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Intravital imaging of dynamic behaviors of leukocytes in UVB-induced skin inflammation

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The University of Toledo

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A Dissertation

entitled

Intravital Imaging of Dynamic Behaviors of Leukocytes in UVB-induced Skin Inflammation

by

Ran Lu

Submitted to the Graduate Faculty as partial fulfillment of the requirements for

Doctor of Philosophy Degree in Biomedical Sciences

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The University of Toledo
May 2013
An Abstract of

Intravital Imaging of Dynamic Behaviors of Leukocytes in UVB-induced Skin Inflammation

by

Ran Lu

Submitted to the Graduate Faculty as partial fulfillment of the requirements for Doctor of Philosophy Degree in Biomedical Sciences

Ultraviolet B (UVB) irradiation diminishes surface densities of Langerhans cells (LCs) in the epidermis of the skin. Other leukocytes, such as neutrophils and macrophages, are recruited to the irradiated sites due to chemo-attracting factors released from the altered microenvironment. However, there is little evidence directly demonstrating the behavior of LCs as well as other leukocytes in response to UVB radiation, and it is also unknown whether cellular interaction occurs during skin inflammation. In the present study, we developed a direct visualization system to observe the dynamic behaviors of both LCs and neutrophils responding to 3000J/m² UVB in the skin. Using intravital confocal microscopy and genetically manipulated transgenic mice, we have found that neutrophils are recruited to inflammatory sites immediately after UVB irradiation. Skin-infiltrating neutrophils can interact with LCs upon activation and can last for several hours. Twenty-four hours after irradiation, LCs migrated to deeper skin layers, resulting in a 30% reduction of LC number in the epidermal compartment, whereas the number of recruited neutrophils increased over time. LCs completely disappeared from the epidermis 4 days after UVB treatment. Depletion of neutrophils by anti-Ly6G monoclonal antibody (mAb)
administration did not affect LCs migration in response to the same UVB radiation, but rather promoted LC precursors’ repopulation to the epidermis after UVB treatment. In addition, depletion of neutrophils greatly diminished the magnitude of UVB-induced ear swelling, suggesting that neutrophils are crucial for UVB induced skin inflammation. Not only do our data unveil the behavior and cell-cell interaction of LCs and neutrophils upon UVB irradiation, they also indicate for the first time that neutrophils play an important role in LC repopulation.
DEDICATION

This work is dedicated to my parents, Rui-shen Lu and Gui-qin Feng, my parents-in-law, Wu-qun Geng and You-jie Zhu, and my sister Yang Lu for their love and support. They always have faith in me and encourage me to pursue this training.

This work is also dedicated to my beloved husband, Shuo Geng, for his full support, understanding, and endless patience. He is not only my wonderful life partner but also my soul mate and makes my life delightful.

To my baby girl, Annie.
ACKNOWLEDGEMENTS

This dissertation and the associated research would not have been possible without the ever-patient guidance of my mentor, Dr. Akira Takashima. Dr. Takashima not only gave me the freedom to make this project my own, but also entrusted me with the task of presenting it at local and national meetings. I am truly grateful for having been given the opportunity to have such valuable learning experiences throughout my training. Also, I would like to acknowledge Colleen Krout, a colleague from whom I learned the basic technique and gained much help in this project’s progression.

I would additionally like to thank Drs. Andrea Kalinoski, Mark Wooten, Kevin Pan, and Anthony Quinn for all their valuable time, constructive suggestions, and criticisms during my study.

Further, for their constant support, I would like to sincerely thank my lab mates Dr. Shuo Geng, Dr. Hironori Matsushima, Yi Yao, Jennifer A Ohtola and Benjamin Chojnacki as well as the many other students, faculty, and staff in the Medical Microbiology and Immunology Department. Thank you all!
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<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>LC</td>
<td>Langerhans cell</td>
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<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>NKT</td>
<td>natural killer T cell</td>
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<tr>
<td>DC</td>
<td>dendritic cell</td>
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<tr>
<td>CLR</td>
<td>C-type lectin receptor</td>
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<tr>
<td>Ep-CAM</td>
<td>epithelial cell adhesion molecule</td>
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<td>CHS</td>
<td>contact hypersensitivity</td>
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<tr>
<td>MHC II</td>
<td>major histocompatibility complex II</td>
</tr>
<tr>
<td>dSEARCH</td>
<td>dendrite surveillance extension and retraction cycling habitude</td>
</tr>
<tr>
<td>EGFP</td>
<td>enhanced green fluorescent protein</td>
</tr>
<tr>
<td>DT</td>
<td>diphtheria toxin</td>
</tr>
<tr>
<td>DTR</td>
<td>diphtheria toxin receptor</td>
</tr>
<tr>
<td>TGFβ</td>
<td>tumor growth factor β</td>
</tr>
<tr>
<td>ID2</td>
<td>inhibitor of DNA binding 2</td>
</tr>
<tr>
<td>RUNX3</td>
<td>runt-related transcription factor 3</td>
</tr>
<tr>
<td>M-CSF</td>
<td>macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>FLT3L</td>
<td>FMS-like-tyrosine-kinase 3 ligand</td>
</tr>
<tr>
<td>HSV</td>
<td>herpes simplex virus</td>
</tr>
<tr>
<td>RANKL</td>
<td>receptor activator of NFκB ligand</td>
</tr>
<tr>
<td>Treg</td>
<td>regulatory T cell</td>
</tr>
<tr>
<td>SCC</td>
<td>squamous cell carcinoma</td>
</tr>
<tr>
<td>BCC</td>
<td>basal cell carcinoma</td>
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<tr>
<td>DTH</td>
<td>delayed type hypersensitivity</td>
</tr>
<tr>
<td>IL-1</td>
<td>interleukin-1</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor-α</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>PET</td>
<td>positron emission tomography</td>
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<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
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<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
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<td>i.v.</td>
<td>intravenous</td>
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<tr>
<td>LN</td>
<td>lymph node</td>
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<tr>
<td>WT</td>
<td>wild type</td>
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<tr>
<td>MED</td>
<td>minimal erythema dose</td>
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INTRODUCTION

In the epidermal compartment of skin, LCs represent a typical immature dendritic cell (DC) subset that is characterized by the extension of long dendritic processes (Banchereau and Steinman, 1998; Romani et al., 2003; Larrengina and Falo, 2005). A major task of LCs in the steady state is to capture surrounding materials (including antigens) by macropinocytosis, endocytosis, and phagocytosis. LCs are generally believed to serve as the primary antigen-presenting cells in the skin (Steinman and Banchereau, 2007; Ginhoux et al., 2006b; Sakaguchi et al., 2008). On the other hand, LC depletion studies with genetically-engineered mouse lines expressing diphtheria toxin (DT) or diphtheria toxin receptor (DTR) in a langerin-specific manner (Bennett et al., 2005; Kaplan et al., 2005; Kissenpfennig et al., 2005) showed opposite effects on allergic contact hypersensitivity (CHS) responses. It appeared that LC exhibition of different functions depends on mouse lines, timing of LC removal, and skin sensitizing protocol (Kaplan et al., 2008). Thus in vivo functions of LCs still remain controversial.

Most of UVB-induced effects take place in the epidermis due to the fact that the epidermis builds the outmost skin layer and UVB cannot penetrate it. UVB irradiation could affect immune function and result in suppressed skin immunity (Kim et al., 1990; Hart et al., 2002; Granstein and Matsui, 2004; Woods et al., 2005). On the cellular level, UVB irradiation can cause reduction of LCs in the epidermis in both human and mice (Austaad and Braathen, 1985; Vermeer and Streilein, 1990; Duthie et al., 2000). The resulting loss of LCs may be related to UVB-induced immunosuppression. Release of inflammatory cytokines such as interleukin 1 (IL-1) or tumor necrosis factor α (TNF-α)
(Takashima and Bergstresser, 1996) from damaged keratinocytes and infiltrated cells may contribute to UVB-induced sunburn reaction including skin redness, heat and swelling.

Analysis of leukocyte behavior in the skin is essential for understanding cellular mechanisms that contribute to normal and diseased states. Recent advances in mouse genetic engineering and intravital confocal imaging have enabled direct visualization of cellular behaviors in multiple organs in living mice. Working with I-Aβ-enhanced green fluorescent protein (EGFP) knock-in mice, in which the endogenous major histocompatibility complex (MHC) class II I-Aβ chain is replaced by an EGFP-tagged version (Boes et al., 2002), we recorded four dimensional behaviors of EGFP+ LCs in the ear skin (Nishibu et al., 2006). Under the steady state, EGFP+ LCs exhibited a unique motile activity, termed dendrite surveillance extension and retraction cycling habitude (dSEARCH), characterized by repetitive extension and retraction of dendrites. Local skin inflammation augmented both dendrite movements and lateral displacement of cell bodies. By using another transgenic mouse line in which DsRed fluorescent protein is expressed under the control of mouse IL-1β promoter, we directly visualized the behaviors of DsRed+ cells in the skin both in the steady state and after topical application of contact sensitizer, oxazolone (Matsushima et al., 2010). Little DsRed signal was detected in skin under the steady state whereas marked elevated DsRed signal was found upon skin oxazolone treatment. Cell identity analysis showed that most DsRed+ cells were neutrophils and monocytes in both epidermal and dermal compartments. Interestingly, the DsRed+ neutrophils/monocytes preferentially emerged around hair follicles.
Daniel Kreisel et al. reported neutrophil extravasations during pulmonary infection by using two-photon imaging system and LysM-EGFP mice, in which endogenous neutrophils are brightly labeled and monocytes and macrophages are labeled to a lesser extent. They found that the lungs of LysM-EGFP mice contained a significant pool of tissue neutrophils in the steady state and during inflammatory conditions induced by bacterial challenge. Neutrophils were rapidly recruited from circulation and distributed in the interstitium of the lung (Kreisel et al., 2010).

The skin constitutively encounters numerous insults from the outside including bacterial, viral and fungal infections, mechanical trauma stimulation, and ultraviolet irradiation from sunlight. Cellular traffic as well as cell-cell interactions in the skin in response to inflammatory stimuli have not been studied.

The aim of our study is to better understand the cellular traffic and interactions between different cell types in the skin under inflammatory conditions induced by UVB irradiation. For this purpose, we recently developed a new transgenic mouse line by cross breeding I-Aβ-EGFP knock-in mice with pIL1-DsRed transgenic mice. In this double mutant strain, we can directly visualize the dynamic behavior of EGFP+ LCs and DsRed+, activated neutrophils in skin under the steady state and during UVB-induced skin inflammation. We believe that direct information gained from intravital imaging could help us to better understand UVB related skin biology.
LITERATURE REVIEW

Skin Immune System and LCs

As the primary interface between the body and the environment, the skin provides the first line of defense against microbial pathogens and physical and chemical insults. A growing body of data supports the notion that the skin, which was previously recognized as a passive barrier, now has essential immunological functions that exist during not only tissue homeostasis but also various pathological conditions.

Human skin has two main compartments: the epidermis and the dermis. The epidermis is the outmost layer and contains four strata. The bottom layer of epidermis is the stratum basale and is responsible for constant cell renewal of the epidermis. This layer contains just one row of undifferentiated cells known as basal keratinocytes that divide constantly. Basal keratinocytes not only differentiate and move to the upper layer, known as the stratum spinosum, to start a differentiation process, but also can divide to renew the basal layer. Keratinocytes in the stratum granulosum are characterized by dark cytoplasmic material and actively produce keratin proteins and lipids. The stratum corneum is the final differentiation state of keratinocytes, and as the outmost layer of the epidermis, it is largely responsible for the barrier function of the skin. Cells in the stratum corneum layer are dead keratinocyte–derived cells without organelles. They provide a barrier that protects against toxic agents and prevents skin dehydration (Proksch et al., 2008). Specialized cells in the epidermis include melanocytes, which produce the pigment
melanin, and LCs, which are the main skin-resident immune cells and locate in the suprabasal layer of skin. In mice epidermis, LCs usually can be found at 15-25µm beneath the surface (see skin structure below). In addition, T cells, mainly CD8^+ T cells, can be found in the stratum basale and stratum spinosum (Krueger and Stingl, 1989).

The epidermis has a relatively simple structure, but the underlying dermis is more complex, with greater cell diversity. It contains many specialized immune cells, including DCs, macrophages, CD4^+ T cells, γδ T cells, natural killer T (NKT) cells, mast cells, fibroblasts, and some nerve-related cells. Lymphatic vessels as well as blood vessels are also located in the dermal layer to support migrating cell traffic.

![Skin Structure Diagram](image)

Characterization of LCs
LCs are named after Paul Langerhans, who reported the presence of dendritic, non-pigmentary cells in the epidermis. At that time, LCs were regarded as intra-epidermal receptors for extracutaneous signals of the nervous system (Langerhans, 1868). This concept persisted for over a century until the recognition of LCs as leukocytes derived from bone marrow belonging to a DC population located in the epidermis (Schuler and Steinman, 1985). Epidermal LCs account for 3-5% of all cells in the epidermal compartment of mice and humans, and they continuously reside in the suprabasal layers with even distribution among keratinocytes. In human skin, LCs are the only hematopoietic cells in the epidermis, although mice have an additional population of epidermal γδ T cells (Tigelaar et al., 1988). The reason for this striking difference between human and mice remains to be determined.

**Surface molecules expressed by LCs**

Human and mouse LCs can be easily identified in the epidermis based on their expression of CD45 and MHC II molecules (Stingl and Shevach, 1991). A unique feature for LCs is their expression of intracytoplasmic organelles known as Birbeck granules. These organelles were identified in 1961, but since then, their role has remained poorly understood (Djavaheri-Mergny et al., 1994). Traditionally, LCs have been distinguished from other cells by the expression of langerin in mice and CD1a in humans (Fithian et al., 1981). Langerin is a type II C-type lectin receptor (CLR) that binds mannose and related sugars in a calcium-dependent manner through its carbohydrate-recognition domain (Valladeau et al., 2000). Binding of surface langerin on human LCs by a langerin-specific antibody induces the internalization and trafficking of langerin to the Birbeck granules.
A human homologue of langerin in mice has also been identified on the surface of mouse epidermal LCs, and its engagement also induces the formation of Birbeck granules (Valladeau et al., 2002; Dupasquier et al., 2004). Human and mouse LCs constitutively express E-cadherin, an adhesion molecule that anchors LCs to their neighboring keratinocytes (Tang et al., 1993). E-cadherin binds to CD103 (also known as αE-integrin), which can be found in epithelial T cells and a subset of DCs (Siddiqui et al., 2010). Epithelial-cell adhesion molecule (EpCAM) is another adhesion molecule expressed by human and mouse LCs (Borkowski et al., 1996). LCs also constitutively express CD205 (also known as DEC205), a lectin that is probably involved in antigen capture and processing (Witmer-Pack et al., 1995; Jiang et al., 1995). Human, but not mouse, LCs express high levels of CD1a.

Migration of LCs

LCs migrate to draining lymph nodes in the steady state (Hemmi et al., 2001), and their migration rate increases during inflammatory conditions (Stoitzner et al., 2005; Stoitzner et al., 1999; Stoitzner et al., 2003). After leaving the epidermis, LCs travel through the dermis, drain into lymphatic vessels, and arrive at the T cell areas of the lymph nodes (Knight et al., 1998). On the way to draining lymph nodes, LCs increase the expression of surface maturation marker--MHC II (Larsen et al., 1990; Larsen et al., 1994; Pierre et al., 1997). Other co-stimulatory molecules such as CD40 and CCR7, which is essential for LCs migration to draining lymph nodes, are also up-regulated (Ruedl et al., 2000; Ohl et al., 2004). On the other hand, E-cadherin is down-regulated. This molecule normally functions as an anchor of LCs to the neighboring keratinocytes in the epidermis, and the
decreased expression of this molecule facilitates LCs moving to the deeper layer from the epidermis. Migratory LCs also increase the expression of CD205 and form the characteristic MHC II$^{hi}$/CD11c$^{+}$/CD40$^{hi}$/CD205$^{hi}$/langerin$^{+}$ cells that can be found in the T cell zone of skin draining lymph nodes (Inaba et al., 1995).

The process of LC maturation has been well characterized in the context of inflammation. It also has been reported that LCs undergo maturation in the steady state (Reis e Sousa, 2006). Evidence suggests that the decreased expression of E-cadherin on bone marrow derived DCs in the absence of inflammatory stimuli causes the phenotypic maturation and up-regulation of CCR7 (Lukas et al., 1996). The maturation process in the steady state shows similar phenotypic changes in DCs as during inflammation; however, the overall effect each process has on the immune response is different (Reis e Sousa, 2006). DCs that mature in response to microbial infection release pro-inflammatory cytokines that promote the induction of an immune response, whereas E-cadherin-involved DC maturation probably leads to immune tolerance in vivo (Riedl et al., 2000). Because LCs express E-cadherin in vivo in the steady state, it is important to study whether E-cadherin participates in LC migration to skin draining lymph nodes, as well as its role in the induction of tolerogenic response in vivo.

Many groups chose to use in vitro generated CD34$^{+}$ hematopoietic progenitor cells or isolated primary epidermal LCs to study the biology of LCs in vitro. Some key markers of LCs such as langerin expression have been identified by using in vitro systems. However, it remains unclear how in vitro culture conditions affect the maturation process of LCs. Therefore, it is difficult to study the factors that influence LC maturation in vitro.
Purification of LCs from human or mouse can be achieved through specialized methods, however, these methods also can trigger the maturation of LCs. It is important to consider the source of LCs when studying their biology, as the maturation of LCs can greatly affect the expression of surface markers (Reis e Sousa, 2006).

**Origin and homeostasis of LCs**

LCs repopulate locally in the steady state. The origin of epidermal LCs has remained a subject of debate for many years since their discovery. LCs were once regarded as part of the peripheral nervous system, as related to the melanocytes, or as ectodermal in origin (Stingl et al., 1978). An important study verifying the hematopoietic origin of LCs was published in 1979. This milestone study showed that in mice that had been lethally irradiated and reconstituted with donor allogenic hematopoietic progenitor cells, a portion of LCs were found to be derived from the donor origin after transplantation (Katz et al., 1979). These observations led to the idea that epidermal LCs are replenished by circulating hematopoietic precursor cells during adult life. However, many studies contradict this notion. In mice that have been lethally irradiated and reconstituted with congenic bone marrow cells, LCs were partly depleted by irradiation. Repopulation of LCs in the skin was independent of circulating precursor cells and remained of host origin throughout life; yet, most lymphoid tissue DCs and DCs in the airways and vaginal mucosa were replaced by circulating donor-derived precursor cells after transplantation (Holt et al., 1994).

Another study using BrdU labeling of the epidermal sheet confirmed the ability of LCs to repopulate themselves locally, independently of circulating precursor cells. The rate of
BrdU incorporation in transplanted mice indicated that host LCs were proliferating in situ and were not coming from bone marrow-derived progenitor cells (Merad et al., 2002). Parabiosis experiments in which two congenic mice share one blood system also further confirmed the ability of LCs to repopulate independently from locally circulating precursors. In such an experiment, up to 30% mixing of DCs were found in the spleen and lymph within 2 months; yet, LCs failed to mix in parabionts up to 6 months. This also supports the notion that LCs have a unique feature of renewing and maintaining themselves locally in the steady state (Merad et al., 2002).

Taken together, these studies show that epidermal LCs have unique origins in the steady state. In contrast to other DC populations in the spleen or mucosal epithelium, epidermal LCs are maintained either through self-renewal or from some local radio-resistant proliferating host progenitors that reside in the skin.

The exact half-life of LCs in the skin has been clearly established by using in vivo imaging of LCs that express enhanced green fluorescent protein (EGFP). The turnover rate of epidermal LCs is slow in the steady state, with an estimated half-life of 53-78 days (Vishwanath et al., 2006) which is consistent with earlier in vivo labeling studies (Ruedl et al., 2000; Kamath et al., 2002). LC conditional ablation by using langerin-DTR transgenic mice showed that repopulation of LCs was slow, requiring several weeks for reconstitution (Kissenpfennig et al., 2005; Bennett et al., 2005). In contrast, conventional DCs in the spleen or lymph nodes only need several days to reconstitute.

Studies about LC origin in humans are much more difficult to perform but data suggest human LCs share similar properties with mice. LCs from human and non-human
primates proliferate in situ (Hayashi et al., 1993), and donor LCs were shown to remain of host origin in human skin that was transplanted onto immunodeficient mice (Krueger et al., 1983). In addition, it was reported that in one patient who had received a limb graft, LCs remained of host origin and were not replaced by donor-derived precursor cells for over one year after transplant (Kanitakis et al., 2004). Another study reported that in the absence of graft-versus-host disease, host LCs can remain in the skin despite receiving allogenic hematopoietic stem cell transplants (Merad et al., 2004). These results established that similar to mice LCs, human LCs can renew locally and persist in the skin even in radiation-based transplantation.

**Regulation of LC homeostasis in the steady state**

The mechanisms underlying LC homeostasis in the steady state still need to be fully elucidated. Studies using transmission electron microscopy (Czernielewski et al., 1985; Czernielewski and Demarchez, 1987; Collin et al., 2006; Miyauchi and Hashimoto, 1987) and fluorescent microscopy following Ki67 staining, have demonstrated that proliferating LCs can be observed throughout the epidermis. These results show that 2-3% of LCs are actively dividing in the mouse and human epidermis. Although this may be enough for LCs to maintain their number in the steady state, some studies propose the existence of specialized LC precursors that reside in the skin and are activated following skin injury. The bulge region of hair follicles in the dermis serves as a niche for keratinocytes, melanocytes, and mast cell progenitors (Kumamoto et al., 2003; Blanpain and Fuchs, 2006). There is evidence suggesting that LCs can be repopulated from hair follicles alone following skin injury of epidermis, but not dermis where hair follicles are located.
(Gilliam et al., 1998). Based on these results, it is reasonable to propose that differentiated LCs that are generated through self-renewal and from specialized local precursor cells could both contribute to the homeostasis of LCs depending upon different physiological conditions.

A growing body of studies has identified developmental requirements for LCs. Mice lacking tumor growth factor β-1 (TGFβ-1) do not contain LCs because of failure of LCs to differentiate and survive (Strobl et al., 1996). TGFβ-1 can be released from both keratinocytes and LCs; however, an autocrine source of TGFβ-1 is required for LC development (Kaplan et al., 2007). In humans, TGFβ-1 is crucial for the differentiation of CD34+ hematopoietic progenitor cells and might be important for human LC development (Strobl et al., 1996). Mice deficient for inhibitor of DNA binding 2 (ID2), which is a TGFβ-1 induced inhibitor of helix-loop-helix transcription factors, also lack LCs (Hacker et al., 2003), but the exact role of ID2 in LCs development is still unclear. Run-related transcription factor 3 (RUNX3) is another molecule required for LC development. RUNX3 is expressed by mature DCs and mediates DC response to TGFβ-1 while deficiency in RUNX3 leads to a lack of LCs in mice (Fainaru et al., 2005).

In addition to TGFβ-1, some groups have recently shown that the receptor for macrophage colony-stimulating factor (M-CSF) is required for the development of LCs, but not for DCs in the spleen (Onai et al., 2007). By contrast, mice that carry a mutation in the gene encoding M-CSF have normal numbers of LCs (Witmer-Pack et al., 1993), suggesting the existence of another ligand for the M-CSF receptor that could compensate for the absence of M-CSF. Consistent with this possibility, interleukin-34 has only been
recently identified as a ligand for M-CSF receptor in mice and humans (Lin et al., 2008), and now it is now clear that interleukin-34 is essential for the development of LCs as well as microglia (Wang et al., 2012). FMS-like-tyrosine-kinas 3 ligand (FLT3L) is a cytokine important for the development of DCs in spleen and lymph nodes; however, LCs are not affected by the deletion of FLT3L (Cherr et al., 2001).

Taken together, these results demonstrated that LCs arise along a unique developmental pathway that depends on M-CSFR and TGFβ-1, but not FLT3L.

**LC homeostasis under inflammatory conditions**

LCs use different renewing mechanisms during skin inflammation compared to that in the steady state. UVB irradiation can lead to skin inflammation and severe LC loss (Ginhoux et al., 2006a; Merad et al., 2002; Merad et al., 2004). Repopulation of LCs is mediated by circulating monocytes expressing Gr-1 and CD115 (Ginhoux et al., 2006a; Merad et al., 2002; Merad et al., 2004). Repopulation of LCs in inflamed skin depends on M-CSFR signaling, but also requires CCR2 and CCR6 expressed by circulating cells (Merad et al., 2002; Merad et al., 2004; Bogunovic et al., 2006). However, mice that lack CCR2 or CCR6 have normal numbers of LCs in the skin. Studies using human cells in vitro also had similar observations (Vanbervliet et al., 2002). CD34+ hematopoietic progenitor cells give rise to LCs with increased expression of CCR2 and CCR6. Monocytes seem to respond to CCR2 ligands expressed in vascular region of the dermis and then differentiate into LCs in response to CCR6 ligands released from the keratinocytes at the epidermal-dermal junction (Vanbervliet et al., 2002). Circulating monocytes (Geissmann et al., 1998) and CD14+ dermal cells (Larregina et al., 2001) isolated from human skin
can differentiate into CCR6+/langerin+ cells in vitro in response to TGFβ-1. In one study of human skin transplantation, researchers found higher numbers of donor LCs in patients with skin injury due to graft-versus-host disease compared to patients without skin inflammation (Collin et al., 2006). These results clearly indicate that factors affecting LC homeostasis depend on skin conditions. In intact skin, LCs maintain themselves locally through self-renewal, through specific host precursors, or by both. Under inflammatory conditions in which specific host precursors have been eliminated, LCs are replenished by circulating monocytes through a M-CSFR-dependent pathway.

**Role of LCs in skin immunity**

Previous in vitro studies demonstrate that LCs can prime T cells, suggesting that LCs serve as antigen presenting cells in the skin (Schuler and Steinman, 1985). However, a more recent study showed that migratory LCs in skin draining lymph nodes failed to prime antigen-specific CD8+ T cells after herpes simplex virus infection. This finding came as a surprise and prompted further investigation of the role of LCs in vivo. Another study using lentiviral intradermal injections showed that migratory skin DCs have a crucial role in the induction of antigen-specific CD8+ T cells (He et al., 2006). The role of skin-derived DCs in priming viral-specific T cells in response to lentiviral vector but not herpes simplex virus (HSV) infection suggests that cytolytic virus may have the ability to inhibit the capacity of LCs to prime specific CD8+ T cell responses. Supporting evidence for this hypothesis can be found in one study in which the authors used cytolytic viral vaccine and HSV1 to infect mice and found that these viruses could induce apoptosis of DCs and limit the ability of skin-derived DCs to prime T cell responses.
Taken together, the contribution of LCs or dermal DCs to anti-viral immunity in the skin varies based on the different virus models that have been used.

The function of human LCs has been studied only in vitro. LCs isolated from human skin can efficiently induce CD4+ T cell responses and cross-present soluble antigens to CD8+ T cells. On the other hand, dermal DCs are more potent in initiating another subset of CD4+ T cells-follicular T-helper cells-and assisting B cell immunoglobulin class switching (Klechevsky et al., 2008). These results are consistent with a study in which langerin-DTR-EGFP knock-in mice were used to trace migratory langerin+ DCs to the lymph nodes. The data showed that langerin+ dermal DCs and langerin− dermal DCs travel to different areas in the lymph nodes; the former population is preferentially located in the paracortex area where T cells most reside, whereas the latter population remained near B cell follicles in lymph nodes (Kissenpfennig et al., 2005). The exact role of dermal langerin+ DC and langerin− DC has not yet been tested.

**Role of LCs in contact dermatitis**

Studies concerning allergic contact dermatitis usually use a model of CHS response to haptens. The role of LCs in this disease model has been studied extensively, but as yet, a conclusive notion regarding their specific role has not been established. The use of a LC conditional ablation mouse model, in which the DTR is expressed under the control of the langerin promoter, greatly enhances the process of direct assessment of LC functions in vivo.

Initial studies using langerin-DTR transgenic mice showed that dermal DCs are required for CHS whereas LCs are dispensable. In these studies, they immunized the mice either
by skin sensitization or with a gene gun containing DNA-coated gold particles in the ear, allowing the haptens or antigens to skip the epidermal compartment and reach to the dermal layer of the skin (Kissenpfennig et al., 2005; Stoecklinger et al., 2007). However, more recent studies have shown that LCs are required for cutaneous sensitization to haptens, but only when the antigen is administered to the epidermis and no diffusion occurs to the dermal layer (Wang et al., 2008; Stoecklinger et al., 2007). It is also surprising that when mice lack LCs, cutaneous sensitization to hapten was found to be enhanced. This suggests that other mechanisms might compensate LC function and regulate skin immunity in a LC deficient mouse model (Kaplan et al., 2005). These results emphasize that it is critical to choose the right model to study the contribution of LCs or any other specific DC subset in skin immune responses.

**Role of LCs in the induction of tolerance**

LCs can induce skin tolerance to peripheral antigens in the steady state. The capacity for LCs to transport and present skin antigens to the draining lymph nodes under the steady state was reported in a study in which mice deficient in TGFβ-1 possessed higher numbers of melanocytes and melanin granules in the epidermis and lacked melanin granules in the lymph nodes. Because LCs are the only skin DCs that require TGFβ-1 for their development, these results suggest that LCs can transport melanin granules into the draining lymph nodes in the steady state (Borkowski et al., 1996). The role of LCs in induction of tolerance was also suggested in another study. Receptor activator of NFκB ligand (RANKL) was over-expressed by keratinocytes in a transgenic mouse strain, where LCs were found to have decreased expression of co-stimulatory molecules and
increased numbers of systemic CD4\(^+\) CD25\(^+\) regulatory T cells (Treg). These results led the authors to propose a potent role for LCs in induction of Treg cells in response to RANKL expression during inflammation (Loser et al., 2006). In addition, LCs were found almost completely absent from epidermis in a mouse model in which keratinocytes over-expressed CD40 ligand. These mice developed spontaneous inflammatory skin lesions that resembled chronic autoimmune disease, indicating that in situ activation of LCs can lead to systemic autoimmune disease and further suggesting that skin tolerance to antigen is disturbed when LCs are activated and migrate to draining lymph nodes (Mehling et al., 2001).

**Ultraviolet Radiation and the Skin**

The sun emits a wide spectrum of electromagnetic radiation that includes very short wavelength radiation such as X rays and gamma rays, as well as very long wavelength radiation such as microwave radiation. The wavelengths of sunlight span between ionizing radiation (<100 nm) and visible light (400-800 nm). UV wavelengths are further divided into UVC (100-290 nm), UVB (290-320 nm), and UVA (320-400 nm) (Brash et al., 1996). Solar radiation is filtered by the stratospheric ozone layer so that all ionizing and UVC radiation and the majority of UVB radiation are blocked from reaching the earth’s surface. Thus, we receive a natural exposure composed of primarily UVA, some UVB, visible light, and infrared radiation (>800 nm).
When solar light reaches the earth’s surface and comes into contact with human skin, UVC and UVB can be absorbed by DNA of the skin cells and have mutagenic effects. Of the solar radiation reaching the earth surface, UVB is the most carcinogenic and is responsible in producing sunburn and delayed tanning. By contrast, UVA is considered effective to produce immediate tanning. It is believed that some photoreceptors such as proteins, lipids, and nucleic acids in skin may absorb UVB, UVA, and visible radiation. After absorbing photons, these receptors achieve an exited state that transforms them into specific photoproducts such as DNA pyrimidine dimers (Simon et al., 1994) and oxidized membrane lipids (Mittal et al., 2003). Photoprodutcs trigger a cascade of events that may include phosphorylation, signal transduction, and transcription factor activation leading
to protein synthesis (e.g., melanin and cytokines) or activation of enzymes (e.g., repair of DNA damage). This in turn produces cellular changes such as apoptosis, mitosis, differentiation, or transformation, which may result in inflammation (sunburn), melanogenesis (tanning), carcinogenesis, or autoimmunity (Cleaver and Crowley, 2002).

**UV-induced skin cancer**

Human skin is exposed daily to UV from the sun, occupational light sources, and phototherapy systems. In photobiology, the joule (J) is the unit of energy most frequently used to describe the amount of light energy. The energy of light applied to skin is called fluence and is expressed in J/cm$^2$ or J/m$^2$. The rate of UV delivery is called irradiance or intensity and is expressed in W/cm$^2$ or W/m$^2$. Fluence and irradiance are related by the following equation: Fluence (J/m$^2$) = Irradiance (W/m$^2$) $\times$ Time (s) (Anderson and Parrish, 1981).

Skin cancer is by far the most common kind of cancer diagnosed in many western countries and ultraviolet radiation is the most important risk factor for cutaneous squamous cell carcinoma (SSC) and basal cell carcinoma (BCC) (Granstein and Matsui, 2004). There are about 200,000 new cases of SCCs diagnosed each year in the USA, with between 1300 and 2300 deaths per year from metastatic carcinomas (Hodges and Smoller, 2002). Solar irradiation can mutagenize DNA, often producing UV-signature mutations (C$\rightarrow$T or CC$\rightarrow$TT) usually via cyclobutan dimers. When these mutations affect the function of sufficient oncogenes, tumor-suppressive genes, and some important housekeeping genes that cause the cell cycle to become out of control, transformation of keratinocytes and melanomacytes occurs (Kramer et al., 1990). Rather than mutating
DNA directly, UV-induced alteration in signal transduction may affect mutation frequency indirectly such as by altering the cell cycle to allow less time for DNA repair or by reducing the levels of enzymes that protect cells from UV damage (Daya-Grosjean et al., 1995).

During the development of UV-induced skin cancer, variable alterations of the oncogenic, tumor-suppressive, and cell-cycle control signaling pathways occur. These pathways include (a) mutated PTCH (in the mitogenic Sonic Hedgehog pathway) and mutated p53 tumor-suppressor gene in basal cell carcinomas, (b) an activated mitogenic Ras pathway and mutated p53 in squamous cell carcinomas, and (c) an activated Ras pathway, inactive p16, and p53 tumor suppressors in melanomas.

**UVB-Induced Immunosuppression**

**Immune suppression induced by UV irradiation**

Photoimmunology is a discipline that deals with the effects of light on the immune response. It includes elements of photobiology, immunology, and dermatology. The notion of UV light affecting the immune function grew from the studies originally done by Margaret Kripke. The focus of her work was to study the biology of UV-induced skin cancers. Tumors were induced by chronic exposure to UV radiation provided by a bank of fluorescent sunlamps. The tumors were then transplanted into normal age and sex-matched recipient mice. Surprisingly, none of the tumors grew progressively in the normal immunocompetent recipient mice. Apparent tumor growth was observed only in
recipient mice that had been immunosuppressed. This study indicated that tumors induced by UV irradiation were highly antigenic. They were rejected by a normal immune system and would only grow progressively if the recipient mice were immunocompromised. The recognition of the antigenic property of UV-induced skin tumors explains why tumors were rejected by the normal immune system of recipient mice; however, how these tumors developed in the first place was still questionable. One interpretation is that chronic exposure to UV radiation may have two outcomes: skin cancer induction and immune suppression. Experimental proof of this hypothesis was provided the observed progressive growth of transplanted tumors in mice that were exposed to a sub-carcinogenic dose of UV irradiation (Kripke, 1974). Subsequent studies by Kripke and co-workers (Fisher and Kripke, 1982; Kripke et al., 1979; Ullrich, 1994) and Daynes and Spellman (Roberts et al., 1989) indicated that exposure of mice to UVB suppressed alloantigen-induced hypersensitivity by stimulating antigen-specific suppressor T cells, and transfer of these T cells to intact mice induced immune suppression in the recipient mice. Moreover, they found that suppressor T cells in the lymph nodes of UV-irradiated mice were critical for the induction of skin cancer (Magee et al., 1989). A recent study indicated UV-induced suppressor T cells that control skin cancer rejection belong to a population of IL-4 secreting, CD1-restricted, immune regulatory T cells known as NKT cells (Moodycliffe et al., 2000).

Data derived from humans also support the hypothesis that UV-induced immune suppression plays a critical role in skin cancer induction. Many reports have documented that patients who receive immunosuppressive therapy have an increased risk of skin cancer (Ullrich, 2007; Streilein et al., 1994; Granstein and Matsui, 2004; Euvrard et al.,
Most skin tumors appear on sun-exposed sites, suggesting that immune responses are involved in the process of UV induced skin cancer. Other groups also made the observation that UV suppresses the induction of immunity in human volunteers (Cooper et al., 1992; Tie et al., 1995; Kelly et al., 1998). Moreover, the correlation between UV-induced immunosuppression and skin cancer induction has been documented. When healthy age-matched volunteers were sensitized through UV-irradiated skin, about 40% of them were susceptible to the effects of UV-induced immunosuppression; on the other hand, nearly all patients diagnosed with squamous or basal cell carcinomas were susceptible to immunosuppressive effects of UVB irradiation (Yoshikawa et al., 1990). Other studies also confirmed that immune function of patients with skin cancer were suppressed (Czarnecki et al., 1995). Based on data generated from both animal experiments and human patients, it is safe to say that immune suppression induced by UV radiation is a major risk factor for skin cancer development.

In addition to tumor immunity, UV exposure has been shown to suppress a variety of immune reactions, including CHS to chemical haptens (Applegate et al., 1989; Cruz and Bergstresser, 1992) and delayed type hypersensitivity (DTH) to viral (Howie et al., 1986), bacterial (Jeevan et al., 1992), and fungal antigens (Denkins et al., 1989).

In the epidermis, LCs form a network that functions to capture invading microorganisms, ingest them, and then process antigen into a form that can be recognized by T cells. During migration out of the epidermis and into the skin-draining lymph node, LCs undergo maturation with increased surface expression of MHC II as well as co-stimulatory molecules. After arriving at the T cell area of the draining lymph node, LCs
present processed antigens to specific T cells, thus initiating a protective immune response against threats to the host.

UV exposure alters epidermal LC function and destroys the DC network. Mice sensitized through UV-irradiated skin are unable to generate a CHS response to contact allergens (Toews et al., 1980). These mice also fail to react to the application of the same hapten at a later time, at a distant site. Moreover, antigen-specific suppressor cells were found in the spleen of mice sensitized through UV-irradiated skin. (Elmets et al., 1983)

Some studies have provided evidence that LCs are the targets of UV irradiation, and their function is altered after irradiation (Cruz et al., 1990). LCs isolated by flow cytometry were exposed to UV irradiation, conjugated with hapten, and injected into intact mice. Results showed that the response found in mice injected with UV-irradiated LCs was significantly suppressed compared to mice injected with non-irradiated LCs, indicating that LCs mediated the induction of immunosuppression by UV irradiation. Depletion of LCs by UV exposure could attenuate CHS, as evidenced by inhibition of sensitization and inefficient T cell priming (Kaplan et al., 2005). In one study using Langerin-DTR mice to examine UV-induced immunosuppression, LCs were selectively depleted by injection of DT into mice 10 days before UV exposure and hapten sensitization. These mice were resistant to UV induced immunosuppression and CD4+ CD25+ Treg cells did not arise (Schwarz et al., 2010). These results showed that LCs are required for UV-induced immunosuppression. In contrast, another study using LC-depleted Langerin-DTR mice reported that LCs are not required for UV-induced immunosuppression (Wang et al., 2009). Most likely, the controversial results reflect different doses of UV used in
the two experiments. The latter applied too low of UV dose to remove LCs from the epidermis. Another piece of evidence supporting the critical role for LCs in UV-induced immunosuppression is that when Tregs from UV-irradiated mice were transferred to recipient mice after co-culture with LCs, the recipient mice did not develop CHS (Gomez de et al., 2012).

The immediate response to UV is mediated by several mechanisms including direct effects on keratinocytes to release pro-inflammatory cytokines such as IL-1 and TNF-α (Wood et al., 1996); effects of photons on DNA damage (Brash et al., 1996; Cleaver and Crowley, 2002; Simon et al., 1994); depletion of antioxidants and generation of reactive oxygen species (ROS) including hydrogen peroxide, hydroxyl radical, singlet oxygen and peroxyl radicals (Lavker, 1979; Rhie et al., 2001); and generation of prostaglandins and other inflammatory mediators such as histamine and leukotriines by mast cells in skin (Hart et al., 2002). At the cellular level, UV irradiation can trigger cytokine production (Takashima and Bergstresser, 1996), induce surface expression of adhesion molecules (Krutmann and Grewe, 1995) and induce cellular mitosis, apoptosis, and necrosis (Bielenberg et al., 1998). UV irradiation leads to epidermal keratinocyte damage, depletion of LCs, dermal edema, endothelial swelling, mast cell degranulation and cellular infiltration of neutrophils and monocytes into the dermis and epidermis. Skin aging induced by UV irradiation demonstrates decreased epidermal turnover, diminished inflammatory response to UV, and impaired immune function.

**Release of inflammatory mediators in response to UV**
UV radiation, tape stripping and even mechanical stress of skin can cause release of IL-1 from keratinocytes to initiate the inflammatory reactions within the living layers of skin (Wood et al., 1996; Murphy et al., 2000). In addition, UV irradiation stimulates TNF-α release from keratinocytes into the blood stream, suggesting epidermal derived cytokines may mediate systemic inflammatory reactions (Kock et al., 1990). These two primary cytokines can in turn induce the synthesis and release of other pro-inflammatory cytokines in response to UV irradiation (Takashima and Bergstresser, 1996; Hirao et al., 1996; Shreedhar et al., 1998). UV irradiation also dramatically induces the production and secretion of IL-1, IL-3 and IL-6 that are normally low in intact skin. Additionally, UV irradiation of human skin also induces the expression of IL-1, IL-10, and IL-7 in vivo (Brink et al., 2000). IL-10 and IL-12 were reported to be differentially regulated by UV irradiation--UVB up-regulates both IL-10 and IL-12; however, UVA appears to only induce IL-10, but not IL-12, in keratinocytes (Kondo, 1999).

Phagocytic cells such as neutrophils and monocytes infiltrate into skin from capillaries in response to UV irradiation. Cytokines such as IL-1 and TNF-α are released from keratinocytes in the UV irradiated skin. In addition to keratinocytes, the phagocytes themselves secrete cytokines (IL-1, TNF-α, IL-8, etc.) that further enhance the recruitment of inflammatory cells (Ullrich, 1995). Phagocytes recruited and activated by these cytokines generate reactive oxygen species (ROS) and nitric oxide (NO) as part of their defense mechanism. In addition, these cells generate a variety of other oxygen species that help in the killing of microorganisms. Phagocytes induce keratinocytes to synthesize and release elafin, an inhibitor of human neutrophil elastase, and this eventually limits the damage caused by inflammatory neutrophils to skin. Elafin is highly
induced in inflamed skin such as in psoriasis, after skin wounding, and in skin tumors (Pfundt et al., 2001).

**Intravital Imaging Technology**

A central feature of the immune system is the migratory behavior of cellular components. To fully understand the generation and maintenance of immune responses, we must consider how hematopoietic cells home to, interact within, and exit from secondary lymphoid organs as well as peripheral tissues. Recent advances in in situ imaging techniques permit us direct observation of these events in the settings that resemble physiological environments and provide results with high resolution.

Our knowledge of immune cell interactions has primarily come from in vitro studies of various culture systems, ex vivo examination of consequences of cellular interactions in situ, and static histological analyses of lymphoid tissues. All of the above techniques are dispensable for our understanding of immune cell interactions (Jenkins *et al*., 2001). However, these techniques do not allow us to examine the dynamic processes at the single cell level and in a temporal and spatial fashion within the physiologic environment of lymphoid tissues. In the past 10 years, advances in imaging instruments, software, fluorescent reagents, and animal manipulation tools have begun to address these limitations.

The first dynamic imaging method is the use of epifluorescence-based video intravital microscopy (MacPhee *et al*., 1992; Mempel *et al*., 2003). This application provided new insight into cell adherence to and penetration of microvasculature endothelium but did
not allow analysis of cells within densely organized lymphoid tissues. New micro-PET (positron emission tomography) (Herschman, 2003), luminescence (Hardy et al., 2001) and micro-MRI (magnetic resonance imaging) (Kircher et al., 2003) methods have allowed us to study the distribution of lymphocytes in animals, but with limited resolution that does not reveal single cell behavior (Choy et al., 2003). These limitations have been overcome in mouse models by the application of conventional confocal microscopy and by the more powerful approach of two photon laser scanning microscopy. Both confocal microscopy and two photon microscopy limit the collecting light to a small z-direction distance, thereby improving the resolution of objects in three-dimensional space. These techniques have the ability to sequentially collect several colors of x-y data at multiple z levels in a thick specimen. Repetition of this volume imaging over time allows us to track the shape, movement, molecular patterning, and even gene activation responses of multiple cell types simultaneously in a dynamic process with high resolution (Bousso et al., 2002; Bousso and Robey, 2003; Cahalan et al., 2002; Mempel et al., 2004; Stoll et al., 2002).

In the case of two photon microscopy, data collection can extend hundreds of microns into native tissues and imaging can be extended over many hours. This is an exciting development that has already begun to provide new understanding of the dynamic nature of the immune system and it is a revolutionary step in progress to renew our concepts about immune cell behavior.

Currently, two methods of tissue preparation exist for in situ confocal microscopy and two-photon laser scanning microscopy: tissue explants microscopy and intavital
microscopy. Miller et al. (Miller et al., 2002) provided the first detailed, quantitative analysis of the movement of adoptively transferred, ex vivo-fluorescent labeled naïve B cells and CD4 T cells in a lymph node tissue. Both B cells and T cells are extremely mobile, moving at 11 and 6µm/min, respectively. A similar high speed motility was also observed for naïve CD8 T cells by Bousso and Robey (Bousso and Robey, 2003) by using an O2-perfused explants method similar to that of Miller et al. Imaging data analysis indicated that up to 500 naïve CD8 T cells probed a single DC every hour in the absence of antigen. A new method of imaging intact lymph node organs in anesthetized animals (intravital microscopy) has been recently developed to provide for more physiological conditions of observation (Miller et al., 2003).

The development and use of fluorescent reporter technology (Zhang et al., 2002) has allowed the investigators to assess specific gene activity (Hu-Li et al., 2001; Mohrs et al., 2001), to observe molecular redistributions such as those occurring during immune synapse formation (Egen and Allison, 2002; Krummel et al., 2000; Wetzel et al., 2002) and to follow intracellular signaling events (Miyawaki et al., 1999; Ting et al., 2001; Delon et al., 2001). Endogenous expression of fluorescent reporters is essential for overcoming the limitation of adoptive transfer of ex vivo manipulated, dye-labeled cells that are problematic with respect to cell biology. The use of CD43-EGFP to reveal DC-T immunological synapse formation in vivo shows the feasibility of this approach (Stoll et al., 2002).

Making use of such technology, we have studied the in vivo LC turnover rate in the epidermis in the steady state by using I-Aβ-EGFP knock-in mice and intravital
microscopy (Vishwanath et al., 2006). Combining these two powerful techniques, we have successfully visualized the dynamic behaviors of LCs in the epidermis in both steady state and under inflammatory conditions such as skin hapten sensitization (Nishibu et al., 2006). LCs amplified their typical movements that we termed “dSEARCH” after skin haptens were painted on the ear surface. This alteration is mediated by inflammatory cytokines such as IL-1 and TNF-α which are released in the painted skin upon sensitization (Austaad and Braathen, 1985). A new transgenic mouse line has also been generated in our lab in which the gene for the red fluorescent protein DsRed is expressed under the control of the 4.1 kb mouse IL-1β promoter. Using this mouse strain and intravital confocal imaging, we have visualized and analyzed the movement of IL-1β producing cells in the ear skin after hapten painting (Matsushima et al., 2010). Imaging technology has greatly improved our understanding of cellular behaviors in immunobiology through high-quality direct evidence.
MATERIALS AND METHODS

Animals

Imaging experiments were conducted in I-Aβ-EGFP knock-in mice (Nishibu et al., 2007; Vishwanath et al., 2006) LysM-EGFP knock-in mice, and langerin-DTR-EGFP knock-in mice (Bennett et al., 2005). Some experiments used C57BL/6 wild type mice. I-Aβ-EGFP/pIL1-DsRed double mutant mice were generated in our laboratory by cross breeding I-Aβ-EGFP and pIL1-DsRed mice. All of the animals were 8 to 16-week old and obtained from Jackson Laboratory and housed in the animal facilities of University of Toledo. The animal experiments were approved by the Institutional Animal Care and Use Committee at the University of Toledo and carried out according to the National Institutes of Health guidelines.

Application of UV irradiation
UVB irradiation (290nm-320nm) was provided by a bank of four TL 20W/01RS lamps (Philips Co, Holland). Mice were anesthetized with anesthetic cocktail (ketamine 100mg/kg, xylazine 10mg/kg, and acepromazine 1mg/kg), and left ears were exposed to single dose of UVB light at 3000J/m². The rest of the mouse body was protected from UVB irradiation by covering with a black sheet (shown in Fig. 1A). The irradiance of UVB was measured by using an IL-1700 radiometer (International Light, Newburyport, Massachusetts) equipped with the SED240/UVB-1/TD photo detector (shown in Fig. 1C). All mice recovered from anesthetization after irradiation.
Fig. 1 Illustration of UVB application on mouse ear skin at 3000J/m². (A) Anesthetized mouse is exposed to UVB only on left ear and the rest of the body is covered. (B) The mouse is fixed on a height changeable stage with the left ear positioned at the center spot where the irradiance of UVB is 3000J/m². (C) Mouse is exposed to a UVB light from a bank of 4 TL 20W/01RS lamps.

Intravital imaging of fluorescent cells by confocal microscopy
Confocal images of fluorescent cells were recorded as described previously (Nishibu et al., 2006). Briefly, mice were anesthetized with intraperitoneal injection of anesthetic cocktail (ketamine 100 mg/kg, xylazine 10 mg/kg, and acepromazine 1mg/kg) with repeated half dose administrations as required. To reduce auto-fluorescent signals associated with hair follicles, the ear skin was treated with Nair (Church & Dwight, Princeton, NJ) 3 days before UVB treatment in all experiments. This pretreatment caused no detectable changes in the number, distribution, or morphology of the cells. After removing hair from the ears and pre-marking the imaging area with quantum dots (Invitrogen, Eugene, Oregon), the mice were placed on an imaging stage. The tip of the ear, ventral side down, was mounted between a glass slide and a glass coverslip immersed with phosphate-buffered saline. Fluorescent images were then acquired by a Leica TCS SP5 confocal microscope or Olympus FV1000 confocal microscope with required excitation wavelength. EGFP/FITC fluorescence was excited using a 488 nm argon laser beam and emitted light was collected between 500 and 580 nm, the appropriate spectral range. Also, for visualizing DsRed/PE signal, a 561 nm DPSS laser was used for excitation during collection of 575 to 610 nm signal emitted from beads. Fluorescence signals were detected using objectives of 20x (for intermittent images and time-lapse movies) or 63x (for LC morphology measurement and time-lapse movies) by scanning at 0.99 μm intervals in the z-axis starting from the supra-basal keratinocyte layer (where > 95% of EGFP+ LCs are found) down to a level 20–25 μm below to include the dermal–epidermal junction. For intermittent imaging experiments, the second set of images was obtained as above in the same pre-marked microscopic fields at 24 h after UVB exposure (Vishwanath et al., 2006). Throughout the observation period, the body...
temperature of the animal was maintained with a heating pad (Gaymar, Orchard Park, NY) while oxygen was delivered via an inhaler to maintain the animal’s respiration.

**Data processing**

All images and movies were generated by either the Leica SP5 Software Package or the Olympus FV10-ASW2.1 Confocal Software package. Data were processed by Metamorph software (Universal Imaging, Downington, PA) and Image J program (National Institutes of Health, Bethesda, MD). Maximum intensity projections of x-y planes of fluorescent signals were collected and covered a total z-axis depth of 20-30 µm for epidermis and up to 60 µm for the whole skin including epidermis and dermis. All the graphs and figures were generated by the GraphPad Prism 5.0 program (GraphPad Software, Inc. CA, USA) and Adobe Illustrator.

**Whole skin immunofluorescent staining**

Dorsal sides of ear skin were prepared and fixed in 4% paraformaldehyde (samples with langerin staining were fixed in cold acetone for 15 min at -20°C), and then blocked with 3% BSA/PBS buffer containing CD16/32 mouse mAb for up to 5 hours at 4°C. Then samples were stained with appropriate fluorophore-conjugated antibodies, and incubated at 4°C overnight. Samples were then extensively washed with PBS 3 times (20 min each time) and mounted on slides with GEL/MOUNT (Biomeda corp. Foster, CA) with the epidermal aspect facing the coverslip.

**Flow cytometric analyses**
The epidermis was separated from ear skin with 0.5% dispase II (Roche Diagnostics, Indianapolis, IN) for 45 minutes at 37°C, then further treated with 0.3% trypsin (Worthington, Lakewood, NJ) in the presence of 0.1% DNase I (Roche Diagnostics) for 10 mins at 37 °C to prepare a single cell suspension. The dermis was minced and incubated with 1,000U/ml collagenase IV (Worthington), and 0.1% DNase I for 1 hour at 37°C. The obtained single-cell suspensions were pretreated with 5mg/ml anti-CD16/CD32 (2.4G2) mAb for 15 min on ice, and then stained with fluorescence-conjugated mAb for 30 minutes on ice. Propidium iodide (5µg/ml) was added before the samples were analyzed with FACSCaliber (BD Biosciences) flow cytometer. Data were analyzed by FlowJo (Tree Star) software.

**Injection of anti-Ly6G mAb to deplete neutrophils**

To deplete neutrophils, mice were injected intraperitoneally with 300 µg of anti-Ly6G mAb (1A8) (BD Bioscences) 1 day before and 2 days after UVB irradiation respectively. Control rat IgG2a (BD Biosciences) was injected at the same dose and schedule.
CHAPTER 1

Intravital Imaging of Langerhans Cell Behavioral Responses to UVB Irradiation

Chapter 1.1

It is well known that UVB irradiation can reduce the surface density of LCs in both mice and humans. The resulting loss of LCs may be related to UVB-induced immunosuppression. However, most studies are based on the static staining of the epidermal sheet with LC specific markers like MHC II or CD11c. How LCs exhibit their motility and respond to UVB irradiation is not known. In order to visualize LCs, we used I-Aβ-EGFP knock-in mice in which LCs can be recognized as EGFP+ cells in the epidermis under confocal microscope. By using this method, we studied the time kinetics of UVB-induced LC depletion in vivo in live animals.

Approach:

Anesthetized I-Aβ-EGFP knock-in mice were irradiated with UVB at 3000J/m² only on the left ears (Fig. 1A). After indicated time periods, ear skin of knock-in mice were fixed on the heating stage of the confocal microscope. Initial images of EGFP+ cells were taken from both ears of one mouse before irradiation, then the left ear of the same mouse was irradiated with UVB at 3000 J/m² and the right ear was left untreated to serve as control. We examined both ears under the confocal microscope on day 1, 2, 3 and 4 after UVB irradiation/sham treatment. By recording over 15 images of EGFP+ cells at 20×
magnifications, we calculated the EGFP\(^+\) cell density in both irradiated skin and non-irradiated skin on each day to determine changes in LC number in response to UVB irradiation.

Results:

**Fig. 2 Time kinetics of UVB induced LCs depletion.** 3000J/m\(^2\) of UVB was applied to the left ears of I-A\(\beta\)-EGFP knock-in mice and the right ears were left untreated to serve as controls. The wavelength for EGFP excitation was 488nm. LC density was measured based on cell numbers counted in images of epidermal EGFP\(^+\) under confocal microscope at 20x magnifications. At least 15 fields were collected from each ear at each time point. Time kinetics of LC density in response to UVB irradiation on each day post exposure was generated (n=3, **p<0.001).
UVB irradiation induced LC depletion in a time-dependent manner compared to control (Fig. 2). An approximate 30% reduction in LC numbers was found 1 day after treatment, and almost complete depletion was observed 4 days after UVB treatment. In the non-irradiated ear, the number of LCs was unchanged, indicating the effects of UVB on LC density were localized to the UVB treated ear only in our experimental system.

Chapter 1.2

During the imaging process, we noticed that the morphology of LCs underwent significant changes after UVB irradiation. Most LCs began to show fewer dendrites compared to their normal shape without any treatment.

In order to quantitate these morphological changes in LCs, we introduced the “shape factor” value into our analysis. The “shape factor” value defines the complexity of an object and is measured based on the ratio of its circumference to the area. Thus, this value increases as a cell loses its dendrites and assumes a more perfectly circular shape. We used Metamorph software analysis to measure these values of individual cells. This measurement required some modification of the images in order to distinguish the cells from the background signal. At each indicated time point, we measured the shape factor values of at least 400 cells in each sample.

Results:
Fig. 3 Morphological changes in LCs after UVB irradiation. Complexity of LC shape was examined by imaging epidermal EGFP$^+$ cells at 60x magnification. (A) Shape factor values for each cell, as indicated by the numbers in each representative image, were measured by Metamorph software. (B) More than 400 cells were measured at each time point for each group. Respective shape factors of individual cells at 6 h, 24 h, and 8 wk after UVB irradiation were compared to the control group and analyzed by Mann-Whitney U test (n=3, ***p<0.001).
LCs became rounded up 24 h after UVB irradiation (Fig. 3A). When we examined LCs 8 weeks later, LC morphology resembled that of control. By measuring more than 400 cells in this way, we found that at 6 h, shape factor values were comparable in the UVB-irradiated and control groups. A remarkable increase of shape factor values was observed in UVB-irradiated ears compared to control ears at 24 h post-irradiation. These results indicate that LCs retract their dendrites between 6 h and 24 h. When we examined these values in the irradiated ear 8 weeks later, shape factor values had returned to control level, indicating reversibility of morphological changes of LCs (Fig. 3B).

Chapter 1.3

After observing the morphological changes in LCs, we asked whether these changes were due to the alteration of LC behaviors after irradiation. Then we tried to record the in vivo behavior of LCs in UVB-irradiated skin to see if there is any alteration compared to control.

Previously, we reported dynamic behaviors of LCs both in the steady state and after hapten stimulation (Nishibu et al., 2006). We found unique behaviors of LCs, characterized by repetitive extension and retraction of the dendrites, termed “dendrite surveillance extension and retraction of cycling habitude” (dSEARCH). However, LC response to UVB irradiation is still unknown. To answer this question, we recorded time lapse movies of EGFP+ cells in I-Aβ-EGFP knock-in mice using the confocal microscope. We took images every 2 minutes and generated movies up to 30 minutes in length. In this
way, we could directly visualize movements of LCs in vivo. Movies were recorded at 63× magnification to examine fine dendrite motility.

Results:

**Fig. 4** LCs retain movement of dendrites after UVB irradiation. Anesthetized I-Aβ-EGFP knock-in mice received UVB irradiation on left ears or sham treatment on the right ears. 6 h, 24 h, 8 wk later dynamic behaviors of EGFP+ LCs in the irradiated sites were visualized. Actual behaviors are shown in movie 1, 2, 3. Data shown are overlaid images of EGFP+ LCs recorded at time 0 (pseudo-colored in green), 15 min (pseudo-colored in red), and 30 min (pseudo-colored in blue). Scale bar = 50 μm. Images are representative of three experiments.

Fig. 4 demonstrates the LCs dendrite movements at 6 h, 24 h and 8 wk after UVB irradiation or sham treatment, respectively. Colored images indicate dendrite movements are active during 30 minutes, whereas cells with white color indicate limited behavior of their dendrites during the same period. In the UVB-irradiated group, dendrite movements
of LCs were elevated to a comparable level at both 6 h and 24 h post irradiation, whereas 8 weeks later, they became sessile again. In the steady state, LCs exhibit dSEARCH motion of their dendrites (Movie 1). Six hours after UVB irradiation, we found dSEARCH motion of dendrites was still detectable (Movie 2), and 24 h later, in those cells showing dendritic processes, dSEARCH motion was clearly detectable (Movie 3). These results suggest that LC retained their unique behaviors even after UVB irradiation.

Chapter 1.4

Given the morphological and behavioral changes in LCs after UVB irradiation, we next wondered how these changes related to the LC number depletion in the epidermis upon UVB irradiation. Retraction of dendrites and rounding up of LCs may greatly facilitate detachment from the neighboring keratinocytes and increase migration to the dermal compartment.

It has been well established that LCs have the ability of self-renewal in steady state, and about 2% of LCs are replaced by new cells every 24 h. The half-life of LCs in the epidermis ranges from 53-78 days. In order to determine whether UVB irradiation alters LC immigration or emigration, we employed the intermittent imaging protocol to study the influx and efflux rate of LCs in both treated and control ears.

Intermittent imaging approach:

Before irradiation, we recorded EGFP⁺ signals and pseudocolored them in green. 24 h later, we recorded a second set of images in the same microscopic field and pseudocolored them in red. By overlaying these two sets of images, we can calculate the

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LC influx and efflux during 24 h. Cells present in the same location between the observed time points will appear in yellow in the overlaid picture. Single red cells are those that appeared at 24 h, representing cell influx; single green cells are those that disappear during 24 h, representing cell efflux.
**Fig. 5 UVB irradiation promoted LCs efflux rate from the epidermis without affecting their influx rate.** Two sets of montage pictures were generated from confocal images recorded on day 0 (pseudo-colored in green) and 1 day (pseudo-colored in red) after UVB irradiation on left ears, or sham treatment on right ears in the same pre-marked microscopic field of anesthetized I-Aβ-EGFP knock-in mice. Overlaid images are shown in the right panel. Images in the boxed area are shown at a higher magnification in (B). Single green cells (representing LC efflux) or single red cells (representing LC influx) are indicated with arrows or arrowheads, respectively. Bar = 100µm. Images are representative of three experiments.

Fig. 5A shows the montage images of EGFP⁺ LCs before (green) and 24 h after (red) UVB irradiation, and the right panel contains overlaid images to determine cell efflux and influx in UVB-irradiated and control ears. Boxed areas in Fig. 5A are magnified and shown in Fig. 5B. Arrow heads indicate LC entry to the epidermis, whereas arrows indicate LC exit from the epidermis in 24 h. As shown in the overlaid images on the right, no difference was observed in UVB irradiated skin compared to control skin in terms of LC influx. LC efflux, on the other hand, was enhanced remarkably as demonstrated by a great number of single green cells in the overlaid image after UVB irradiation compared to control.

**Table 1. Estimation of LC efflux and influx rates in response to UVB irradiation.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Experiment</th>
<th>Influx/Efflux</th>
<th>Total number of cells</th>
<th>Number of &quot;single-red/green&quot; cells</th>
<th>Influx/Efflux rate (%)</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-) UVB</td>
<td>1</td>
<td>Influx</td>
<td>1564 (time 24h)</td>
<td>17 (single-red/green cells)</td>
<td>1.1</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Influx</td>
<td>1117 (time 24h)</td>
<td>14 (single-red cells)</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Influx</td>
<td>1169 (time 24h)</td>
<td>11 (single-red cells)</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>(+) UVB</td>
<td>1</td>
<td>Influx</td>
<td>1133 (time 24h)</td>
<td>23 (single-red cells)</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Influx</td>
<td>1138 (time 24h)</td>
<td>12 (single-red cells)</td>
<td>1.1</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Influx</td>
<td>873 (time 24h)</td>
<td>15 (single-red cells)</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>(-) UVB</td>
<td>1</td>
<td>Efflux</td>
<td>1596 (time 0)</td>
<td>32 (single-green cells)</td>
<td>2.1</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Efflux</td>
<td>1121 (time 0)</td>
<td>18 (single-green cells)</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Efflux</td>
<td>1181 (time 0)</td>
<td>15 (single-green cells)</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>(+) UVB</td>
<td>1</td>
<td>Efflux</td>
<td>1702 (time 0)</td>
<td>516 (single-green cells)</td>
<td>45.7</td>
<td>34.3 ± 5.0**</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Efflux</td>
<td>1538 (time 0)</td>
<td>412 (single-green cells)</td>
<td>26.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Efflux</td>
<td>1232 (time 0)</td>
<td>274 (single-green cells)</td>
<td>30.4</td>
<td></td>
</tr>
</tbody>
</table>

Total number of EGFP⁺ LCs examined at 0 or 24 h after UVB/sham treatment.
The number of EGFP+ LCs that became undetectable at 24 h (single green cells representing LC efflux) or that were detectable only at time 24 h (single-red cells representing LC influx).

Mean efflux or influx rates of LCs based on 3 experiments. Efflux or influx rates were assessed by student t test (P = 0.007).

By counting more than 3000 cells in 3 animals in this way, we calculated LC influx and efflux rates (Table 1). As expected, the LC influx rate was comparable in UVB-treated and control ears. We concluded that the reduction of LC number after UVB was due to their significantly increased efflux rate from the epidermis. It is conceivable that the morphological changes and amplified dendrite behaviors facilitate their emigration after UVB irradiation.

Since we found a 30% reduction in LC number after UVB exposure, we attempted to record the process of LC exit from the epidermis by time lapse movie. Starting from 16 h post irradiation, we successfully recorded one cell disappearing from the epidermis over a 90 min time span (Movie 4).
Fig. 6 LC emigration from the epidermis after UVB irradiation. A 90 min time lapse video was recorded at a UVB-irradiated site at 16 h post UVB treatment. Data shows representative static images of one EGFP+ LC disappearance as indicated by the arrow. Bar = 50µm.

Fig. 6 shows the static images extracted from the movie and illustrates the cell locations at indicated time points. An arrow indicates a cell that exited from the epidermis during the 90 minutes.

The real time process of one cell disappearing from the epidermis can be viewed in Movie 4, in which we noticed that the outgoing cell disappeared while maintaining its dendritic shape. Other cells that already retracted their dendrites were still located in their original position throughout the imaged periods. This result indicates that it is not necessary for LCs to retract their dendrites to leave the epidermis.
Chapter 1.5

The observation of LC disappearance from the epidermis led us to consider the fate of LCs that disappeared after UVB irradiation. There may be two possibilities: LCs undergo apoptotic process upon irradiation, or they migrate to deeper layers of the skin.

We first examined whether LCs undergo apoptosis after irradiation. C57BL/6 wild type mice were irradiated on the left ear with 3000 J/m² and the right ear was untreated. After 48 h, ears were harvested and epidermal single cell suspensions were prepared. Cells were fluorescently labeled with anti-MHC II antibody and caspase-8 staining kit. Apoptosis of LCs were determined according to caspase-8 activation in the MHC II⁺ population by FACS analysis. The efficiency of caspase-8 detection kit was tested by using thymocytes treated with dexamethasone for 4 h at 37 °C. In three independent experiments, we failed to detect increased caspase-8 activation in LCs in UVB irradiated ears. Annexin V staining of epidermal single cell suspensions also failed to show any significant increase in Annexin V⁺ LCs in irradiated ears compared to sham-treated ears (data not shown). Based on these observations, we concluded that UVB irradiation may not directly induce apoptosis of LCs, or it induced the LC apoptosis but in a caspase-8 independent manner.

To determine whether UVB induces vertical migration of LCs, we attempted to track LCs by staining the whole ear with LC-specific markers, MHC II and langerin, and compared vertical locations of LCs in irradiated and control ears.
Briefly, we stained the whole ear skin (including both epidermal and dermal compartment) with APC-anti-langerin and PE-anti-IA/IE antibodies to track LCs, and compared their locations between irradiated and non-irradiated skin.

Results:
Fig. 7 Identification of LCs in deeper layers of skin after UVB irradiation. Ear skin (including epidermis and dermis) from C57BL/6 WT mice were harvested and stained with FITC-anti-MHC II and APC-anti-langerin antibodies 1 day after UVB or sham treatment. LCs were identified as MHC II⁺/langerin⁺ cells in the skin. (A) Panels show static images of LC locations in vertical direction. UVB promoted LC vertical migration into deeper layer of skin is indicated with an arrow. (B) Statistical analysis of LC vertical locations after UVB/sham treatment. Red dots represent LCs located in significantly deeper layers of skin (>mean +3SD of sham control group, χ² test, p<0.001).

MHC II⁺/langerin⁺ LCs were evenly distributed at the suprabasal keratinocyte level (depth: 20µm from the stratum corneum) in skin samples harvested from sham-treated skin (Fig. 7A). After UVB irradiation, LCs became detectable at a variable range of z-axis levels. To illustrate the vertical displacement of some of these LCs, x-y plane images of the same field are shown at 10µm from the stratum corneum and every 5 µm below. Two LCs showing deeper vertical locations are indicated with arrows (Fig. 7A). Sequential x-y images scanned at different z-axis levels can be viewed in Movie 5A and 5B.

Fig. 7A indicates the vertical locations of LCs in non-irradiated and UVB-treated ears. Starting from the top surface of epidermis, most MHC II⁺/langerin⁺ LCs are located at a 20µm depth in intact skin. On the other hand, the majority of LCs in UVB irradiated skin can be found at 20µm but there are still a number of cells located in the deeper layers as shown in Fig. 7A (arrows). Some MHC II⁺/langerin⁺ cells can be found as deep as 40µm. We measured the vertical locations of over 120 double positive cells from random areas of UVB or sham treated ear as shown in Fig. 7B. We defined the cells located deeper than the mean + 3SD of control depth as migrating LCs. Subsequently 37 cells out of 162 total cells in the irradiated ear and none of the cells in the sham ear were scored as
migrating LCs. The $\chi^2$ test demonstrated the difference is highly significant between the two groups ($p < 0.001$). Scanning of the skin in the vertical direction can be viewed in Movie 5A and 5B. In these two movies, a volume of skin was scanned step by step in 1 $\mu$m intervals, reaching up to 60 $\mu$m deep in the dermis. In the UVB-irradiated skin, many cells were located around 20 $\mu$m in depth; however, a number of langerin$^+/\text{MHC II}^+$ cells were found at deeper positions that included the dermal compartment. This phenomenon was not seen in intact skin (Movie 5A).

Because we failed to detect LC apoptosis after UVB irradiation, tracking of LCs in the whole skin by anti-langerin$^+/\text{MHC II}^+$ staining may partially explain the reduction of LCs in the epidermis after UVB. We propose that UVB irradiation induces LC migration to the deeper layers of the skin, thus reducing surface density of LCs in the epidermis. In fact, we found MHC II$^+/\text{langerin}^+$ cells in the epidermal-dermal junction area of irradiated skin, whereas no such cells were found in non-irradiated skin.

By using intravital imaging and I-A$\beta$-EGFP knock-in mice, we studied early behavioral responses of LCs to 3000 J/m$^2$ UVB treatment. We found that UVB radiation reduces surface densities of LCs by promoting their migration into the deeper layers of the skin without inducing apoptosis. As early as 6 h, LCs began to retract their dendrites and exhibit amplified dendrite movements. These movements may facilitate cells in detaching from neighboring keratinocytes, and we speculate that the process of cell migration may involve some cell membrane molecules that normally connect two neighboring cells. However, whether the increased dSEARCH motion of LCs is related to the regulation of these molecules needs further investigation. We noticed that it is not necessary for LCs to
retract their dendrites to go to deeper layers of the skin because LCs found in the deeper layers still demonstrated a dendritic morphology. Complete depletion of LCs from the epidermis was seen at approximately 4 days post UVB irradiation.

To the best of our knowledge, this is the first study demonstrating the dynamic behaviors of LCs in response to UVB irradiation.
CHAPTER 2

Neutrophils Migrate into UVB-Irradiated Skin and Interact with LCs

Chapter 2.1

We studied LC behaviors in normal skin and their dynamic responses to UVB irradiation. Next we sought to determine how other cells respond to UVB radiation during the skin inflammation. In order to answer this question, we employed LysM-EGFP knock in mice to study the behavior of other leukocytes in the ear skin. It has been reported that the EGFP signals in LysM-EGFP mice can be found in myeloid cell derived leukocytes. To study the effects of UVB on these cell types, we treated anesthetized LysM-EGFP mice with the same UVB dose as before (3000 J/m²), and then examined movements of EGFP⁺ cells in response to UVB irradiation at different time points under the confocal microscope.

Results:

In the steady state or without UVB radiation, very few EGFP⁺ cells can be observed in the ear skin under 20× magnification (Movie 6). We were able to distinguish two populations of EGFP⁺ cells based on their differential EGFP signal intensities. One population expressed high EGFP signal and traveled very fast in the intact ear skin. The other population exhibited lower levels of EGFP signal and included larger but immobile cells (Fig. 1, left panel). We also examined the movements of EGFP⁺ cells in the same
mouse ear skin at 0.5 h and 24 h after UVB radiation (Fig. 1 middle and right panel respectively). As early as 0.5 h after UVB irradiation, we detected many EGFP+ cells in the irradiated skin. These cells showed rapid movements as observed in time lapse Movie 7. When we examined irradiated ear skin 24 h later, an even larger number of EGFP+ cells were found in the irradiated site, and interestingly, many of them were found preferentially around hair follicles (Fig. 1)

![Image of time point comparison](image)

**Fig. 1 Emergence of EGFP+ leukocytes in the skin after UVB irradiation.** At the indicated time points after UVB irradiation, irradiated ears of LysM-EGFP knock-in mice were examined under the confocal microscope. Data shown are confocal images at 20× magnifications. Bar = 20µm.

In order to determine the identity of these EGFP+ cells that emerged after UVB irradiation, we first examined the surface markers of these cells in both the epidermis and dermis using flow cytometry analysis. In the dermal compartment, almost all EGFP+ cells were CD45+ and CD11b+, indicating these cells were myeloid leukocytes. The EGFP+ population can be further divided into two populations based on the level of their EGFP expression which was also consistent with what we observed under the confocal
microscope. We found that \( \text{EGFP}^\text{high} \) cells uniformly expressed both Ly6G and Ly6C on their surface, indicating that they were neutrophils in the dermal compartment. \( \text{EGFP}^\text{low} \) cells were F4/80 positive, indicating that they were macrophages/monocytes (Fig. 2A). This result is consistent with the observation from the confocal movies showing that brighter cells travel faster and exhibit relatively small cell size. They expressed specific marker for neutrophils, i.e., Ly6G. On the other hand, the \( \text{EGFP}^\text{low} \) cells are immobile and exhibit larger cell sizes. They expressed F4/80, a marker of macrophages and monocytes.

Fig. 2B displays the phenotype analysis of \( \text{EGFP}^+ \) cells in the epidermis of irradiated skin. Different from the dermal cells, only \( \text{EGFP}^\text{high} \) cells were present in the epidermis. These cells were identified as neutrophils based on their surface expression of Ly6G and Ly6C. From the above results, we concluded that the cells that we observed in the epidermal compartment under confocal microscopy were mostly neutrophils that infiltrated into the inflamed skin after UVB irradiation.
**Fig. 2 Surface phenotype of EGFP⁺ cells of LysM-EGFP knock-in mice in the ear skin after UVB treatment.** (A) Dermal cell suspensions were prepared from the ear skin of LysM-EGFP knock-in mice 18 h after UVB irradiation and examined for EGFP expression (x-axis) and indicated surface markers (y-axis). Cells circled in red indicate EGFP^{high} cells, whereas the green circle indicates cells with EGFP^{low} cells. (B) Epidermal cell suspensions were also prepared at the same time from the ear skin of LysM-EGFP knock-in mice and examined for the expression of EGFP and indicated surface markers.

To further confirm the identities of EGFP⁺ cells in LysM-EGFP mice after UVB irradiation, we also stained the whole ear skin with PE-conjugated mAb against the neutrophil marker, Ly6G (as described in the Materials and Methods).

In the dermal compartment (Fig. 3A), we observed that approximately 40% of EGFP⁺ cells were Ly6G⁺, which is consistent with the results from FACS analysis in which not all EGFP⁺ cells were Ly6G positive in the dermis of irradiated skin. In the epidermal compartment, we found virtually all EGFP⁺ cells co-expressed Ly6G, indicating that those green cells in the epidermis were mostly neutrophils. From these results, we concluded that in LysM-EGFP mice, EGFP⁺ cells emerging in the epidermis after UVB irradiation were mostly neutrophils, whereas EGFP⁺ cells in the dermal compartment were composed of neutrophils and macrophages/monocytes, which are distinguishable based on their speed of movement and EGFP expression levels.
Fig. 3 Identification of neutrophils in the dermal and epidermal compartment in LysM-EGFP knock-in mice after UVB irradiation. LysM-EGFP knock-in mice ear samples were harvested at 16 h after UVB irradiation. The samples were stained with PE-anti-Ly6G mAb to identify neutrophils. Data shown are images acquired by confocal microscope in the dermal compartment (A) and epidermal compartment (B). Bar = 20µm.

Chapter 2.2

Since we identified that the EGFP+ cells emerging in the epidermis after UVB irradiation were neutrophils, we next sought to determine the time kinetics of neutrophil infiltration into the epidermis after UVB irradiation. To address this question, we followed neutrophil emergence in response to UVB irradiation by recording time lapse movies in ear skin of LysM-EGFP mice immediately after UVB until 22 h post exposure. Immediately before confocal imaging, we intravenously injected Alexa-fluor-546 dextran (30 mg/ml, 100 µl/mouse) into the mice to label the blood vessels in the ear skin.

Results:
Fig. 4 Extravasations of EGFP+ neutrophils in ear skin of LysM-EGFP knock-in mice after UVB irradiation. Ear skin of anesthetized LysM-EGFP knock-in mice was irradiated with UVB and immediately examined under the confocal microscope. Alexa-fluor-546 dextran was i.v. injected into the mice before imaging to label blood vessel in the skin. Data shown are static images at indicated time points after UVB irradiation of the same field at 10× magnifications. The process of EGFP+ neutrophils extravasation can be viewed in Movie 8A, and 8B.

Fig. 4 demonstrates a series of images of EGFP+ neutrophils migrating into the UVB-irradiated skin starting from time 0 post exposures. At the beginning, all the EGFP+ cells were traveling along inside the blood vessels. At later time points, increasing numbers of EGFP+ cells moved out of the vessels, scattering in the irradiated skin. At 8 h after irradiation, a large number of EGFP+ neutrophils were found outside of the blood vessel.
The real-time process of neutrophil extravasation during the initial 8 h can be viewed in Movie 8A. We noticed some “hot spots” along the blood vessels from which neutrophils preferentially exited. Around these areas, neutrophils exhibited rolling and tethering motions typical during extravasations. By using this method, we recorded a total of 5 movies that covered the whole time period of up to 22 h post irradiation.

We calculated the number of extravascular neutrophils at each hour based on the static images we recorded. Fig. 5 shows the density of extravascular EGFP+ cells at different time points after UVB irradiation. We found that the number of extravascular EGFP+ cells increased in a time-dependant manner. Seven hours after UVB irradiation, neutrophil density in the epidermis had reached 80 cells/mm². The number of EGFP+ cells that migrated out of blood vessels increased in a time-dependent fashion, and the density of extravascular cells reached up to 140 cells/mm² at 22 h.
Fig. 5 Time kinetics of extravascular EGFP+ cell emergence during 22 h after UVB irradiation. Five LysM-EGFP knock-in mice were irradiated on the ear skin and examined at indicated time periods as shown with different colors. AF-546-dextran was i.v injected into the mice just prior to confocal imaging. The density of extravascular EGFP+ cells was calculated based on the number of EGFP+ cells outside of blood vessels in static confocal images at each indicated time point.

Neutrophils can react in a very quick manner to UVB irradiation and the number of neutrophils that infiltrated into the skin from the blood vessel increased progressively for 24 h. When we examined neutrophil behaviors at later time points after UVB (Movie 9),
we noticed that the number of EGFP\(^+\) cells 7 days post-irradiation was remarkably decreased, and their movements were much slower than that on day 1. Based on cell size and their slow movements, it was very likely that these cells were macrophages residing in the inflamed skin but not neutrophils. Seventeen days after UVB irradiation (Movie 10) we only observed the macrophage-like cells, only they were much less mobile than the cells observed at earlier time points. These observations indicated that neutrophils responded to UVB irradiation in a quick manner and peaked in number within 48 h. Subsequently, they disappeared from the inflamed sites. The fate of neutrophils after UVB still needs to be examined.

Chapter 2.3

Since we observed that neutrophils responded to irradiation in a prompt manner, we next wanted to know the activation status of neutrophils upon UVB irradiation. In other words, we sought to determine whether UVB irradiation could induce not only the extravasation but also the activation of these neutrophils during inflammation. The activation of neutrophils can be characterized by their expression of IL-1\(\beta\) promoter. Thus, we generated a pIL1-DsRed transgenic mouse line expressing the gene for the red fluorescent protein DsRed under the control of the mouse IL-1\(\beta\) promoter. We have reported that many DsRed\(^+\) cells emerged under skin inflammation induced by hapten application to the ears of pIL-1-DsRed mice. The majority of DsRed\(^+\) cells in the epidermis are CD11b\(^+\)/Gr-1\(^+\)/MHC II\(^-\) indicating that they are neutrophils in the epidermal compartment of inflamed skin (Matsushima et al., 2010). In the present study,
we used this transgenic mouse line to investigate the activation of neutrophils under UVB irradiation.

In order to determine whether UVB irradiation can induce the activation of neutrophils, we treated the ear skin of the transgenic mice with the same dose of UVB as before. Fig. 6 shows the examination of the same mouse skin before and after UVB radiation. There was little DsRed signal in the skin without UVB exposure (Fig. 6, left panel). Twenty-four hours after exposure, we noticed many DsRed\(^+\) cells in the irradiated skin that exhibited a similar pattern of movement as previously observed with EGFP\(^+\) cells in LysM-EGFP mice after UVB irradiation. Thus, the same dose of UVB exposure can cause not only the accumulation of neutrophils but also their activation in the inflammatory sites.
Although both LCs and neutrophils responded to the same dose of UVB, they exhibited different reactions to the exposure—LCs migrated to the deeper layer of the skin whereas neutrophils became activated and migrated out of blood vessel into the epidermal compartment of the irradiated skin. Since both LCs and neutrophils were found in the same location (i.e. epidermis) after irradiation and since they both showed active movements, we hypothesized that these two cell types may interact with each other. In order to directly visualize the existence of such interaction, a specific transgenic mouse line allowing us to visualize both LCs and neutrophils was required. We generated a new transgenic mouse line by breeding I-Aβ-EGFP knock-in mice with pIL1-DsRed transgenic mice. We then examined the ear skin of the offspring mice in the steady state and during skin inflammation induced by UVB under the confocal microscope. In the intact mouse ear skin, we can only observe the EGFP+ LCs in the epidermis with typical dendritic shape and limited dSEARCH motions. The situation of LC movement resembled what we observed in I-Aβ-EGFP knock-in mice under normal conditions (Movie 11). Then, we applied the same dose of UVB on the ear skin as before. Twenty-four hours after treatment, we examined the cell movement under the confocal microscope. In Movie 12A, we observed EGFP+ cells in the epidermal compartment that were more active than in the steady state. At the same time, we found DsRed+ neutrophils also emerged in the epidermis. In this 3 h time lapse movie, we noticed that neutrophils...
were much more mobile than LCs after UVB irradiation and it seemed that they were attracted to LCs. Some neutrophils kept “communicating” with LCs during the entire recording time (Fig. 7A, Movie 12A). We tracked the migratory path of each DsRed+ cell during the 3 h recording period (Fig. 7B). It was noticeable that DsRed+ cells were preferentially moving to or around the EGFP+ LCs (Movie 12B). In order to confirm whether neutrophils preferentially interact with LCs, we divided the entire observation area into two parts: one part contained LCs, termed “LC territory”; the other part that contained no LCs, termed “non-LC territory”. The “LC territory” was 0.037 µm², whereas the “non-LC territory” was 0.37 µm². We then counted the numbers of neutrophils in the “LC territory” versus “non-LC territory” every 6 min and calculated the frequency of neutrophils located in either area during 3 h (Fig. 8A). The frequency of neutrophils located in the “LC territory” was 727 ± 91 cells/mm², whereas their frequency in “non-LC territory” was 29 ± 7 cells/mm². These results indicated that neutrophils were preferentially found around LCs after UVB irradiation (p<0.001) (Fig. 8B).
Fig. 7 Emergence and behavior of DsRed⁺ cells as well as EGFP⁺ LCs at 16 h after UVB irradiation. I-Aβ-EGFP/pIL1-DsRed transgenic mice were anesthetized and irradiated on the ear skin and examined 16 h later under the confocal microscope. Ear skin was recorded as confocal images every 6 mins for 3 h to observe the dynamic behaviors of both EGFP⁺ LCs and DsRed⁺ activated neutrophils. (A) Static images represent the location of DsRed⁺ cells as well as EGFP⁺ LCs at indicated time point. (B) Migratory paths of individual DsRed⁺ cells in 3 h are shown. (C) LC-territory was indicated by those circles containing LC and Non-LC territory was the rest of area containing no LC.
Fig. 8 Distribution of DsRed⁺ cells to the LC territory after UVB irradiation. (A) Number of DsRed⁺ cells in LC-area versus non-LC area at the indicated time frames during 3 h of recording time. Static confocal images were divided into 2 areas based on the EGFP signal. LC territory represents the area containing EGFP⁺ LCs covered. Non-LC territory represents the remaining area. We counted the numbers of DsRed⁺ cells in the two areas every 6 minutes. (B) Density of DsRed⁺ cells in LC versus non-LC territory. Data indicate DsRed⁺ cells are preferentially located in the LC territory (p<0.001).

To visualize interaction between neutrophils and LCs, we generated a 3-dimensional rotating movie; it demonstrated intimate contact between a single activated neutrophil and LC (Movie 13). Fig. 9 shows the visualization of interaction between one LC (green)
and one activated neutrophil (red) from different angles. Therefore, intimate physical interaction between activated neutrophils and LCs indeed occurs after UVB radiation.

Fig. 9 DsRed⁺ activated neutrophil interacts with EGFP⁺ LC in the epidermal compartment after UVB irradiation. Data shows the interaction of one EGFP⁺ LC with one DsRed⁺ activated neutrophil from different orientations. Yellow color indicates their intimate association area. The rotating process can be viewed in Movie 13.

By using the I-Aβ-EGFP/pIL1-DsRed double-mutant mouse, we were able to observe different cell responses in the skin upon UVB irradiation. Under inflammatory conditions, LCs became more active than in the steady state and some of them began to migrate out of the epidermis. Neutrophils, on the other hand, were recruited immediately from the blood into the irradiated sites. During their migration to the epidermis, neutrophils also became activated and interacted with LCs. This is the first documentation of a direct interaction between LCs and neutrophils upon UVB irradiation.
CHAPTER 3

Effects of Netrophil Depletion on LCs and UVB induced Skin Inflammation

Chapter 3.1

Although we observed that neutrophils and LCs interact during UVB induced inflammation, the biological significance of this kind of association still remained unclear. To address this important question, we deleted the neutrophils by a standard protocol with anti-Ly6G mAb before UVB irradiation and examined the consequence of neutrophil depletion.
Anti-Ly6G mAb was intravenously injected into I-Aβ-EGFP knock-in mice one day before and two days after UVB irradiation. EGFP⁺ cell numbers were examined in anesthetized mice under the confocal microscope at 20× magnification. In control IgG injected mice, EGFP⁺ cells started to disappear on day 1 and almost no EGFP⁺ cells remained in the epidermis on day 4 (Fig. 1, top panels). By contrast, significant numbers of EGFP⁺ cells remained detectable in the epidermal compartment in anti-Ly6G mAb treated mice (Fig. 1, bottom panels).

Based on the images acquired by using confocal microscopy, we counted the numbers of EGFP⁺ cells on each day after UVB irradiation and analyzed the time kinetics of UVB induced changes in the number of EGFP⁺ cells in the mice with anti-Ly6G mAb treatment or control IgG treatment (n = 5) (Fig. 2).
Fig. 2 Impact of neutrophil depletion on the number of EGFP+ epidermal cells in UVB-irradiated skin. Densities of EGFP+ cells were calculated based on the confocal images acquired at 20× magnification at indicated time points (***, p<0.0001, (+) UVB/anti-Ly6G mAb group and (+) UVB/control IgG group were compared.)

We compared LC numbers after UVB/sham treatment in anti-Ly6G treated and control antibody treated mice. Before UVB irradiation, the density of EGFP+ cells of all groups was comparable. On day 1 and day 2 after UVB irradiation, we observed a similar reduction of EGFP+ cells in both anti-Ly6G mAb and control IgG treated groups.
Significant differences between the two groups occurred on day 4: control antibody injected mice showed complete disappearance of EGFP+ cells, whereas anti-Ly6G mAb treated mice had significant numbers of EGFP+ cells still remaining in the epidermal compartment. Even on day 7, EGFP+ cells were readily detectable in the epidermis of anti-Ly6G mAb-treated mice. The size of EGFP+ cells remaining in the anti-Ly6G mAb-treated mice was relatively small, and some of these cells showed irregular shape.

Chapter 3.2

If those EGFP+ cells remaining in the epidermis after UVB radiation represent “residual” LCs, they should express langerin, which is the prototypic marker for LCs. To test this, we used Langerin-DTR-EGFP knock-in mice and performed the same experiments as we performed with I-Aβ-EGFP knock-in mice (see details in Methods).
As shown in Fig. 3, we observed that in langerin-DTR-EGFP mice, EGFP+ LCs showed slightly different morphology from those observed in I-\(\alpha\)β-EGFP mice. This is because langerin molecules are preferentially expressed along the dendritic processes. LC
numbers decreased in a time-dependent fashion, and we observed complete disappearance of EGFP+ cells from the epidermis on day 4 in control antibody-treated mice. In the anti-Ly6G mAb treated mice, UVB radiation resulted in almost complete disappearance of EGFP+ cells. This result indicated that the EGFP+ cells remaining in the epidermis of neutrophil-depleted I-Aβ-EGFP mice did not express langerin. Thus, we concluded that they were not bona fide LCs that remained in the epidermis. Alternatively, those EGFP+ cells expressing I-Aβ molecules might be LC precursors.

According to the literature, LCs can be depleted from the epidermis by UVB irradiation, and LC precursors derived from blood monocytes repopulate the irradiated skin and differentiate into LCs. These pre-LCs express Gr-1 molecules on their surface at the early stage of differentiation (Ginhoux et al., 2006b). Another study about LC self-renewal described that pre-LCs could also express CD115 on their surface (Ginhoux et al., 2006b).

In order to identify whether the EGFP+ cells remaining in the epidermis after UVB irradiation are pre-LCs, we used FACS analysis to test the expression of Gr-1 and CD115 on EGFP+ epidermal cells in I-Aβ-EGFP knock-in mice with or without neutrophil depletion.
Fig. 4 Identification of EGFP+ cells remaining in I-Aβ-EGFP knock-in mice 7 days after UVB irradiation. Seven days after UVB irradiation or sham treatment with anti-Ly6G mAb or control IgG injection, ear samples were harvested. Epidermal single cell suspensions were prepared and labeled with PE-anti-Gr-1 (A) and APC-anti-CD115 (B) monoclonal antibodies. FACS data shows the identity of EGFP+ cells in indicated treatments. Red boxes indicate EGFP+/Gr-1+ (A) or EGFP+/CD115+ (B) cell population. Green box indicates EGFP single positive cells. Numbers in the boxed area show the percentage of cells in the whole epidermal cell population.

Fig. 4 shows the profile of Gr-1 (Fig. 4A) and CD115 (Fig. 4B) expression on EGFP+ cells in I-Aβ-EGFP knock-in mice. In the control mouse without neutrophil depletion, LCs, which were EGFP positive, were negative for both Gr-1 and CD115 before UVB irradiation. Seven days after UVB irradiation, no EGFP+ cells were observed in the epidermal single cell suspension, consistent with what was observed using confocal microscopy. In the anti-Ly6G mAb-treated mice, EGFP+ cells were detectable with the similar frequency to control IgG-treated mice and did not express Gr-1 or CD115 before UVB treatment. However, after 7 days of UVB, we found around 5% cells in the epidermis of anti-Ly6G treated mice were EGFP+, and a small population (around 10%) of these EGFP+ cells were Gr-1+ with about 30% expressing CD115. Thus, small portions of EGFP+ cells remaining in the epidermis after UVB irradiation exhibited the phenotype of pre-LCs.

Chapter 3.3

The overall outcome of neutrophil depletion on UVB irradiation was evaluated by measuring ear swelling. We measured ear thickness of each mouse by using a mitsutoyo micrometer (Fig. 5).
Fig. 5 Time kinetics of ear swelling in I-Aβ-EGFP knock-in mice after UVB/sham treatment and anti-Ly6G/control IgG injection. Mice received anti-Ly6G/control IgG injection and UVB irradiation as previously described. Ear thickness was measured by using a mitsutoyo micrometer at the indicated time points and ear swelling was calculated (*p<0.05, **p<0.01, ***p<0.001, groups between (+) UVB/anti-Ly6G panel and (+) UVB/control IgG panel were compared, n = 5).

In control IgG treated mice without UVB irradiation, ear thickness remained unchanged, whereas in the irradiated skin, ear thickness remarkably increased in a time-dependent fashion with a peak on day 7. On the other hand, ear swelling after UVB irradiation in
anti-Ly6G mAb-treated mice was moderately less severe compared to control IgG-treated mice (p<0.05 to p<0.001). The results strongly indicate that neutrophil infiltration was at least partially responsible for UVB induced skin inflammation.
DISCUSSION

Our study has unveiled dynamic behavioral responses of LCs and neutrophils in UVB induced skin inflammation in vivo in living animals. LCs and activated neutrophils exhibit physical interactions during the early stage (within 24 h) of inflammation. UVB irradiation causes changes in LC behavior as well as their morphology. UVB irradiation at 3000 J/m² causes depletion of LCs from the epidermis and infiltration of activated neutrophils into the epidermis. The novel aspect of our study is that we have directly visualized the physical interactions between these two cell types in the living animal under inflammatory conditions. The biological significance of this type of interaction was tested by depletion of neutrophils through anti-Ly6G antibody injection. We have found that in the absence of neutrophils, UVB is unable to completely deplete EGFP⁺ cells in I-Aβ-EGFP knock-in mice. At the same time, depletion of neutrophils significantly diminished the UVB-induced ear swelling response. Some of the EGFP⁺ cells remaining in the epidermis expressed CD115 and Gr-1, which are normally expressed by inflammatory monocytes. Merad et al. have shown that blood circulating monocytes serve as pre-LCs to replenish the epidermis after UVB irradiation (Ginhoux et al., 2006b). These pre-LCs are Gr-1 and CD115 double positive during the early phase of LC recovery but reduced the expression level of Gr-1 at later stage (Ginhoux et al., 2006b). Our results have shown that around 30% of EGFP⁺ cells remained in the epidermis of I-Aβ-EGFP knock-in mice after neutrophil depletion are CD115 positive suggesting that they are pre-LCs. Only small numbers (about 10%) of EGFP⁺ cells expressed Gr-1,
perhaps because we examined the skin at later stages. Thus, we have concluded that the infiltration of neutrophils into epidermal compartment prevents the entry of pre-LCs into UVB-irradiated skin.

Reduction of LC numbers by UVB irradiation or other stimuli has already been studied by many groups. However, none of those previous studies examined dynamic behaviors of LCs after UVB radiation. The number of LCs was usually counted by staining epidermal sheets of skin with LC specific markers such as MHC II, ADPase, and langerin. This approach usually requires sacrificing multiple animals in order to measure the densities at different time points. Our approach, however, enabled the examination of number, morphology, and motility of LCs at different time points in the same animals.

With regard to the fate of LCs after UVB irradiation, it has been suggested that they migrate to the skin draining lymph node (Richters et al., 1996). However, supporting evidence in this study was rather weak because only increased numbers of total DCs were found in the draining lymph node of UVB-irradiated mice as compared to non-treated mice. Whether this increased number of DCs was the result of LCs that migrated from the irradiated epidermis was not clear because dermal DCs also have the ability to migrate to draining lymph nodes after UVB irradiation (Moodycliffe et al., 1992). We also tried to determine if LCs actually travelled to the draining lymph nodes after UVB irradiation by examining the LC frequency in the lymph nodes with Ep-CAM and MHC II staining. However, we failed to reach a conclusive result since there are many subtypes of DCs that share the similar surface markers making it technically challenging to distinguish LCs from the other DC populations.
Whole mount immunofluorescent staining with anti-MHC II and anti-langerin antibodies allowed us to test whether LCs migrate to deeper layers beneath the epidermis upon UVB irradiation. Skin whole mount IF staining is a new technique used to detect vertical locations in the skin up to 60 µm under confocal microscope. Once we define the skin surface of the sample based on the typical honeycomb structure of the stratum corneum using DIC, we can locate each cell’s vertical position by scanning the sample at 1µm intervals using the confocal microscope. By using this new technique, we confirmed that many LCs migrated into much deeper layers of skin after UVB radiation. We also observed that a few LCs were located at dermal-epidermal junctions. These results strongly suggest that LCs migrate to underlying dermis upon UVB treatment.

We also tested another possibility for LC’s fate—apoptosis, by staining the epidermal cells with caspase-8 and Annexin V in both UVB treated and control group. We did not detect any increased percentage of caspase-8+ cells or Annexin V+ cells in UVB treated ears. However, with these results we are still not sure whether LCs undergoing apoptosis or not since it is not feasible if the case LC’s apoptosis is caspase-8 independent.

UVB-exposed keratinocytes modulate the adhesion molecule ICAM-1 and synthesize a whole set of cytokines including IL-1β, IL-6, IL-8, TNFα, and PGE2. These cytokines are responsible for the onset of inflammation, providing chemotactic signals to neutrophils and macrophages in the skin (Cooper et al., 1993; Hammerberg et al., 1994). Neutrophils are the dominant effector cells of the innate immune system and function as the first line of defense to external insults such as bacterial, viral, and fungal infection. Neutrophil infiltration into UVB-irradiated sites has been well known for many years;
however, most of these studies quantified time kinetics of neutrophil infiltration using conventional histological methods. Our new approach of intravital imaging to examine the process of neutrophil extravasation upon UVB irradiation in real time provides us with a more direct view of the exact situation of neutrophil recruitment to the irradiated sites. By recording images for up to 24 hrs after UVB irradiation, we have found that neutrophils start to migrate into the epidermis very promptly. At 0.5 h post-irradiation, we have already observed neutrophils migrating out of the blood vessels. This is different from the previous studies demonstrating that demonstrate neutrophils are first seen at 4 h after UVB (Hart et al., 2002). Our approach can show much earlier events because using confocal microscopy and transgenic mice, we can scan the whole irradiated area and find out the earliest cells coming to the examined areas. By using 5 mice examined at different time points after UVB irradiation, we established the real-time neutrophil extravasation kinetics. This should provide us with more accurate information of neutrophil response to UVB irradiation.

Under physiological conditions, neutrophils and LCs are localized in different compartments; neutrophils are mainly present within the blood, whereas LCs appear as sentinel cells within the epidermal compartment of skin. When skin receives UVB exposure, cytokines such as IL-1β, TNFα, IL-8, and IL-12 (Takashima and Bergstresser, 1996; Pentland and Mahoney, 1990) derived from the irradiated keratinocytes attract neutrophils to UVB-irradiated skin sites. At the same time, LCs also undergo morphological and behavioral changes upon UVB radiation. Cytokines mentioned above also regulate LC responses to irradiation. Moreover, infiltrating neutrophils release chemokines and chemoattractants, including MIP-1α, MIP-1β (Kasama et al., 1993), IL-8,
and α-defenisins (Yang et al., 2000). These factors have been reported to attract immature DCs (Yang et al., 2000). In turn, DCs have been reported to transiently produce the neutrophil chemoattractant IL-8 shortly after receiving a maturation stimulus (Sallusto et al., 1999). Thus, DCs and neutrophils are likely to attract each other at peripheral sites during inflammation. Actually, Klaas et al. (van Gisbergen et al., 2005) have been able to demonstrate that DCs and neutrophils establish physical contact in vivo in colonic mucosa during chronic inflammation in patients with Cohn’s disease. They also demonstrated that cellular interactions between DCs and neutrophils (especially activated neutrophils) modulated DC maturation. Neutrophil-induced DC maturation depended on cellular interactions with DC-SIGN and Mac-1. A recent report has also shown that TNF-α produced by neutrophils triggers maturation of DCs (Bennouna et al., 2003). Our results have provided the direct evidence of activated neutrophils interacting with LCs in UVB-induced skin inflammation, and their interaction can last at least 3 h or longer.

We examined the potential contribution of neutrophils to UVB-induced skin inflammation by depletion of neutrophils with anti-Ly6G mAb injection (Daley et al., 2008). 1A8 mAb specifically depletes blood neutrophils, without affecting Gr-1 expressing blood monocytes. Using this protocol, we have found that neutrophils are at least partially responsible for UVB-induced skin inflammation as measured by ear swelling. Moreover, we have observed that the presence of infiltrating neutrophils prevents the entry of pre-LCs (blood derived monocytes) into UVB-irradiated skin sites.
Depletion of epidermal LCs in langerin-DTR-EGFP mice by administration of diphtheria toxin (DT) led to virtually complete depletion of epidermal and dermal langerin+ cells within 24–48 h, as previously reported (Schwarz et al., 2010). Careful examination of epidermal sheets revealed that langerin+ epidermal “colonies” slowly increased over time but LC did not fully reconstitute the epidermis within the 6-week observation period. Reconstitution of epidermal LCs after conditional depletion is a slow process. LC precursors have been investigated by Merad’s group (Ginhoux et al., 2006a). They reported the absence of LCs in mice deficient in the receptor for M-CSF in steady-state conditions. Using bone marrow chimeric mice, they established that M-CSF receptor–deficient hematopoietic precursors failed to reconstitute the LC pool in inflamed skin caused by UVB irradiation. Furthermore, monocytes with high expression of the monocyte marker Gr-1 (also called Ly-6C/G) were specifically recruited to the inflamed skin, proliferated locally and differentiated into LCs in M-CSFR-dependent fashions. The LC repopulation by blood circulating monocytes required at least 7 days. Our results have unveiled that depletion of neutrophils during UVB induced inflammation partially promotes the entry of LC precursors to the epidermis. 7 days after UVB irradiation, we saw many Gr-1+/EGFP cells (indicating neutrophils) in the epidermis even in neutrophil depleted mice. We think these cells are delayed neutrophils that come to the irradiated skin on day 7, since the depletion of neutrophil by anti-Ly6G injection was temporary.

In summary, we have studied behavioral responses of both LCs and neutrophils in the UVB-induced skin inflammation. UVB irradiation at 3000J/m² depleted LCs from the epidermis almost completely in 4 days by promoting their migration to deeper layers of skin. UVB irradiation triggered rapid neutrophil infiltration into the epidermis within 30
minutes. For the first time, we directly observed the interactions between LCs and activated neutrophils under the inflammatory condition in a living animal. Depletion of neutrophils during inflammation resulted in accelerated recruitment of blood circulating monocytes to replenish the LC network in the epidermis, while diminishing the magnitude of UVB induced skin inflammation. Our finding may provide important knowledge for the development of new approaches to prevent sunburn reaction, UVB-induced immunosuppression, and UVB-induced skin cancer development.


Merad,M., Hoffmann,P., Ranheim,E., Slaymaker,S., Manz,M.G., Lira,S.A., Charo,I.,
Langerhans cells before transplantation of donor alloreactive T cells prevents skin graft-

Merad,M., Manz,M.G., Karsunky,H., Wagers,A., Peters,W., Charo,I., Weissman,I.L.,

U. S. A 100, 2604-2609.

Miller,M.J., Wei,S.H., Parker,I., and Cahalan,M.D. (2002). Two-photon imaging of
lymphocyte motility and antigen response in intact lymph node. Science 296, 1869-1873.

from grape seeds prevents photocarcinogenesis in SKH-1 hairless mice: relationship to

during the early recovery phase after ultraviolet-B irradiation. J. Invest. Dermatol. 88,
703-708.

2140.

immunity in vivo with a bicistronic IL-4 reporter. Immunity. 15, 303-311.

irradiation and urocanic acid isomers on dendritic cell migration. Immunology 77, 394-
399.

suppression and skin cancer development: regulation by NKT cells. Nat. Immunol. 1,
521-525.

Murphy,J.E., Robert,C., and Kupper,T.S. (2000). Interleukin-1 and cutaneous
114, 602-608.

TNFalpha in dynamic behavioral responses of Langerhans cells to topical hapten


