750-anti-CD45 (30-F11), FITC- and eFluor 450-anti-MHCII (M5/114.15.2), PE- and Alexaflour 700-anti-CD11c (N418), FITC-anti-CD4 (GK1.5), PECy5-anti-CD8α (53-6.7), PE-anti-CD103 (2E7), PECy7-anti-B220 (RA3-6B2), PercpCy5.5-anti-CD11b (M1/70), PE-anti-CD62L (MEL-14), and PECy5-anti-CD44 (IM7). The following antibodies were purchased from BD Pharmingen, San Diego, CA: PE-anti-CD45 (30-F11), Alexaflour 700-anti-CD86 (GL1), and PECy7-anti-IFNγ (XMG1.2). The following antibodies were purchased from BioLegend: PercpCy5.5-anti-CD40 (3-23), PECy5-anti-CD197 CCR7 (4B12). Alexa 568-anti-Epcam (G8.8) and Alexa 647-anti-CD207 (L31) antibody conjugates were generated as previously described (Gaiser, 2012 14003 /id).The following antibodies were purchased from Cell Signaling technology: p-Smad2 (#3101 for western blotting; #9510 for

FACs analysis), Smad2/3-3102, p53-2524, GAPDH-2118, p21-6246 (Santa Cruz), Actin-1501 (Millipore). Antibodies used for immunohistochemistry: CD45 (BD Pharmingen#550539), CD3-ε (M-20) (Santa Cruz-1127)

3.3.4 Flow Cytometry: Dendritic cells were isolated from the inguinal lymph nodes, the epidermis and dermis as described [45]. Single cell suspensions of DCs were incubated with CD16/32 followed by staining for extracellular surface antigens. For anti-CD207 staining, cells were fixed and permeabilized using fixation/permeabilization buffer (Ebioscience) and incubated with anti-CD207 antibody in 0.2% saponin buffer. For phospho-Smad2 staining, the cells were fixed with 2% paraformaldehyde for 10 minutes followed by staining for surface antigens. Cells were then permeabilized by 90% methanol for 30 minutes and then stained for p-Smad2. Cells were analyzed using a Fortessa LSRII (BD Biosciences, San Jose, CA). Single-cell suspensions were prepared from the inguinal lymph nodes and the UV-exposed dorsal skin as described [46]. Following incubation with PMA/ionomycin and Brefeldin A (eBioscience, San Diego, CA) for 4.5 hours at 37 °C, the cells were stained for surface antigens and then fixed with 4% paraformaldehyde, and permeabilized with 0.2% saponin buffer and stained for intracellular IFNγ. Cells were acquired on FC500 (Beckman Coulter, Indianapolis, IN) and analyzed using the FlowJo software (Tree Star, Ashland, OR).

3.3.5 Ear Explant Cultures: Ears of CD11c-DNR mice and WT mice were excised immediately after a single MED dose of UVB. They were then rinsed in 70% ethanol, and then in a solution containing 200 IU ml^{-1} penicillin and 200 μg ml^{-1} streptomycin for 5 minutes to make them sterile. Only the dorsal halves of the ears were presumed to be exposed to UVB. The dorsal and ventral halves were split from the attached cartilage and the dorsal halves were cultured in complete RPMI media (10% fetal calf serum) for 72 hours at 37 °C. The dendritic cell

populations migrate out of the ear skin and into the media mimicking migration to the SDLN from the skin *in vivo*. The culture media was then harvested to analyze the cells that migrated from the explant, enumerated, stained and analyzed by flow cytometry similar to 3.3.4.

3.3.6 Contact hypersensitivity

WT and CD11c-DNR mice were sensitized with 25 µl of 0.5% DNFB (Sigma) in 4:1 solution of acetone/olive oil on shaved abdominal skin. The mice were challenged 5 days later with 30 µl of 0.3% DNFB on a distant site from sensitization-on both sides of the left ear while the right ear was treated with the vehicle and served as an internal control for baseline thickness. In experiments with UVB irradiation, the shaved abdomen was exposed to single UVB dose of 5.4 kJ/m^2 before sensitization with 0.5 % DNFB with challenge 5 days later. Ear thickness measurements (increase in ear thickness relative to the vehicle-treated ear) were done 24 hours following challenge, in a blinded fashion. Ear tissue was also collected for histology at 24 hours following challenge.

3.3.7 Statistical Analysis: All statistical analyses were performed using the GraphPad Prism software and the values are expressed as Mean±SEM (GraphPad Software, La Jolla, CA). A two-tailed Student's *t*-test was performed to compare the groups. P values of significance were represented as: * $p<0.05$, ** $p<0.01$.

3.4 RESULTS

3.4.1 Blockade of TGFβ signaling in skin CD11c^{+} DCs suppresses UV-induced migration to the skin draining lymph node (SDLN)

We used mice expressing a CD11c^{+} promoter driven dominant negative TGFβ type II receptor transgene (CD11c-DNR) [24] to block TGFβ1 signaling in dendritic cells and determine effects on their UVB response. To test the effect of UV irradiation on the TGFβ1 pathway, we treated the skin of 7 week-old CD11c-DNR mice with UVB at the minimum erythema dose (MED) of 5400 J/m2. 2 hours after UVB IR, there was an increase in the percent of p-Smad2^{+} dermal DCs similar to the UVB treatment of SKH1 mice described in Chapter 2 (Figure 3.1). However, the difference was not significant due to inadequate number of control mice used in the experiment.

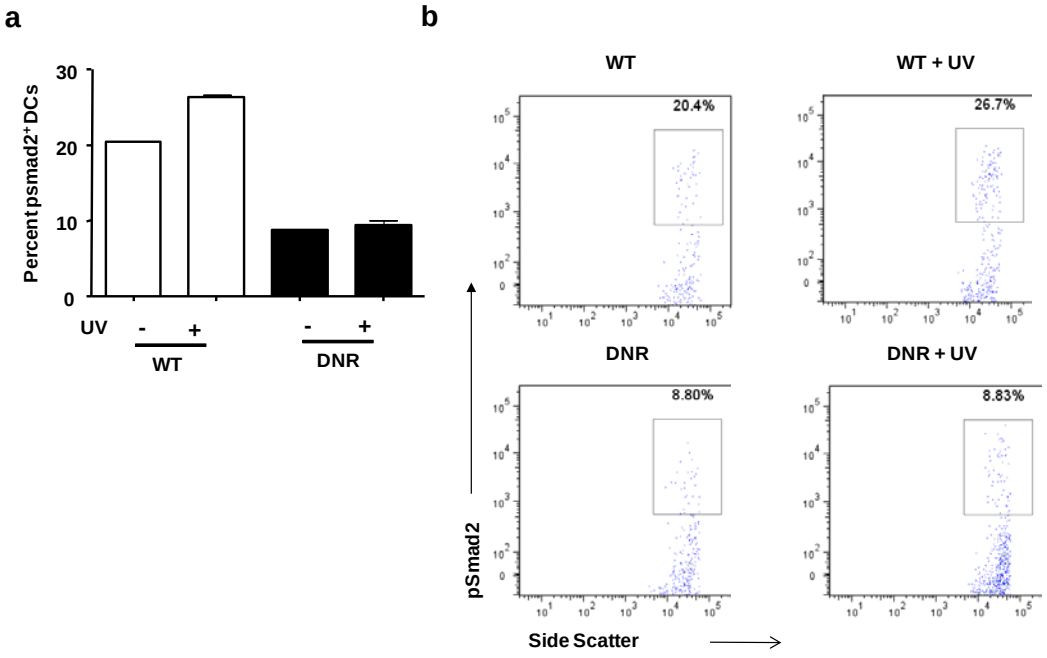

Figure 3.1: UV-induced Smad phosphorylation is blocked in CD11c-DNR dermal DCs. (a) Quantitation and (b) representative FACs profile of p-Smad2^{+} subset of MHCII^{Hi} CD11c^{+} dendritic cells (DCs) isolated from the dermis of WT and CD11cDNR mice 2 hours after UVB IR

In DNR mice, the steady state p-Smad2 levels were markedly less and the UVB-induced increase in p-Smad2 was completely blocked.

To test if CD11c-specific genetic blockade of TGFβ1 blocked the UVB-induced activation and migration of skin resident DCs similar to pharmacological inhibition, we analyzed migratory DC subsets in the SDLNs 72 hr post-UVB irradiation. There was an increase in percentage of MHCII^{high} CD11c^{+} cells with UVB IR which was blocked in the DNR although

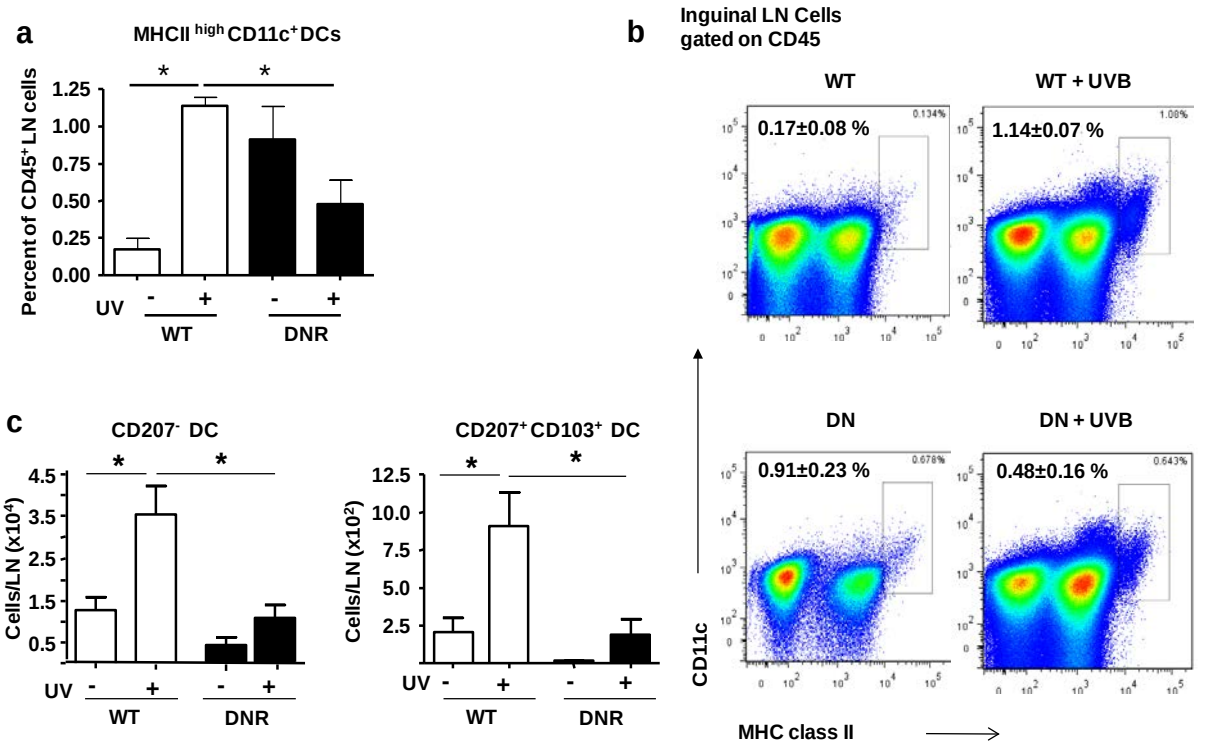

Figure 3.2: Blockade of TGFβ signaling in dermal CD11c+ cells suppresses UV-induced migration (a) Percentages and (b) representative FACs profiles of MHCII ^{high} CD11c^{+} DCs out of CD45 $^{+}$ cells in the Skin draining lymph nodes (SDLN) of wildtype (WT) or CD11c-DNR transgenic mice (DNR) 72 hours after ultraviolet irradiation (UVB IR). (c) Numbers of CD207- and CD207+ CD103+ Dendritic cells (DC) in SDLNs of WT or CD11c-DNR after UVB IR. Error bars = ±SEM. *P<0.05 relative to indicated group; **P<0.01 relative to indicated group.

there was some variability in the steady state percentages (Figure 3.2 a, b). After gating on MHCII^{high} CD11c^{+} cells and excluding LN resident MHCII^{high} CD8^{+} DCs and MHCII^{high} B220^{+}

DCs, the numbers and percentages of the three skin DC subsets: $CD207^-$, $CD207^+CD103^+$ ($CD207^+$ dDCs), $CD207^+CD103^-$ were analyzed in the SDLNs. In untreated steady state conditions the number of $CD207^+$ dDC and $CD207^-$ DC subsets in the SDLN in DNR mice were reduced to 10 % and 30% respectively compared to wildtype (WT) controls (Figure 3.2 c).

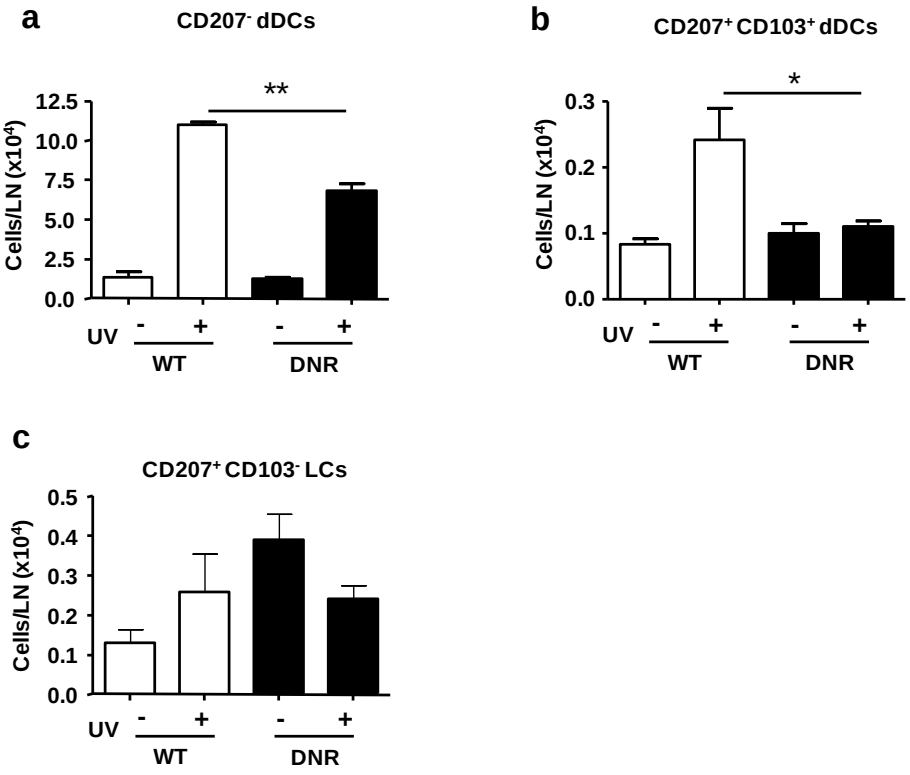

Figure 3.3: Chronic UVB-induced migration of dermal DC subsets is suppressed in CD11c-DNR mice. WT and CD11c-DNR mice were treated with UVB treatment on alternative days for 1 week. Lymph nodes were harvested 40 hr post last UVB treatment and multicolor flow cytometry was used to immunophenotype dendritic cells by gating for $MHCII^{high}$ $CD11c^+$ cells, $B220^-$ and $CD8^-$. (a) Cell numbers of the $CD207^-$ dDC subset in SDLN. (b) Cell numbers of the $CD207^+$ $CD103^+$ dDC subset in the SDLN. (c) Cell numbers of the $CD207^+$ $CD103^-$ dDC subset in SDLN. Error bars = ±SEM. *P<0.05 relative to indicated group; **P<0.01 relative to indicated group.

Although the fold increase in $CD207^+$ dDC and $CD207^-$ DC was similar in UVB treated DNR mice, the total numbers of each subset in the SDLN after UVB IR was significantly reduced by 2

and 3.5 fold, respectively (Figure 3.2c). We obtained similar results with chronic UVB in which mice were irradiated 3X with UVB on alternate days over 1 week.

The $CD207^-$ DC subset migration increased by 6-fold and the $CD207^+$ dDC subset increased by 1.5 fold in the UV-treated WT mice. There was a 3-fold decrease in $CD207^-$ DC subset and 1.5 fold decrease in the $CD207^+$ dDC subset in the DNR mice with UVB treatment compared to the UV-treated WT mice (Figure 3.3). Importantly, there was no meaningful difference in the $CD207^+$ $CD103^+$ DC (LC) numbers in the lymph node with chronic UVB.

3.4.2 CD11c-specific blockade of TGFβ1 signaling suppresses UV-induced activation and migration in an *ex vivo* ear explant assay

To further analyze the requirement of TGFβ1 signaling for UVB induced migration, we performed paired ear explant cultures with UVB and measured the migration of skin DC subsets into the media after 72 hours. Although there was no significant difference in steady state or UVB-induced migration of the $CD207^+$ $CD103^+$ DC subset between DNR and WT mice in ear explants cultures, steady state $CD207^-$ DC migration was decreased in DNR ear explants and UVB-induced migration was blocked (Figure 3.4).

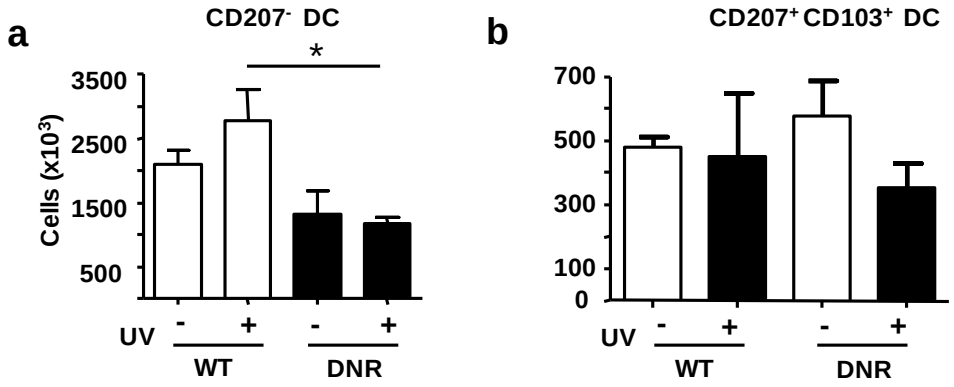

Figure 3.4: UV-induced migration of $CD207^-$ subset is suppressed in ear explant culture of CD11c-DNR mice.

Ears of CD11c-DNR (DNR) and wildtype (WT) mice were harvested immediately after a single MED UVB and split and floated in complete RPMI media and cultured for 72 hours. Cell numbers of the (a) $CD207^-$ and (b) $CD207^+$ $CD103^+$ subsets in media of ear explant culture are shown. *$P<0.05$ relative to indicated group.

There was a reduction in steady state numbers of LC in the SDLN in the CD11c-DNR mice but a similar fold increase in response to UVB IR (Figure 3.5a). LC migration in ear explants cultures was not significantly different between CD11c-DNR and WT mice (Figure 3.5b).

Finally, to test whether defective TGFβ1 signaling prevented activation of dDC we analyzed expression of CCR7 and CD86 24 hours after UVB as increased expression of these is associated

with activation of skin DC in response to inflammatory stimuli [47] [48] [6]. Although CCR7 was undetectable, we observed a UVB IR-induced increase in CD86^{high} DC in the dermis that was significantly reduced in CD11c-DNR mice (Figure 3.6). This suggests that UV-induced activation of dDC is impaired by blocking TGFβ1 signaling in these cells.

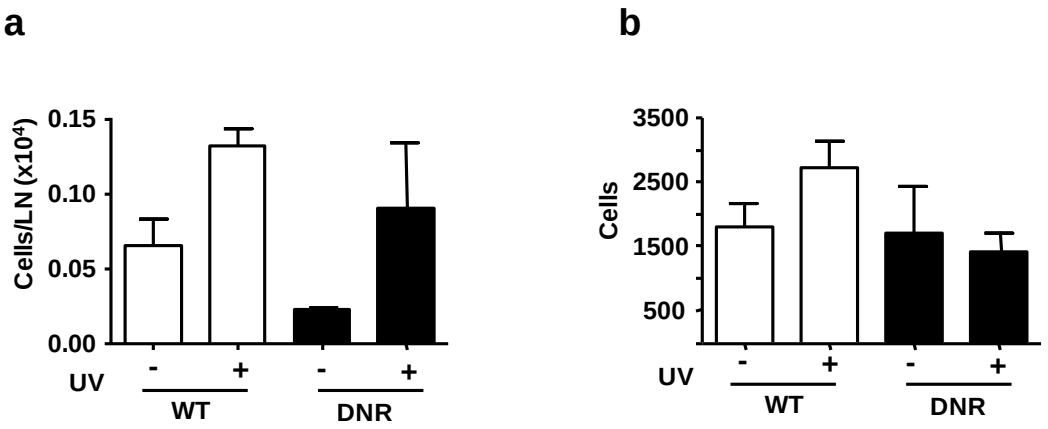

Figure 3.5: Langerhans cell migration in UVB irradiated wildtype and CD11c-DNR mice
(a) Lymph node cell numbers of CD207^{+} CD103^{-} DC subset in CD11c-DNR transgenic mice (DNR) or wildtype (WT) 72 hours post UVB. (b) Cell numbers of CD207^{+} CD103^{-} DC subset in media after 72 hours of ear explant culture with and without UVB irradiation.

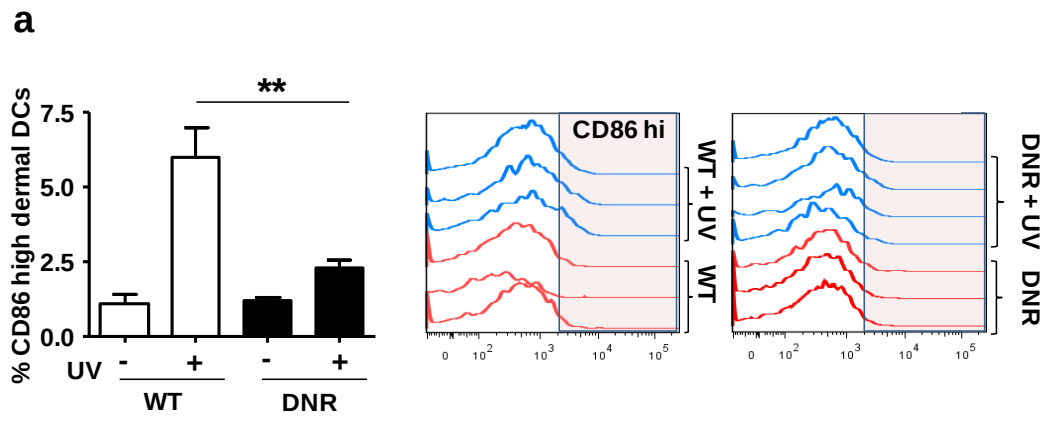

Figure 3.6: Blockade of TGFβ signaling in dermal CD11c+ cells suppresses UV-induced activation in dermis

(A) Expression of activation marker CD86 in wildtype and CD11c-DNR dermal DCs 24 hours after UVB IR. Mean Fluorescence Intensity (MFI) of CD86 was established by flow cytometry and the percentage of CD86^{High} subset of dermal DCs was plotted for the different treatment groups. n=2-3 for controls and n=3-4 for UVB IR groups. Error bars = ±SEM. *P<0.05 relative to indicated group.

3.4.3 Genetic Blockade of DC-specific TGFβ1 signaling suppresses chronic UVB-induced activation of T cells in the skin draining lymph nodes

To analyze if genetic blockade of TGFβ1 signaling in DCs, resulted in suppression of T cell activation, which is the key downstream event for UVB induced cutaneous inflammation, we irradiated WT and CD11c-DNR mice with UVB at the dose of 5.4 kJ/m^2 3X on alternative days for 1 week. The SDLNs were harvested and analyzed for T cell markers by flow cytometric analysis.

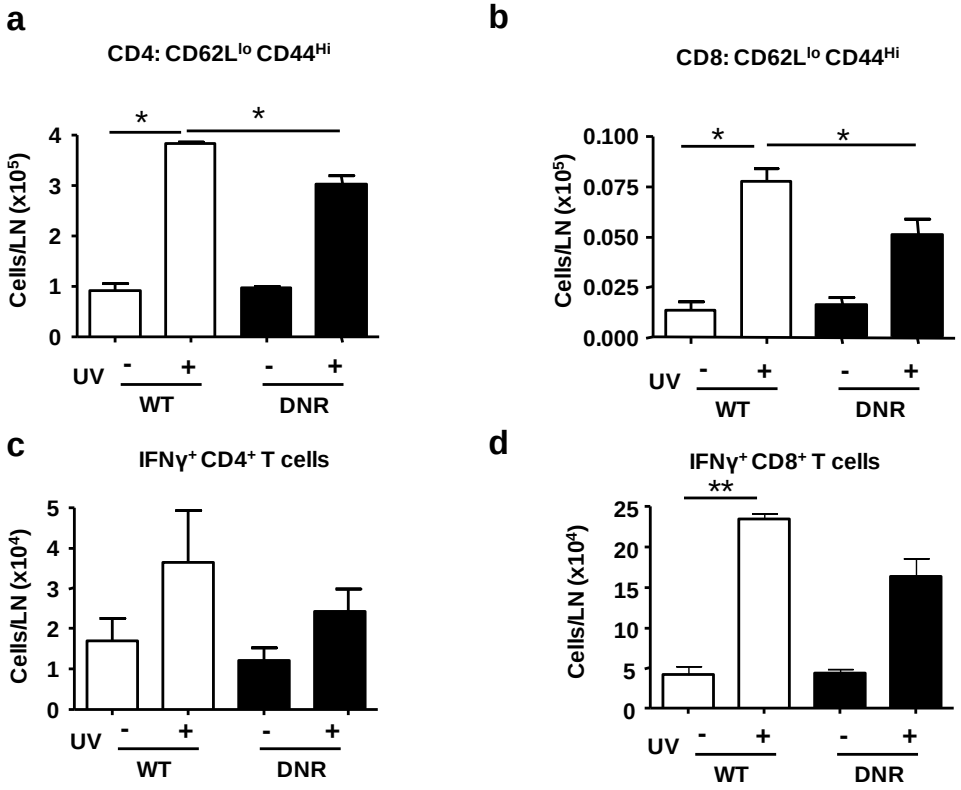

Figure 3.7: DC-specific defect in TGFβ1 signaling suppresses UVB-induced T cell activation in lymph node

WT and CD11c-DNR mice were treated with 3X UVB treatment on alternative days for 1 week. Lymph nodes were harvested 40 hr post last UVB treatment and multicolor flow cytometry was used to T cell populations. (a and b) Numbers of effector CD4$^+$ and CD8$^+$ T cells (CD44^{Hi} CD62L^{Lo}) from SDLN of mice. (c and d) Numbers of IFNγ$^+$ CD4$^+$ and CD8$^+$ T cells in SDLN of mice treated as in a. n=3 for controls and for UVB irradiated groups . . Error bars = ±SEM. *P<0.05 relative to indicated group.

There was a significant increase in $CD44^{+}CD62L^{low}$ central memory $CD4^{+}$ and $CD8^{+}$ T cells from by 3-fold and 6-fold respectively (Figure 3.7 a, b). In DNR mice suppressed these respective populations by 25 % and 31 %. UVB also caused a small increase in $IFN\gamma^{+}CD4^{+}$ T cells and a 5-fold increase in $IFN\gamma^{+}CD8^{+}$ T cells, which was reduced in DNR mice (Figure 3.7 c, d). These results show that defective DC TGFβ1 signaling in DNR mice reduces the UVB-induced activation of T cell populations in the skin-draining lymph nodes which is important for cutaneous inflammation.

3.4.4 DC-specific defect in TGFβ1 signaling suppresses the synergistic increase in Contact Hypersensitivity (CHS) responses with UVB IR

Since blockade of TGFβ signaling in DCs reduced their steady state migration to the SDLNs, we hypothesized that CHS responses would also be reduced in DNR mice. After initial sensitization with 0.5 % DNFB and then challenge with 0.3 % DNFB 5 days later, the difference in ear thickness was measured between the DNFB and vehicle treated ear 24 hours post challenge. Then, the mean ear thickness in hapten-treated WT group was normalized to 1 fold. Compared to the WT mice, there was a small but statistically significant reduction in ear thickness with defective DC TGFβ signaling in DNR mice in CHS model (Figure 3.8).

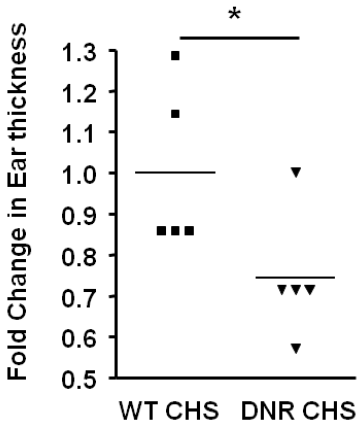

Figure 3.8: Defective TGFβ1 signaling in DCs suppresses hapten responses in a Contact Hypersensitivity (CHS) bioassay

WT and CD11c-DNR mice were sensitized with 0.5 % DNFB. 5 days after sensitization, the ears were challenged with vehicle and 0.3 % DNFB and difference in ear thickness was analyzed 24 hours post challenge. . Error bars = ±SEM. *P<0.05 relative to indicated group.

As UVB IR increased the migration of dermal DC subsets to the SDLN, we hypothesized that UVB would significantly enhance the response to hapten in the CHS bioassay. To assess the role of TGFβ signaling on UV-induced dermal DC migration, we performed a single UVB treatment

before sensitization with 0.5 % DNFB in WT and DNR mice. When control (wildtype) mice were challenged 5 days later, there was a statistically significant increase of 0.4 fold in ear thickness with UVB irradiation (Figure 3.9). Consistent with increased ear thickness, there was a

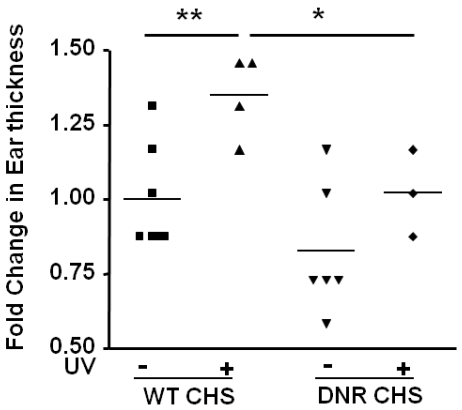

Figure 3.9: DC-specific defect in TGFβ1 signaling suppresses the synergistic increase in Contact Hypersensitivity (CHS) responses with UVB IR

WT and CD11c-DNR mice were irradiated with 1 MED UVB before sensitization with 0.5 % DNFB. 5 days after sensitization, the ears were challenged with vehicle and 0.3 % DNFB and difference in ear thickness was analyzed 24 hours post challenge. Error bars = ±SEM. *P<0.05 relative to indicated group; **P<0.01 relative to indicated group

small increase in the CD4 and CD8 memory effector population with UVB (Figure 3.10 a, b). In addition, there was a marked increase in $IFN\gamma^{+}CD4^{+}$ T cells from 0.006 to 0.04 million and $IFN\gamma^{+}CD8^{+}$ T cells from 0.08 to 0.25 million (Figure 10 c, d). There was also a 3.3 fold increase in $IL17^{+}CD4^{+}$ T cells, 2.5 fold increase in $IL17^{+}CD8^{+}$ T cells, 4.5 fold increase in $IL2^{+}CD4^{+}$ T cells and 5 fold increase in $IL2^{+}CD8^{+}$ T cells with UVB (Figure 3.10 e, f, g, h). The significant increase in IL2 secretion correlated with an increase in total SDLN numbers from 1.4 to 9.1 million with UVB IR (Figure 3.10 i). These results suggest that the synergistic increase in T cell activation with UVB treatment is due to the increased migration of dermal DCs to the SDLNs during the initial sensitization phase rather than any role of DCs in the elicitation phase.

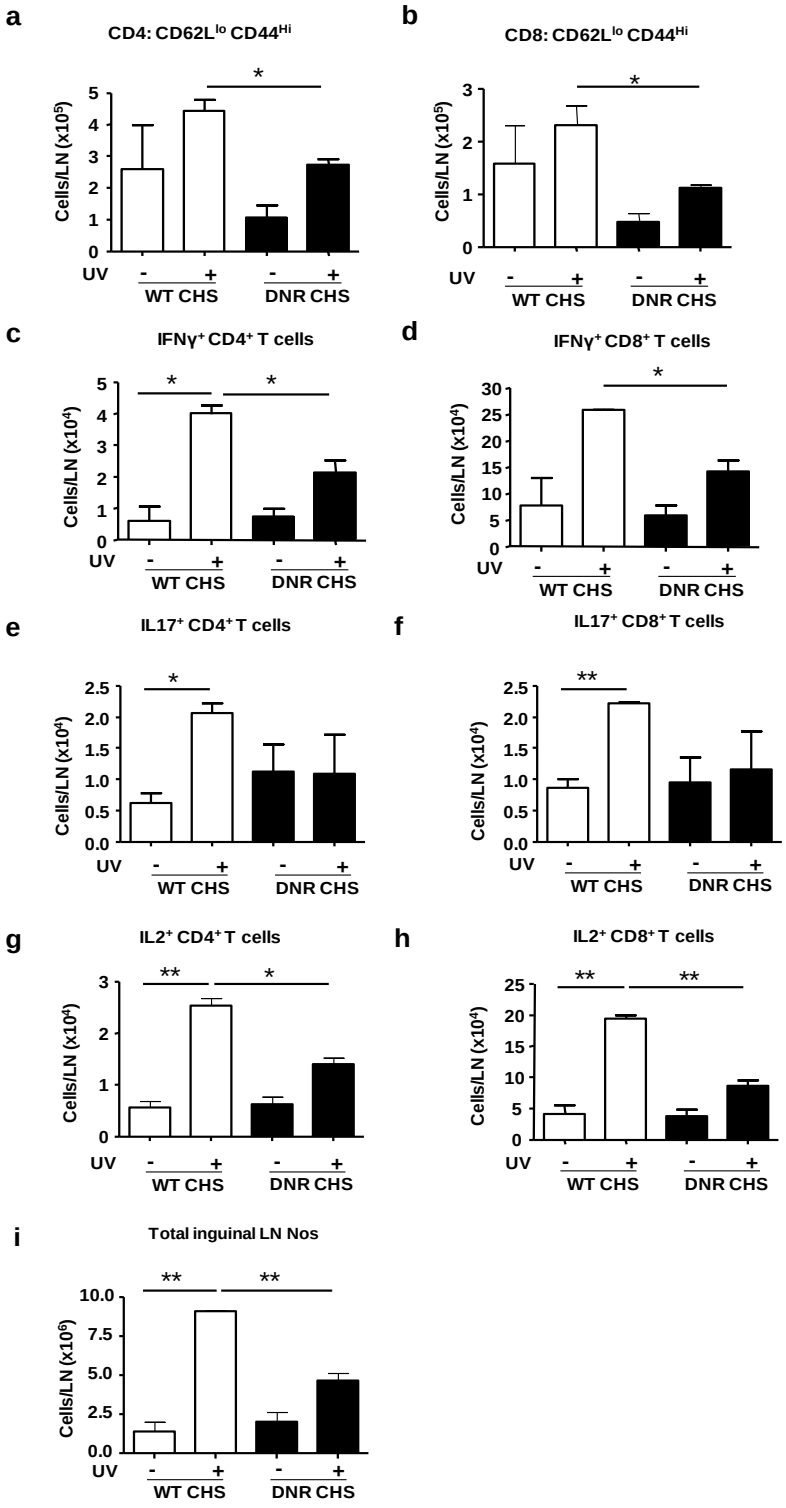

Figure 3.10: DC-specific defect in TGFβ1 suppresses the synergistic increase in Contact Hypersensitivity (CHS) responses with UVB

WT and CD11c-DNR mice were irradiated with 1 MED UVB before sensitization with 0.5 % DNFB. 5 days after sensitization, Skin-draining lymph nodes (SDLNs) were harvested 5 days post sensitization and analyzed by flow cytometry for (a and b) $CD4^+$ and $CD8^+$ effector T cells (c and d) $IFN\gamma^+$ $CD4^+$ and $CD8^+$ T cells (e and f) $IL17^+$ $CD4^+$ and $CD8^+$ T cells, (g and h) $IL2^+$ $CD4^+$ and $CD8^+$ T cells (i) Total SDLN numbers. n=3 for non-UV and UV IR groups. Error bars = ±SEM. *P<0.05 relative to indicated groups.

In contrast, there was no significant UVB induced increase in ear thickness in DNR mice over the untreated group. In addition, the total SDLN numbers reduced from 9.1 million with UVB in the WTs to 4.6 in the DNR (Figure 3.10 i). There was also a reduction in CD4 effector population and CD8 effector population close to non-UV treated WT controls (Figure 3.10 a, b). The $IFN\gamma^{+}CD4^{+}$ T cells and $IFN\gamma^{+}CD8^{+}$ T cells reduced to half relative to UV treated group (Figure 10 c, d). Further, $IL17^{+}CD4^{+}$ T cells $IL17^{+}CD8^{+}$ T cells $IL2^{+}CD4^{+}$ T cells and $IL2^{+}CD8^{+}$ T cells also reduced to half of the UVB treated group (Figure 10 e, f, g, h). These results clearly suggest that blockade of TGFβ1 signaling in DCs impairs the initial sensitization even and consequently inflammatory response to hapten in a CHS bioassay.

3.5 DISCUSSION

UV has been shown to inhibit cutaneous immune responses at low doses either by directly downregulating the function of Langerhans cells or by inducing regulatory T cells, [1-3;49] which in turn, reduces the CHS response to haptens [50;51]. However at the MED doses that we use, UVB acts as an inflammatory stimulus potently inducing migration of dermal DC subsets in a TGFβ dependent manner.

Consistent with the pharmacological model of TGFβ1 signaling inhibition with SB, we observed a significant reduction in the migration of $CD207^-$ dDCs and $CD207^+$ dDCs in DNR mice with acute UVB both *in vivo* and in an *ex vivo* ear explant culture and this was maintained through chronic UVB treatment for 1 week. The decrease in dermal DC migration also correlated with decreased T cell activation in terms of memory effector differentiation and IFNγ secretion in CD4 and CD8 T cells. More importantly, our results indicate that the steady state migration of $CD207^+$ dDCs as well as $CD207^-$ DCs was reduced with genetic inhibition of TGFβ in DCs suggesting a greater role for TGFβ signaling in maintaining dermal DC homeostasis in the skin. Whether the defective DC migration we observe in CD11c-DNR mice reflects direct requirement of TGFβ1 signaling in dermal DC for activation or an indirect effect through synergy with other inflammatory pathways remains to be determined. Our studies on TGFβ1+/+ and TGFβ1+/- mice have shown that there is reduced TNFα induction by UVB in TGFβ1+/- mice, which is a key downstream target of UVB-induced NFκB pathway activation mediated via Smad3. These results suggest that there might be a cross-talk between UVB-induced TGFβ signaling and UVB-induced inflammatory pathways such as NFκB. Consistent with their reduced migration, CD86 upregulation on dDCs in response to UVB was suppressed in DNR mice. This suggests that

TGFβ1 signaling is important in activation of dermal DC in response to UVB, but direct effects on migratory ability have not been ruled out.

$CD207^+$ $CD103^+$ dDCs are important in adaptive immune responses, [12;52]and in contact hypersensitivity are the main DC subset to cross-present keratinocyte derived antigens for priming naïve CD8 T cells in the lymph nodes [6]. $CD8^+$ cytotoxic T lymphocytes (CTLs) are key effectors of the elicitation phase of CHS and CD8 CTL-derived IFNγ, perforin/granzyme and Fas-L are crucial for clinical manifestation of CHS [30;53-55]. Our results indicate that the migration of $CD207^+$ dDCs was reduced with genetic inhibition of TGFβ in DCs. Coupled with the significant suppression of UV-induced $CD8^+$ T cell activation in the SDLN and suppression of enhanced CHS responses by UVB these data strongly implicate TGFβ1 signaling in $CD207^+$ dDC for the observed CD8 T cell activation in UV-induced cutaneous inflammatory responses. Although the $CD207^-$ dDCs form a majority subset in the dermis as well as the SDLNs, it forms a heterogeneous subset and its contribution to cutaneous immune responses is largely uncharacterized [56]. A main reason for this is the absence of a useful marker to knockdown this population specifically.

The UV + CHS irradiation experimental model in which the skin is irradiated with UVB on the sensitization site immediately before the hapten exposure addresses a specific hypothesis: whether the UVB induced increase in the migratory subsets of $CD207^-$ dDCs and $CD207^+$ dDCs has physiological relevance and can be demonstrated in a $INF\gamma^+$ $CD8^+/CD4^+$ T cell driven pathology such as CHS. As expected, with UVB, we observed a synergistic increase in WT mice in ear thickness and lymph node activation of CD4 and CD8 T cells measured in terms of IFNγ and IL-17 secretion and memory effector phenotype with skin hapten treatment, which was significantly reduced in the absence of DC-specific TGFβ signaling. The observation that the

reduction in ear thickness measured in DNR mice with hapten+UV compared to WT was reflected in reduced IFNγ and IL-17 cytokine profile suggests that DC TGFβ signaling is important for the initial sensitization phase of CHS although any contribution to the elicitation phase cannot be entirely ruled out. Both IL-17 and IFNγ have been shown to be essential for the elicitation phase of CHS [57-59]. Our data strongly suggest that in this CHS model TGFβ signaling in DCs is needed for the establishment of the optimal inflammatory cascade.

Consistent with reduced steady state migration of DC subsets in the DNR mice, we observed a reduction in ear thickness in CHS when compared to the WT mice indicating that defective DC TGFβ signaling in the DNR mice is physiologically relevant, which is consistent with the decrease in baseline p-Smad2^{+} dermal DC subset between WT and DNR mice. However, in our UVB irradiation model, we observed an increase in p-Smad2^{+} dermal DCs in response to UVB inflammatory stimulus with complete blcokade in the CD11c-DNR skin. A critical question is whether UVB p-Smad2 activation is specific to an inflammatory stimulus such as UVB or is a more common mechanism and is associated with differences in CHS responses as well.

Active trafficking of the hapten by Dendritic cells to the draining lymph node is essential for immune responses in CHS model [60];Allenspach, 2008 14657 /id} and passive draining of the hapten through the lymphatics to the skin draining lymph nodes is not sufficient to elicit a response [60]. Distinct from dermal DC subsets, Langerhans cells have been shown to be suppressive in an IL-10 dependent manner during the sensitization phase and genetic depletion resulted in enhanced CHS responses [61-65]. The contribution of LCs to CHS responses in the absence of UVB remains to be characterized in our model. However, in response to UVB IR, since we do not observe any increases in migratory LC population between WT and DNR mice, it is possible that the enhanced CHS response with UVB irradiation in WT and DNR mice is

directly due to increased migration of $CD207^-$ and $CD207^+$ dermal DCs with UVB IR in WTs. Indeed, studies have reported that CHS responses are greatly diminished in mice, which have both their LC, as well as $CD207^+$ dermal DCs ablated suggesting a greater role for $CD207^+$ dermal DCs in CHS responses [66-68]. However, some groups have also shown that Langerhans cells and $CD207^+$ dermal DCs have redundant functions in CHS model [69;70], while one study suggest that $CD207^+$ dermal DCs are dispensable for contact hypersensitivity responses in a Batf3 knockout mice, suggesting that the largely uncharacterized $CD207^-$ subset might play a greater role in CHS. Although it has to be demonstrated conclusively by specific ablation of LCs, our results link TGFβ signaling in the two dermal DC subsets with the ability of UVB irradiation to enhance the CHS response.

Taken together, our results suggest a common model of inflammation in the skin mediated by TGFβ signaling in dermal DC, which is a prerequisite for mounting a potent immune response to cutaneous inflammatory insults such as UVB and hapten exposure in CHS.

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Chapter 4

DISCUSSION

In our study, we have focused on the UVB induced activation of T lymphocytes and inflammation within the skin and how that functions as a tumor promoter in UVB carcinogenesis in relationship to TGFb1 signaling. TGFβ1 pathway inhibition with the small molecule ALK5 kinase inhibitor SB431542 (SB) in a UVB carcinogenesis model led to fewer tumors than the vehicle-treated group, which correlated with decreased infiltration and activation status of $CD4^+$ and $CD8^+$ T cells. Our results strongly suggest that TGFβ signaling in DCs plays an important role in this inflammatory response.

We found that long-term inhibition of TGFβ1 signaling with SB as well as defective TGFβ signaling in CD11c-DNR mice led to decreased steady-state migration of all 3 cutaneous DC subsets, thus providing evidence that TGFβ1 signaling is required for homeostatic migration of epidermal LCs and dermal DCs, $CD207^-$ and $CD207^+$ $CD103^+$ dDC subsets. Further, inhibition of TGFβ signaling with SB in skin led to decreased migration of dermal DC subsets, $CD207^-$ and $CD207^+$ $CD103^+$ in response to UVB IR. It was associated with a significant decrease in p-Smad2 levels in dermal DCs compared to vehicle group. Consistent with this, UVB induced migration of dermal DC subsets, $CD207^-$ and $CD207^+$ $CD103^+$ was also reduced in CD11c-DNR mice compared to WT and it correlated with a decrease in DC activation marker, CD86. Thus, TGFβ1 signaling is required for UVB-induced migration of dermal DC subsets, $CD207^-$ and $CD207^+$ $CD103^+$ but not LCs to the skin draining lymph nodes**.**

Consistent with decreased migration of dermal DCs with SB inhibition as well as DNR mice in response to UVB, there was suppression in activation of $CD4^+$ and $CD8^+$ T cells in terms of the memory effector phenotype and IFNγ secretion relative to WT mice. In DNR mice, this

suppression of T cell activation resulted in reduced responses to hapten in Contact Hypersensitivity model leading to reduced ear thickness compared to WT mice. Thus, TGFβ1 signaling mediates UVB-induced inflammation within the skin.

4.1 UVB and immune responsiveness:

A number of studies have documented the immunosuppressive properties of Ultraviolet B radiation (UVB) on skin resulting from keratinocyte-Langerhans cell crosstalk. However, the immunoresponsive properties of the skin to UV light have to be interpreted with specific reference to the UVB dose employed in the experimental model. Minimal Erythema Dose (MED) is defined as the minimum dose of UVB radiation required for manifesting a visible erythemal response characterized by vascular dilation followed by an influx of leukocytes, which appears as reddening of the skin commonly referred to as Sunburn. The MED for C57Bl/6 mice is 5.4 kJ/m^2 and MED for skin hairless mice (SKH1) is 2.4 kJ/m^2. UVB irradiation of C57Bl/6 mice at a dose of 1 kJ/m^2 [1-4] much lower than the MED, results in systemic immunosuppression leading to reduced ear thickness in a Contact Hypersensitivity (CHS) model mediated through modulation of antigen presentating cells [4-7].

CHS has been a commonly used experimental model to study the cellular immune responses to UVB radiation. CHS is dependent upon an active uptake of hapten by antigen presenting cells in the skin and migration to the SDLNs to activate naïve T cells. It is a T cell driven pathology. Langerhans cells (LCs) have been recognized as the primary mediator of UVB mediated immune responses [6-8]. Low dose UV exposure has been shown to inhibit activation markers such as MHCII, CD80/86 and ICAM1 on LCs [8-11] Low dose UV also induced the secretion of IL-10 from keratinocytes which promoted the tolerogenic differentiation of LCs [7]. As a result of this

defective maturation, when LCs migrated to the SDLNs, they promoted the differentiation of antigen-specific suppressor T cells now identified as $T_{regulatory}$ cells. This immunosuppresion could be transferred by injecting splenocytes from irradiated donors to naïve mice [4;6;7]. These reports implicate LCs and T_{reg} cells as key mediators of the immunosuppression to low dose UV.

When a dose response was performed with UVB radiation, increasing doses of UVB increased the migration of LCs from epidermal sheets over steady state, although beyond a threshold, the numbers started decreasing with very high doses presumably due to an increase in LC apoptosis. Further, two distinct populations of LCs were identified based on their activation status, their expression of IL-1β, IL-6, and TNF-α, and their capacity to stimulate T cells [12;13]. Another study also highlighted a disparity in the immunostimulatory status of epidermis-resident DCs and the DCs that migrated out of epidermal sheets clearly indicating distinct populations of DCs are generated with UVB irradiation [14]. Hence, the activation status of DCs in response to UV light is dependent on two main factors: 1) the specific DC subset and 2) the UVB dosage.

In chapter 2, using SKH1 mice, where we chronically irradiated the mice with MED dose of UVB over a one-week period, we observed a pronounced activation of $CD4^+$ and $CD8^+$ T cells in the lymph nodes measured in terms of both their effector phenotype and $IFN\gamma^+$ status. This clearly indicates that UV exposure at the dose we employed was a potent inflammatory stimulus rather than an immunosuppressant. The response was consistent with two weeks of UVB as well as in the UVB carcinogenesis study. These higher doses of UVB are also relevant for UVB carcinogenesis because, at higher doses, UVB generated tumors more rapidly than at lower doses [15]. Further, when a syngeneic UV-induced skin carcinoma cell line (T51/6.53) was implanted onto SKH hairless mice after different doses of UV exposure, either 0.5 MED or 1 MED, there was accelerated implant acceptance with 1 MED dose relative to 0.5 MED highlighting the

positive correlation between UV dosage and tumor growth [16;17]. There is also evidence for increased inflammation with high UVB doses which in turn acts as a tumor promoter resulting in increased number of tumors [18-20]. In addition, increased exposure to UV light is the single most important risk factor for Non melanoma skin cancer, and skin tumors most often occur in sun exposed head and neck areas of the human body as opposed to the less exposed areas [21-23].Thus, high UVB doses act as a complete carcinogen- causing mutations in DNA and promoting tumor outgrowth through inflammation.

In addition, in our CHS model with local UVB irradiation during the sensitization phase led to a pronounced ear thickness in C57BL/6 WT mice. Consistent with this, there was an enhanced activation of T cells in the SDLN, both $CD4^+$ and $CD8^+$ subsets with an increased cytokine profile 5 days post UVB and hapten sensitization. These results, along with the chronic UVB model in SKH1 mice clearly demonstrate that UVB acts as a potent inflammatory stimulus within the skin.

4.2 The distinct functions of the different cutaneous DC subsets in skin immunity

4.2.1 Langerhans Cells

When we investigated what effect the MED dose had on the migration of cutaneous DC subsets, we did not observe any appreciable increases in the migration of LCs. The lack of immunosuppression with UVB in our model could be directly correlated to 1) the lack of UVB induced increase in migration of LCs and 2) the higher UVB dose used in our dosage regimen. However, UVB has been shown to induce apoptosis of LCs as well [24;25]. This may the lack of increase in LC migration with UVB could possibly due to increased LC apoptosis, but we did not

address this issue experimentally. LC apoptosis could be verified by Annexin V staining of LCs in conjunction with TUNEL assay by flow cytometric analysis [26].

The contribution of LCs to CHS responses is under intense investigation. Three groups have obtained three different results with LC deficiency in CHS response with DNFB: diminished reaction, enhanced reaction and unchanged response. Kaplan and colleagues reported that the ablation of LCs resulted in an enhanced CHS response suggesting not only that other cutaneous DC subsets can compensate for the absence of LCs but also that LCs play a regulatory role in the response rather than active antigen presentation [27]. Another group showed that this regulatory role is mediated by the induction of Treg cells [28]. In contrast, two other groups reported that LCs are required for optimal hapten priming of T cells in CHS since LC absence led to reduced CHS responses [29;30]. There are also additional reports showing that LCs are completely dispensable for the CHS responses in the skin [31;32]. The difference in LC function has been partially attributed to the differences in the timing of the inducible ablation of the LCs before the sensitization phase and to certain variations in the strain of the mice used for generating the transgenic lines.

With respect to UV IR effects on CHS, LCs were found to be dispensable and the CD8 response generated was dependent primarily upon $CD207^+$ dDCs [33]. While a general consensus has not evolved for the function of LCs in antigen presentation responses in the CHS model, the role of dermal DC subsets has gained increasing prominence in cutaneous immunity and there are many reports which suggest that dermal DC subsets especially $CD207^+$ dDCs play non-redundant roles in cutaneous antigen trafficking and presentation [32;34-36].

4.2.2 Dermal Dendritic cells

Consistent with the importance of dDC in the UV response, an early study suggested that UV irradiation suppressed the migration of Langerhans cells, and promoted the migration of dermal DCs [9]. In our study, we observed a statistically significant increase in migration of the $CD207^-$ and $CD207^+$ $CD103^+$ dermal DC subsets to the skin draining lymph node following UVB irradiation. Our results also show an enhanced activation of $CD8^+$ effectors and $CD8^+INF\gamma^+$ cells following UVB, strongly linking the increased migration of CD207+ dermal DCs with UVB to naïve CD8 T cell activation. Cross-presentation is essential for the priming of $CD8^+$ T cell responses and involves the presentation of exogenous as opposed to endogenous antigens on MHC class I molecule. Cross-presentation is mainly carried out by specific dendritic cell (DC) subsets through an adaptation of their endocytic and phagocytic pathways [37]. The most important reported function of $CD103^+$ dDCs is cross-presentation and are specialized in the cross-presentation of cell-associated self and viral antigens similar to LN resident $CD8alpha^+$ DC [38;39]. $CD207^+$ dDCs are also capable of cross-presenting OVA peptide [34] and keratinocyte derived antigens without the requirement for LCs [32]. In the lung, $CD207^+$ dermal DCs are required for the clearance of influenza virus infection [40] and are also capable of cross-presentation [41].

There are also reports which suggest that one of the roles of the migratory DCs is to carry antigens from the dermis and epidermis and transfer them to lymphoid tissue resident DCs which then perform the actual antigen-presentation [32].Dermal DCs can interact with LC-derived antigenic peptides and migrate to the SDLNs to amplify the antigen presentation event. However, dermal DCs are a very heterogeneous population with respect to CD103 and CD11b expression in addition to CD207 (Langerin). CD103 expression alone cannot be used to identify

$CD207^+$ $CD103^+$ dDCs because only half of them express the marker. $CD207^+$ dDCs are negative for CD11b but the larger $CD207^-$ subset is heterogeneous for CD11b. Hence, there is no exclusive marker for dDCs that allows useful sorting by flow cytometry for functional assays. The Diphtheria toxin (DT)-based inducible transgenic models [32] provide a way to circumvent this issue where the difference in tissue reconstitution of LCs vs dermal DCs can be exploited to study the functional significance of $CD207^+$ dermal DCs in the selective absence of LCs immediately after DT treatment.

The Batf3 knockout is another useful model as it lacks $CD103^+$ $CD11b^-$ DCs. Batf3 belongs to AP-1 family of transcription factor involved in the lineage differentiation of mainly CD8α+ conventional DCs. Hence, they are also deficient in $CD8\alpha^+$ cDCs which are also important in cross-presentation [32] although this subset is a LN-resident subset and is not skin-derived allowing functional dissection of $CD103^+$ $CD11b^-$ DCs, of which $CD207^+$ dermal DCs form a constituent subset. It is important to note the specific contribution of each subset to cutaneous antigen presentation is very complex and DT based transgenic mouse models in conjunction with bone marrow chimera models where the LCs are of host origin and dermal DCs are of donor origin have to be employed to reliably interpret data specific for each subset along with elaborate phenotypic characterization of each subset for example, simultaneous analysis of CD11b, CD207 and CD103 for $CD207^+$ dermal DCs.

Our data provide evidence for the role of $CD207^+$ dDCs and $CD207^-$ dDC subset in the UVB-induced inflammation in the skin. With acute UVB, we observed an increased migration of these key subsets from the skin to the SDLNs and in ear explant culture, which was significantly suppressed with abrogation of TGFβ signaling. This increased migration was associated with an increase in Smad2 phosphorylation in these subsets absent with ALK5 inhibition. Further, CD86

upregulation in these subsets was associated with functional TGFβ signaling. In addition, we showed that not only is TGFβ signaling essential for UVB-induced migration but also for the steady-state migration for these subsets. We recently showed that overexpression of TGFβ1 by keratinocytes caused rapid migration of dermal DC subsets and not LCs causing an immunostimulatory response in a CHS assay [42]. However, TGFβ signaling has been shown to be dispensable for the development as well as function of dDC subsets [35;43]. Further, the abrogation of TGFβ signaling did not alter the p-Smad^{+} LCs or their migration in response to UVB however, their steady state migration was dependent on TGFβ signaling. Studies on LC function have shown TGFβ1 signaling to be essential for development and maintenance of LC in the epidermis in an immature state [43-45].

In order to definitively demonstrate the relative contribution of each skin DC subset and to establish the role of TGFβ1 signaling in this response, crosses of DT-transgenic mice for Langerin have to be made on the CD11c-DNR background and a combination of approaches described above should be employed. In addition, the specific DC populations have to be sorted using FACs and subjected to functional T cell stimulation assays such as allogenic stimulation of T cells followed by assessment of proliferation or cytokine stimulation of T cells. Alternatively, the DC subsets can be loaded with ovalbumin antigen and stimulated with OTII-TCR transgenic naïve T lymphocytes to determine their proliferation with ova stimulation [46]. These mice have a T cell receptor specific for ovalbumin.

4.3 Inflammation associated with skin cancer

The dual role of TGFβ in carcinogenesis has been well-documented [47-49]. In our UVB carcinogenesis study, SB suppressed the tumor numbers at all time-points after 20 weeks when the tumors started increasing in size and in number. There was an associated reduction in tumor

infiltrating CD4 and CD8 lymphocyte population with reduced IFNγ secretion. This was consistent with previous studies from our lab that showed the function of TGFβ as a tumor promoter and reduced inflammation associated with carcinogenesis in TGFβ heterozygous mice or ALK5 inhibition. [50;51]. In addition, similar to the two-stage chemical carcinogenesis model, ALK5 inhibition with SB enhanced the number of SCC and poorly differentiated SCC. This is in agreement with studies in literature that also reported enhanced SCC with conditional deletion of the TGFβ pathway [52-55]suggesting a role for TGFβ signaling in inhibition of metastatic phenotype. However, this is in contradiction to the classical view of TGFβ as an inhibitor of tumorigenesis in the early stages while enhancing EMT at the later stages with associated invasive and metastatic phenotype [56-60]. Part of this biphasic and context-dependent role of TGFβ can be explained by the multifaceted role of TGFβ signaling in modulating the plasticity of the tumor-associated immune responses [61-64].

As discussed in the introduction, DCs, Tumor associated macrophages (TAMs) and Tumor infiltrating lymphocytes (TILs) form major constituents of tumor immune infiltrates. The differentiation status, immunogenic vs tolerogenic, of DCs within a tumor greatly influences the type of tumor immune response [65]. Immature DCs are linked to a predominantly suppressive environment in basal cell carcinoma (BCC) with increased levels of IL-4 and IL-10 [66]. These cells, characterized as myeloid $CD11c^{+}$ DCs are associated with immunosuppression in squamous cell carcinoma as well. Increased expression of IL-10 and TGFβ around the tumor tissue was correlated to the suppressive function of these DCs [66;67]. TGFβ has been commonly linked to decreased tumor infiltration, activation and migratory capabilities of DCs polarizing them towards a tolerogenic phenotype [68-74]. However, importantly, DCs from both the tumors and the peritumoral nonlesional skin were both phenotypically mature and they

differed only in their ability to stimulate allogenic T cell population emphasizing the relevance of a functional assay in conjunction with phenotypic analysis to determine the differentiation state of DCs. The presence of TAMs in BCCs has been associated with a more aggressive cancer phenotype [75]. However, in SCCs, TAMs had a more heterogeneous phenotype with pro as well as anti-tumor characteristics [76].

In our UVB carcinogenesis study, there were no significant differences in the $CD11b^+$ $ly6G^+$ subset representing neutrophils as well as $CD11b^+$ $Ly6G^-$ $F4/80^+$ cells representing TAMs between SB treated tumors and the vehicle tumors. Since we did not determine the specific cytokine profile of macrophages, it is possible that there could be a phenotypic difference M1 vs M2 between the SB and control tumors which would dictate the role of TAMs infiltrating the tumors even if the relative frequencies were the same. With respect to TILs, we noted that the majority population of infiltrating lymphocytes to be $CD4^+$ (average of 38 % out of viable leukocytes) compared to 12.5 % of $CD8^+$ cells within the vehicle treated tumors which were both significantly reduced with SB treatment. Further, the $CD4^+$ infiltrating the SB treated tumors were also defective in their capability to secrete IFNγ.

In a study analyzing lymphocytes infiltrating SCC and actinic keratosis (AK), $CD8^+$ TILs were reported to be a minority population with cytotoxic phenotype [77]. $CD8^+$ cytotoxic T lymphocytes are effector cells of the immune system with potent anti-viral and anti-tumor functions [78]. They perform their cytolytic function through two distinct mechanisms, one secretory and the other by cell-cell contact. The secretory pathway, mediated by perforin and granzyme, directly forms a pore in the target cell-membrane and effects its cytolysis. The nonsecretory pathway is Fas mediated and involves the engagement of CTL membrane ligand, FasL with apoptosis-inducing Fas on the target cell inducing the programmed death of the target

cell carrying the altered self-antigen. Increased $CD8^+$ cytotoxic infiltration is commonly associated with better prognosis for cancer [79;80]. However, tumor infiltrating CD8 lymphocytes in melanoma have also been reported to be functionally impaired by the tumor microenvironment via upregulation of an immunoinhibitory receptor PD-1 [81;82].

CD4 T lymphocytes, unlike CD8 T lymphocytes, cannot directly recognize cancer cells due to the lack of MHC class II on most solid cancers [83]. However, certain solid cancers such as melanoma can express MHCII constitutively [84]. In addition, class II restricted CD4 T cells specific for tumor antigens have also been detected in melanoma [85], breast [86] and ovarian cancer [87]. $CD4^+$ T cells perform anti-tumor function by providing cytokine help to $CD8^+$ T cells such as type 1 cytokines [88;89] They can also recognize endogenously processed antigens on tumors and help in direct tumor killing [90]. In addition, mice lacking sensitivity to IFNγ developed tumors rapidly with greater frequency implicating a tumor-suppressive role for IFNγ potentially regulating tumor immunogenicity [91;92]. However, IFNγ secreted potently by T_H1 cells has been shown to act as a tumor promoter in a two stage chemical carcinogenesis model with an associated increase in T_H17 population [93-95].

In our studies, from initial short-term UVB irradiation to tumors after 30 weeks, inhibition of TGFβ1 signaling reduced the percentage of $IFN\gamma^+$ CD4 T cells. Tumour-specific T_H1 cells orchestrate the immune response against cancer by secreting cytokines and chemokines for recruiting other immune populations. One possible mechanism is that the action of enhanced proinflammatory cytokine signature in the vehicle tumors increase their outgrowth compared to SB treated tumors. T_H1 cells induce secretion of IL-1β and IL-6 by macrophages [96]. Also ketinocyte-T cell crosstalk has been shown to support T_H1 differentiation in an infection setting by increased keratinocyte secretion of IL-6 [97] IL-1 and IL-6 have been shown to be essential

for tumorigenesis [98-102]. IL-1 is a canonical proinflammatory cytokine, and is important for activation of vascular endothelium and stimulation of T lymphocyte functions thus working in a positive feedback loop resulting in exaggerated inflammation [103;103;104]. This inflammation in turn, functions as a potent tumor promoter [105-107]. Further, UVB is a potent activator of the NFκB transcription program [108] and data from our lab has shown that TGFβ pathway is required for optimal activation of the NFκB pathway {Hogan, 2013 14752 /id}.The NFκB-STAT3 interaction is very important for establishing a proinflammatory cytokine milieu within the tumors promoting the development of various tumors [109-113]. Together, these factors create a predominantly inflammatory microenvironment within the tumors in the presence of functional TGFβ signaling allowing the increased development of tumors.

In addition, due to the functional plasticity of CD4 T cells into the different lineages, the specific function of this subset is complex and is rather dependent on the relative abundance of the various subsets, T_H1, T_H2, $T_{regulatory}$ cells and T_H17. Increased T_{reg} cell infiltration within tumors can inhibit the cytotoxic function and proliferation of tumor-specific CD8 cytotoxicity in a TGFβ dependent manner promoting tumor outgrowth [114;115]. It has also been shown that the progression of SCC is associated with increased $Foxp3^+$ Tregulatory cells resulting in more invasive phenotype [116;117]. Increased T_{reg} cell numbers are linked to worse prognosis in BCC [66] SCC [116;118]and melanoma [119-121].

TGFβ supports the development of T_{reg} cells that in turn creates an immunosuppressive environment allowing the outgrowth of tumors. Another explanation for the reduced tumor outgrowth with SB treatment could be that the ALK5 inhibition inhibits T_{reg} cell differentiation allowing the CD4 and CD8 T cells although in lower frequencies relative to the vehicle group to perform their cytotoxic function more effectively resulting in fewer tumors. Also, the presence of

TGFβ in the tumor environment can be directly cytostatic to T cell function or can upregulate inhibitory molecules such as PD1 on T cells making them functionally impaired. [81;82;122;123]. However, the localization of these tumor-infiltrating T cells, intratumoral or peritumoral along with their functional state remains to be tested more rigorously in order to address this possibility and have to be correlated with the malignant state of the tumor.

UVB-induced acute and short-term inflammation within the skin is primarily mediated by the dermal DC activation in a TGFβ dependent manner as has been already discussed. We have further shown that this migration is physiologically significant and results in T cell activation and skin inflammation. Furthermore, we show that this T cell activation in another cutaneous pathology model, CHS where there is a reduction in CHS responses with defective TGFβ signaling in DCs. However, in UVB carcinogenesis, a host of immune infiltrates are present and the immunomodulation of other immune populations, TAMs, NK cells and lymphocytes by ALK5 inhibition cannot be ruled out [124]. In fact, it is more likely that the reduced tumor numbers with SB inhibition is a sum total of reduced immune infiltration and cytokine profile resulting in an overall reduced inflammatory signature rather than the activation of DCs alone. In order to determine the specific contribution of TGFβ signaling in DCs to the UVB carcinogenesis, an inducible flox-cre model of DC-specific TGFβ ablation has to be used where the pathway can be ablated at different stages of carcinogenesis. However, currently, only a constitutive flox-cre model for DC-specific TGFβ ablation is available [125].

4.4 A common theme for T cell mediated cutaneous pathologies regulated by TGFβ

In our study, we have focused on the UVB induced activation of T lymphocyte responses and inflammation within the skin and how that functions as a tumor promoter in UVB skin

carcinogenesis. Further, we also show that UVB induced activation of T lymphocytes enhances the responses to hapten in another common cutaneous pathology such as CHS. Our results show that dermal DC subsets are key mediators of the T cell response in both cutaneous pathologies and TGFβ signaling in dermal DC is required for this event.

Psoriasis is another chronic autoimmune disease of the skin, primarily mediated by Th1 lymphocytes [126]. It is characterized by the hyperproliferation of keratinocytes associated with chronic inflammation resulting in psoriatic plaques. Latent TGFβ1 overexpression in keratinocytes leads to psoriasiform lesions characterized by epidermal hyperproliferation associated with infiltration of neutrophils, T lymphocytes and macrophages to the epidermis and dermis along with angiogenesis and basement membrane degradation [127]. TGFβ levels are focally increased in psoriatic lesions [128]. Increased TGFβ has also been noted in psoriatic patients implicating it as a biomarker for psoriasis severity[129-132]. A large number of dermal dendritic cells are present in the psoriatic plaques beneath the hyperplasic epidermis [133]. Dermal dendritic cells isolated from psoriatic plaques displayed potent autostimulatory activity with T cells polarized predominantly towards IL-2 and IFNγ characteristic of Th1 phenotype with no IL-4 or IL-10 [133]. Plasmacytoid dendritic cells have also been shown to initiate psoriasis through interferon-α production [134;135]. In addition, overexpression of TGFβ1 by keratinocytes induces the migration of dermal DC subsets to the SDLN and also enhances contact hypersensitivity responses [42]. Thus, dendritic cells especially dermal DCs and plasmacytoid DCs have been shown to be important for psoriasis. It is possible that TGFβ presence in the skin is required for this chronic proinflammatory response associated with psoriasis.

Conclusion

TGFβ has been well-demonstrated to play a dual role in carcinogenesis and studies from our lab in a two-stage chemical carcinogenesis model have shown that TGFβ performs a tumor-promoting role by increasing inflammation while acting as a suppressor of malignant progression. However, there is relatively little information on the role of TGFβ signaling in UVB mediated carcinogenesis which is the primary cause of skin cancer in humans.

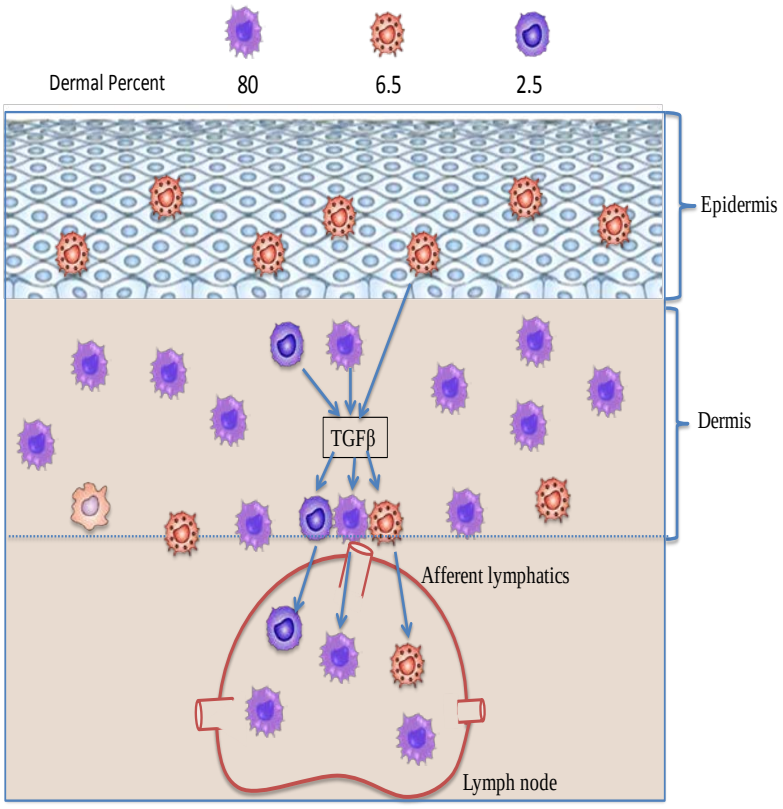
The goal of this dissertation was to understand the role of TGFβ signaling in UVB carcinogenesis bioassay and to identify whether inhibition of TGFβ signaling with a pharmacological inhibitor might have therapeutic potential. Our data support a model where TGFβ, consistent with the chemical carcinogenesis model, promoted tumor outgrowth while suppressing malignancy. This tumor promotion was linked to an overall proinflammatory tumor signature mediated by $IFN\gamma^+$ CD4 and CD8 T cells.

It has been well-recognized that TGFβ is an important immunoregulatory cytokine with a critical role in T cell regulation supported by several *in vivo* genetic studies. However, its effect on dendritic cells especially in response to an inflammatory stimulus such as UVB has not been studied extensively. We used genetic and pharmacological models of TGFβ signaling abrogation and provided evidence for the first time that TGFβ signaling in dermal DCs is essential for their migration to the lymph nodes in response to UVB with an associated activation of the TGFβ pathway. We also show that TGFβ signaling plays a role in homeostatic migration of epidermal langerhans cells and dermal DC subsets. We further show that this modulation of DC function by TGFβ signaling is important for effector T cell differentiation and IFNγ secretion downstream

which is directly relevant for cutaneous pathologies such as carcinogenesis and contact allergic dermatitis.

Thus, TGFβ requirement by dermal DCs for optimal immune function can act as a common theme for manifestation of inflammation within the skin for skin pathologies primarily driven by T lymphocytes. Elucidation of the mechanisms underlying UV-induced inflammation contributes not only to the understanding of biological effects of UV in cutaneous disease conditions but also to clarifying fundamental mechanisms of the immune system.

4.5 Proposed Model:



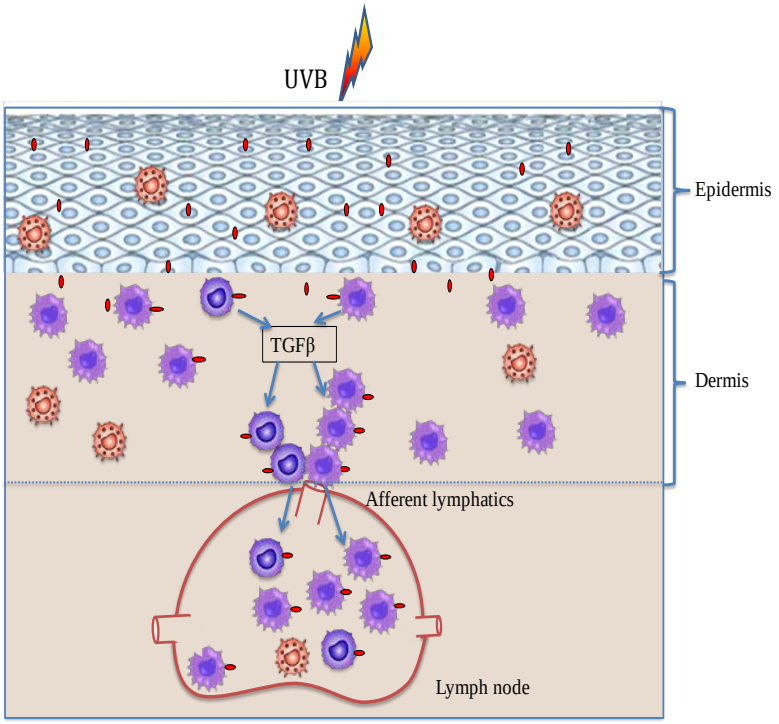
Homeostatic DC migration is dependent on TGFβ

Figure 4.1 **TGFβ1 signaling is required for the steady state migration of epidermal LCs and dermal DCs, CD207$^-$ and CD207$^+$ CD103$^+$ DC subsets:**

Long-term inhibition of TGFβ1 signaling with SB as well as defective TGFβ signaling in CD11c-DNR mice led to decreased steady-state migration of all 3 cutaneous DC subsets.

Legend

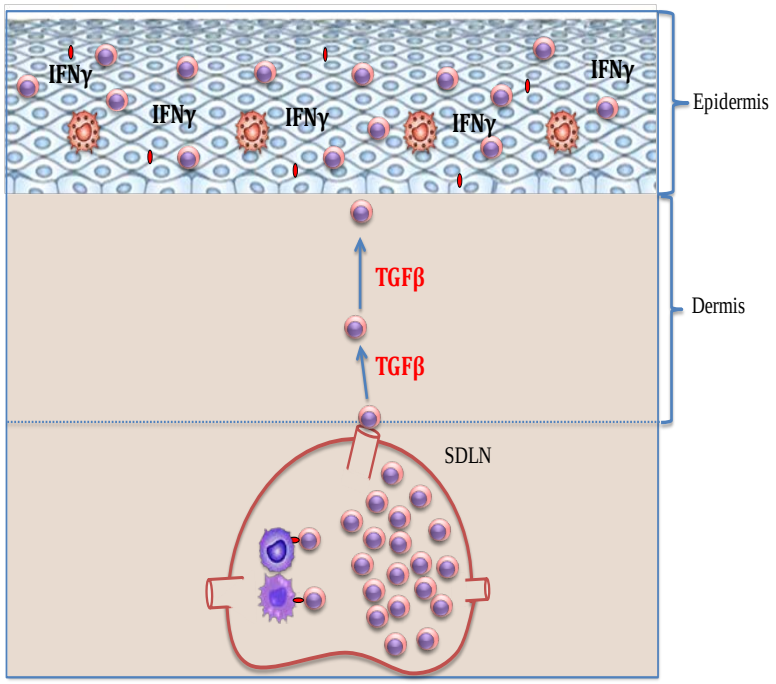
- Antigen
- Langerhans cell
- CD207$^+$ CD103$^+$ dDC
- CD207$^-$ dDC
- Lymphocyte
- Macrophage



UV induced dermal DC migration is dependent on TGFβ

Figure 4.2 **TGFβ1 signaling is required for UVB-induced migration of dermal DC subsets, CD207$^-$ and CD207$^+$ CD103$^+$ but not LCs to the skin draining lymph nodes:**

Inhibition of TGFβ signaling with SB in skin led to decreased migration of dermal DC subsets, CD207$^-$ and CD207$^+$ CD103$^+$ in response to UVB IR. It was associated with a significant decrease in p-Smad2 levels in dermal DCs compared to vehicle group. Consistent with this, UVB induced migration of dermal DC subsets, CD207$^-$ and CD207$^+$ CD103$^+$ was also reduced in CD11c-DNR mice compared to WT and it correlated with a decrease in activation marker-CD86.



UV induced T cell activation and skin infiltration as a result of enhanced dermal DC migration

Figure 4.3 **TGFβ1 signaling is required for UVB-induced inflammation within the skin:**

Consistent with decreased migration of dermal DCs with SB inhibition as well as DNR mice in response to UVB, there was suppression in activation of CD4$^+$ and CD8$^+$ T cells in terms of the memory effector phenotype and IFNγ secretion relative to WT mice.

In DNR mice, this suppression of T cell activation resulted in reduced responses to hapten in Contact Hypersensitivity model leading to reduced ear thickness compared to WT mice.

Further in a UVB carcinogenesis model, TGFβ1 pathway inhibition with SB led to fewer tumors than the vehicle-treated group, which correlated with decreased infiltration and activation status of CD4$^+$ and CD8$^+$ T cells.

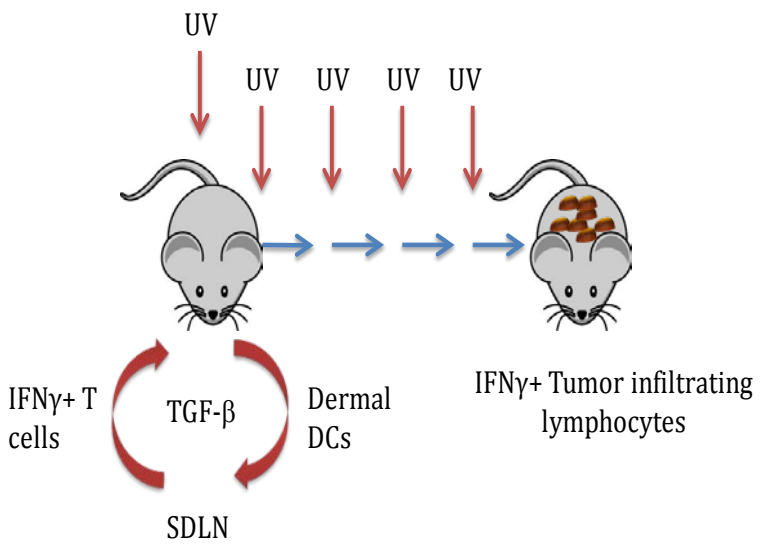


Figure 4.4 **Proposed model: Tumor Promoting Role of TGFβ1 Signaling in Ultraviolet B-Induced Skin Carcinogenesis is Associated with Cutaneous Inflammation and Lymph Node Migration of Dermal Dendritic Cells:**
Chronic UV exposure leads to repeated activation of the DC-T lymphocyte immune response leading to chronic inflammation in the skin which is dependent upon TGFβ signaling. Along with accumulation of mutations in the cells, this inflammatory response ultimately leads to the formation of skin cancers.

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EDUCATION

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HONORS

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2013: Dermal Toxicology Best Abstract award, Society of Toxicology (SOT).
2012: Poster Presentation Award at the Pathobiology Symposium, Penn State University.
2011: NIH Graduate Training Grant Fellowship in "Animal Models of Inflammation".
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2011: Dermal Toxicology Research award, Society of Toxicology (SOT).

PUBLICATIONS

Ravindran A, Mohammed J, Gunderson A, Cui X, Glick AB. Ultraviolet Radiation (UVB)-Induced cutaneous inflammation and lymph node migration of Skin Dendritic Cell subsets is dependent on TGFβ Signaling. *Under review in Carcinogenesis.*

Hogan K, **Ravindran A**, Podolsky M, Glick AB. The TGFβ Pathway is required for NFκB dependent gene expression in mouse keratinocytes. *Cytokine: 2013 Dec;64(3):652-9.*

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POSTER PRESENTATIONS

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