Chapter 26
Live Imaging of the Skin Immune Responses

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26.1 Introduction

Amongst the various organs in the human body, the skin is particularly unique due to its diverse set of roles. From physical to immune protection, thermoregulation for homeostasis and sensory functions, the skin can do it all. This can, however, sometimes be a double-edged sword. Although skin immune cells can confer protection against invading pathogens, they can also become aberrant, leading to autoimmune diseases such as alopecia areata and vitiligo. Histology, flow cytometry and RNA sequencing have been useful tools in the analysis and understanding of immune cell function in the skin in normal and diseased states. These techniques are, however, unable to reveal the dynamics of immune cell migration and cellular interaction in these states in real-time. The technique most suitable for this is the *in vivo* imaging of the skin. In this chapter, we will cover some tools that are utilized for this, and examine key studies that have advanced our understanding of immune responses in the skin.

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26.2 The Skin and Its Key Immune Cells

Histologically, the skin can be divided into two distinct sections, each with their own set of immune cells that function to keep the skin healthy. The upper layer, known as the epidermis, contains keratinocytes and Langerhans cells (LCs), whereas the dermis contains innate immune cells, including macrophages, neutrophils, mast cells, as well as cells for adaptive immune responses, such as dermal dendritic cells (DCs) and T cells (Fig. 26.1).

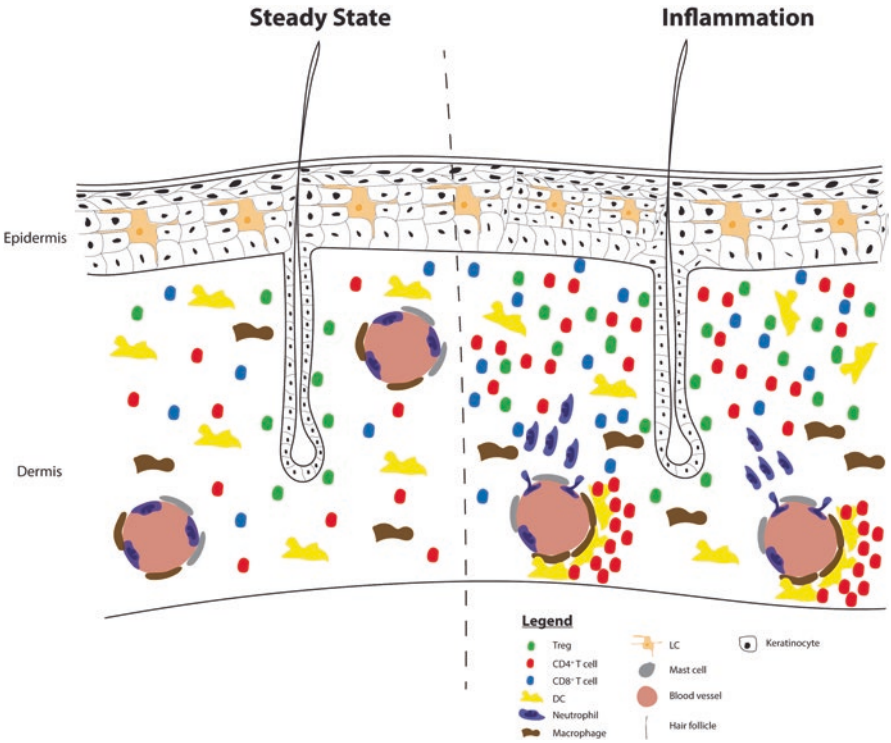

Fig. 26.1 A brief schematic of skin immune cells under steady state and inflammatory conditions

In the steady state, resident populations of LCs, dermal DCs, macrophages, mast cells and T cells exist within the skin. LCs are immobilized between keratinocytes in the epidermis whereas dermal DCs actively migrate around the dermis (Ng et al. 2008). Dermal macrophages are present throughout the dermis as well as around the dermal vasculature where mast cells also typically reside. Resident $CD4^+$ and $CD8^+$ T cells are also present throughout the dermal regions (Carbone 2015; Clark et al. 2006) whereas Tregs preferentially localize around the hair follicles (Ali et al. 2017; Chow et al. 2013)

During inflammation, neutrophils are swiftly mobilized and recruited to the site of inflammation (Goh et al. 2015). T cells are also recruited to the inflamed site albeit at a slower rate. Perivascular macrophages induce the recruitment and formation of DC clusters around dermal vessels that also contain T cells for efficient antigen presentation and activation (Natsuaki et al. 2014)

26.2.1 Dendritic Cells

DCs are specialized antigen-presenting cells (APCs) that are key to the development of immune responses. DCs are a heterogeneous population and, in steady-state skin, exist as LCs in the epidermis (Romani et al. 2010), and as dermal DCs in the dermis (Ginhoux et al. 2009). During inflammation, more DC subsets are recruited and can be found in the skin— these are the monocyte-derived DCs (Leon and Ardavin 2008) and plasmacytoid DCs (Nestle et al. 2009; Wollenberg et al. 2002). Emerging evidence in the field of LC biology highlights the similarities between LC and macrophage ontogeny, leading to the idea that LCs may be a specialized subset of tissue-resident macrophages with the capabilities of DCs (Doebel et al. 2017; Kaplan 2017).

26.2.2 Neutrophils

Neutrophils are short-lived, multi-nucleated leukocytes whose key role is to engulf pathogens. In a human adult, approximately 10^{11} neutrophils are produced in the bone marrow daily, though only 1–2% of these cells are present within the blood circulation (Borregaard 2010; Dancey et al. 1976). A small population of neutrophils has been reported to actively survey uninflamed dermis, a phenomenon that possibly allows for an immediate response to tissue damage (Li and Ng 2012; Ng et al. 2011). Upon initiation of cutaneous inflammation, neutrophils are rapidly recruited to the inflamed site (Phillipson and Kubes 2011). Upon entering the site of inflammation, neutrophils neutralize invading pathogens via phagocytosis and degranulation (Amulic et al. 2012).

26.2.3 Macrophages

Macrophages are important innate immune cells that are capable of a wide array of functions in response to the local microenvironment. Dermal macrophages are derived from blood-circulating monocytes that migrate into the skin (Geissmann et al. 2010; Jakubzick et al. 2013). In addition to phagocytosing invading pathogens, macrophages also play a role in the resolution of inflammation and subsequent wound repair (Lucas et al. 2010; Mirza et al. 2009). These differing abilities are observed with the different macrophage activation states, known as M1 and M2 macrophages or the classically activated macrophage and alternatively activated macrophage, respectively (Sica and Mantovani 2012). During wound repair, the initial pro-inflammatory phase involves M1 macrophages scavenging for, and killing, invading pathogens in the inflamed tissue. The subsequent phase of tissue regeneration involves M2 macrophages producing anti-inflammatory cytokines and growth factors to activate epithelial cells and fibroblasts.

26.2.4 Mast Cells

Deriving from hematopoietic stem cells, mast cells only differentiate to maturity upon entering peripheral tissues (Galli et al. 2005). Mast cells are long-lived immune cells that are particularly present in tissues exposed to the environment, enabling them to be first responders against environmental allergens and antigens (Galli and Tsai 2010). In the skin, mast cells localize around dermal blood vessels in an immotile state (Dudeck et al. 2011). With a spindle-like morphology in the steady state, inflammation results in mast cells taking on a more globular shape. During inflammation, mast cells secrete histamines to increase vascular permeability and promote neutrophil and effector T cell infiltration into the inflamed tissue (Biedermann et al. 2000).

26.2.5 T Cells

T cells are key players in the adaptive immune response, and can be classified into CD4- or CD8-expressing T cells, and natural killer (NK) T cells. CD4 T cells can be further subdivided into helper T cells (Th1, Th2, Th17) and regulatory T cells (Tregs). In a simplistic sense, helper T cells aid other immune cells in mounting an adaptable immune response to a wide variety of pathogens. Th1 cells protect against intracellular pathogens. Th2 cells promote the humoral immune response, stimulating B cells to produce antibodies. Th17 cells help with the recruitment of neutrophils. Tregs, as their name suggests, have a regulatory role and dampen inflammatory responses. CD8 T cells are also known as cytotoxic T cells for their ability to recognize and kill infected host cells.

Normal human skin contains approximately one million T cells per square centimeter of skin, which extrapolates to around 20 billion T cells, close to double the amount present in the blood (Clark et al. 2006). The majority of these T cells express the T cell receptor α and β chains (αβ T cells), and preferentially home to skin with CCR4 and cutaneous lymphocyte antigen (CLA). Most skin-homing T cells consist of CD4 memory T cells, and reside in the dermis. The epidermis, on the other hand, contains a minor population of tissue-resident CD8 memory T cells (Trm) (Carbone 2015). Resident Tregs in the skin preferentially localize around hair follicles (Chow et al. 2013; Gratz et al. 2013; Sanchez Rodriguez et al. 2014), and it has been reported that Treg-expressed Jag1 facilitates the hair follicular stem cell function for hair follicle regeneration (Ali et al. 2017).

The skin houses a special minor population of T cells that expresses the T cell receptor γ and δ chains (γδ T cells). In mice, γδ T cells exist in abundant numbers and are termed dendritic epidermal T cells (DETCs) (Witherden and Havran 2011). The human epidermis, however, does not have γδ T cells such as DETCs, but does have resident γδ T cells in the dermis (Ebert et al. 2006). Unlike the diverse T cell receptor repertoire of αβ T cells, γδ T cells express tissue-specific invariant T cell receptors and possess innate-like functions.

26.3 Tools for *In Vivo* Imaging

26.3.1 Microscopy

Previously, intravital imaging was limited to either the bright-field illumination of transparent tissues (Hickey et al. 1999), epifluorescence imaging of exposed dermal microvasculature (Hickey et al. 2002), or immune responses in the surface layer of the skin, the epidermis (Kissenpfennig et al. 2005). This was due to the poor penetrative ability of visible light. Technological advances led to the use of lasers, making way for a form of microscopy known as multiphoton (MP) microscopy. Also known as two-photon excitation microscopy, this is a process whereby a fluorescent molecule simultaneously absorbs two photons from rapid laser pulses. The benefit of this over conventional single-photon excitation is the deeper penetration into the tissue coupled with the reduced photodamage due to the lower energy transfer. This allows for a greater imaging depth as well as the maintenance of tissue health and viability over long imaging periods. These developments enabled the examination of a variety of fluorescently labelled leukocytes in various tissues in four dimensions (Devi et al. 2010; Gebhardt et al. 2011; Li et al. 2012; Mempel et al. 2006).

26.3.2 Animal Systems and Fluorescent-Cell Labelling Techniques

When using MP microscopy on the skin, structures such as hair shafts and elastic fibers, and collagen fibers, can be visualized due to their intrinsic autofluorescence or second harmonic generation, respectively. The visualization of collagen fibers in the skin distinguishes the epidermal and dermal layers in the skin. The conjugation of quantum dots to antibodies against lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1) can be used to visualize the peripheral lymphatic vessels (Sen et al. 2010). To observe the skin vasculature in the dermis, fluorescently labelled dextran can be used as it is retained within blood vessels for several hours (Egawa et al. 2013). The disadvantage to using fluorescently labelled dextran, however, is leakage due to vascular permeability as a result of inflammation. To circumvent this, a fluorescently labelled antibody against the endothelial cell surface marker CD31 (PECAM-1) can be used (Runnels et al. 2006). Runnels et al. demonstrated that a single administration of anti-CD31 antibody can stain the vasculature for 3–4 days after injection.

To visualize leukocytes endogenously, one can either fluorescently label leukocytes *in vitro* and adoptively transfer them into the animal, or undertake it *in situ* via intravenous injections of fluorescently labelled cell surface markers (Abeynaike et al. 2014; Deane et al. 2012). An alternative, which is currently the gold standard and essential to intravital imaging, is the use of transgenic animals that express fluorescent proteins in specific cells. In the field of immunology, there exists a variety

Table 26.1 List of transgenic mice utilized in *in vivo* imaging

Target cell	Promoter	Reporter	References
Neutrophils	Lysozyme M	eGFP	Goh et al. (2015)
Langerhans cells, Langerin$^+$ dermal DCs	Langerin	GFP	Kissenpfennig et al. (2005)
DCs	CD11c	eYFP	Goh et al. (2015) and Ng et al. (2008)
Tregs	Foxp3	GFP	Chow et al. (2013)
T cells	T cell-specific enhancer	eGFP	Bauer et al. (2014), Manjunath et al. (1999), and Mempel et al. (2006)
		DsRed	
Mast cells	Mcpt5	eYFP	Dudeck et al. (2011)
Ubiquitous (Photoconvertible)	CAG	Kaede	Tomura et al. (2010)
		KikGR	Nowotschin and Hadjantonakis (2009)

of transgenic fluorescent reporter mice (Table 26.1), and their use has contributed significantly to the knowledge of immune responses in the skin (Chow et al. 2013; Egawa et al. 2011; Gebhardt et al. 2011; Goh et al. 2015; Li et al. 2012; Ng et al. 2008; Overstreet et al. 2013). In addition, unique fluorescent proteins, such as KikGR and Kaede have the ability to change their fluorescence via photoconversion by ultraviolet irradiation. These proteins allow researchers to trace cell migration endogenously between peripheral tissues and lymphoid organs (Nowotschin and Hadjantonakis 2009; Tomura et al. 2010).

26.4 *In vivo* Imaging of Skin Immune Responses

In the past decade, intravital MP microscopy has become a vital tool in understanding the behavior of leukocytes in the development and resolution of various skin immune responses.

26.4.1 Sterile Injury

Goh et al. recently employed intravital MP microscopy to investigate dermal DC migration in the skin. Their study was unique in that the transgenic mice they used had two fluorescent cell populations. These LysM-eGFP x CD11c-EYFP mice contained LysM$^+$ neutrophils expressing enhanced green fluorescent protein (eGFP), and CD11c$^+$ DCs expressing enhanced yellow fluorescent protein (EYFP). This allowed Goh et al. to investigate the dynamic responses of the two cell types during sterile injury of the skin (Goh et al. 2015). Their findings demonstrated that the onset of sterile injury to ear skin resulted in a transition in dermal DC motility from a random probing behavior to a highly directional one. This directional motility occurred towards the site of injury with an increase in cell velocity. This transition occurred over a span of 50 min, the outcome of which saw dermal DCs surrounding

the periphery of the injury and a cessation in motility upon arrival. Conversely, neutrophils responded much quicker, arriving at the site of injury within 20 min and infiltrating to the core.

26.4.2 Contact Hypersensitivity

Contact hypersensitivity (CHS) is a commonly used mouse model of contact dermatitis, involving a type IV delayed-type hypersensitivity response. CHS is induced by small chemical compounds known as haptens that, upon binding to self-proteins, form immunogenic structures (Kaplan et al. 2012). CHS is a biphasic response, with the initial sensitization phase composed of these new immunogens activating innate immune cells, such as mast cells, macrophages, and keratinocytes. These innate cells secrete inflammatory mediators that activate resident DCs to capture the haptenated proteins. Following antigen uptake, skin DCs transiently increase their motility (Sawada et al. 2015; Sen et al. 2010) and migrate to the draining lymph nodes for presentation to, and activation of, T cells. Some skin DCs however remain, forming clusters after hapten introduction (Natsuaki et al. 2014). A subsequent exposure to the hapten initiates the second phase of CHS, which is the elicitation phase. Similar to the sensitization phase, innate immune cells are activated and skin DCs take up haptenated proteins. The presence of antigen-specific T cells in the skin generated during the sensitization phase, however, brings about a more robust inflammatory response in the skin.

Using MP microscopy, Natsuaki et al. highlighted the importance of dermal DC clusters for efficient T cell activation in the skin (Natsuaki et al. 2014). Their study showed that dermal DCs localize around perivascular macrophages that are situated on post-capillary venules. Following this, recruited T cells accumulate around these clusters, allowing for activation by proximal antigen-bearing dermal DCs. As these clusters only appear during inflammation, they have been termed "inducible skin-associated lymphoid tissues (iSALT)" (Ono and Kabashima 2015).

MP microscopy in conjunction with the CHS model has also been used to study T cell dynamics in cutaneous inflammation. Honda et al. demonstrated that effector T cells become sessile and form stable contacts with DCs within 10 min of antigen recognition (Honda et al. 2014). These effector T cells successively regain their motility within 6–8 h. Interestingly, Honda et al. discovered an inverse correlation between cytokine production and cell motility whereby these effector T cells only produce cytokines while immobile. Another study by Chow et al. investigated the dynamics of skin regulatory T cells (Tregs) during a CHS response via MP microscopy (Chow et al. 2013). They reported that, unlike the high motility of effector $CD4^+$ T cells, most Tregs were sessile in steady state skin. During the elicitation phase of CHS, however, approximately 40% of Tregs increased their motility. It is possible that migratory Tregs are either increasing their area of regulatory influence via cytokine secretion (Vignali et al. 2008), or are in the process of migrating to draining lymph nodes (Tomura et al. 2010). Sessile Tregs on the other hand, could

be interacting with DC-effector T cell clusters to exert their regulatory control (Onishi et al. 2008).

26.4.3 Infection

Using intravital MP microscopy, Ng et al. investigated the behavior of dermal DCs in ear skin in response to *Leishmania major* injection (Ng et al. 2008). Their study revealed that dermal DCs continuously surveyed the dermis in a highly motile, and G protein-coupled receptor-dependent manner under homeostatic conditions. Upon the introduction of *L. major* parasites to the dermis, local dermal DCs became immotile, initiating parasite uptake into cytosolic vacuoles. These changes in migration suggest that dermal DCs are constantly probing the microenvironment for foreign antigens, and may undergo arrest to process and present these antigens to cells.

Gebhardt et al. utilized MP microscopy to demonstrate the localization and distinct migratory behavior of herpes virus-specific $CD4^+$ (gDT-II) and $CD8^+$ (gBT-I) effector memory T (T_{EM}) cells in mouse skin following resolution of a cutaneous herpes simplex virus (HSV) infection (Gebhardt et al. 2011). Their study describes a slow-moving population of $CD8^+$ T_{EM} cells during the memory phase (30 days post-infection) that were resident in the epidermis, and in close proximity to the site of HSV infection. On the other hand, $CD4^+$ T_{EM} cells were observed to be migrating extensively in a recirculating pattern that was limited to the dermis.

26.4.4 Cancer

Of clinical importance is the role of Tregs in tumor immunology. With the potential to restrict the hosts' anti-tumor immune response, a copious amount of Tregs surrounding the tumor can be a negative prognostic indicator (Tanaka and Sakaguchi 2017). To understand the actions of Tregs in the tumor microenvironment, studies have been conducted using MP microscopy to observe Treg behavior *in vivo*.

Using a mouse model in which influenza HA-expressing tumors were implanted under the flank skin, Bauer et al. documented the interactions of $CD8^+$ T cells and Tregs via MP microscopy (Bauer et al. 2014). By adoptively transferring HA-specific Tregs, tumor-infiltrating $CD8^+$ T cells transitioned to a state resembling T cell exhaustion. Further analysis using MP microscopy revealed that Tregs in the tumor microenvironment were migratory, which was in stark contrast to the surrounding $CD8^+$ T cells. Interestingly, the migratory behavior of the Tregs included moments of arrests to form unstable contacts with $CD11c^+$ APCs. These interacting APCs had a marked reduction in their expression of costimulatory molecules CD80/86, and $CD8^+$ T cell activation by these incapacitated APCs resulted in the expression of inhibitory receptors programmed cell death protein 1 (PD-1) and T cell immunoglobulin- and mucin-domain-containing-3 (TIM-3) on $CD8^+$ T cells. These findings

emphasize the capability of MP microscopy in revealing the mechanism by which Tregs can promote tumor survival.

26.5 Concluding Remarks – Looking Ahead to the Future

Over the last two decades, intravital imaging has proved to be a useful tool in expanding our knowledge on cellular behavior in their native environment. Although techniques such as flow cytometry, immunohistochemistry and RNA sequencing are able to provide insight into cellular function, they are but snapshots. The way in which an immune cell changes shape, moves, and interacts with neighboring cells during various types of immune responses can only be visualized via intravital imaging. Together, these techniques complement each other to not only help us build upon our current understanding of skin immunology, but also potentially discover new facets of leukocyte behavior in the skin.

Currently, MP microscopy is heavily utilized in animal studies, but not in human studies. One key limitation is the thickness of human skin compared to mouse skin, which reduces the penetrative ability of the laser. MP microscopy has been used on humans to evaluate skin tumors, skin aging, and epidermal cells in skin diseases (Klemp et al. 2016; Koehler et al. 2011; Murata et al. 2013; Tsai et al. 2009). For further use on humans, advancements in MP microscopy are necessary. Until then, the development of better cell-labelling systems, novel transgenic mouse systems and optical microscopic systems will drive our continual discovery of skin immunology.

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