Kenji Kabashima *Editor*

Immunology of the Skin

Basic and Clinical Sciences in Skin Immune Responses

Springer

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Preface

The skin is one of the largest organs of our body and is continuously exposed to a variety of external stimuli, such as bacteria, viruses, fungi, ultraviolet light, chemicals, dryness, haptens, and protein antigens. Thus, the skin is an important barrier between the living organism and its environment to maintain our homeostasis. Defending physically against external stimuli, the skin is also an immunological defense.

The immune capacity of the skin involves several cell types: Langerhans cells, dermal dendritic cells, T cells, endothelial cells, keratinocytes, mast cells, basophils, and other cells, all of which participate under certain circumstances in a harmonious manner. Thus, the concept of skin-associated lymphoid tissue (SALT) was proposed in the early 1980s. As a result of immune responses to external stimuli, several inflammatory skin diseases are induced. Therefore, understanding the skin immune responses is essential not only to basic scientists including immunologists but also to clinicians, such as allergologists and dermatologists.

For this book, I prepared two major parts: I. Components of Skin Immune Cells, and II. Immune Systems in the Skin. This thematic division will make the book easily understood by readers. In addition, I have tried to cover each topic in full detail, which will lead to a better, comprehensive understanding of the skin and skin diseases.

To provide a readable and informative presentation, I chose world-renowned authors in each field. I am very glad that they agreed to write their chapters despite their crowded schedules. I hope that this book will be useful to understand the subject of immunology of the skin.

Kyoto, Japan
Autumn 2015

Kenji Kabashima

Contents

1 Overview: Immunology of the Skin 1
Kenji Kabashima

Part I Components of Skin Immune Cells

2 Stratum Corneum 15
Yoshikazu Uchida and Kyungho Park

3 Keratinocytes 31
Koji Sayama

4 Langerhans Cells and Dermal Dendritic Cells 43
Saeko Nakajima

5 T Cells 57
Takashi Nomura and Aya Shinohara

6 γδ T Cells 95
Kazuhiro Kawai

7 B Cells 113
Manabu Fujimoto

8 Mast Cells and Basophils 131
Atsushi Otsuka

9 Neutrophils 147
Rohit Jain, Andrew J. Mitchell, Szun S. Tay, Ben Roediger, and Wolfgang Weninger

10 Macrophages 169
Sho Hanakawa and Akihiko Kitoh

11 Myeloid Derived Suppressor Cells 179
Taku Fujimura and Alexander H. Enk

12 Lymphatic Vessels 193
Satoshi Hirakawa and Michael Detmar

13 Hair Follicles 203
Manabu Ohyama and Keisuke Nagao

14 Platelets 213
Risa Tamagawa-Mineoka and Norito Katoh

15 Adipose Tissues 227
Satoshi Nakamizo and Gyohei Egawa

Part II Immune Systems in the Skin

16 Innate Immunity 241
Takashi Satoh and Naotomo Kambe

17 C-Type Lectin Receptors 255
Nobuo Kanazawa

18 Emergence of Virulent Staphylococci Overriding Innate Immunity of Skin in Communities 275
Junzo Hisatsune and Motoyuki Sugai

19 Viral Infection 295
Tatsuyoshi Kawamura

20 Contact Dermatitis 325
Tetsuya Honda

21 Atopic Dermatitis: Common Extrinsic and Enigmatic Intrinsic Types 339
Yoshiki Tokura

22 Psoriasis 359
Hanako Ohmatsu and James G. Krueger

23 Urticaria 375
Marcus Maurer, Markus Magerl, Torsten Zuberbier, and Karsten Weller

24 Cutaneous Adverse Drug Reactions: Stevens–Johnson Syndrome and Toxic Epidermal Necrolysis 393
Riichiro Abe

25 Pemphigus 405
Jun Yamagami, Hayato Takahashi, and Masayuki Amagai

26 Anaphylaxis 419
Eishin Morita

27 Graft Versus Host Disease (GVHD) 429
Fumi Miyagawa and Stephen I. Katz

28 Rosacea in Skin Innate Immunity 451
Kenshi Yamasaki

29 Cutaneous Lymphomas 463
Simone M. Goldinger, Emmanuella Guenova, Kazuyasu Fujii, and Mirjana Urosevic-Maiwald

30 Photodermatology: Therapeutic Photomedicine for Skin Diseases 477
Akimichi Morita

31 Collagen Vascular Disease 489
Yoshihide Asano

Index 503

Chapter 1
Overview: Immunology of the Skin

Kenji Kabashima

Abstract Skin is a barrier between the living organism and its environment. In addition to defending physically against external stimuli, it also defends immunologically. The immune capacity of the skin involves several cell types: Langerhans cells, dermal dendritic cells, T cells, endothelial cells, keratinocytes, mast cells, basophils, and other cells all participate under certain circumstances in a harmonious manner. Thus, the concept of skin-associated lymphoid tissue (SALT) was proposed in the early 1980s. As a result of immune responses to external stimuli, several inflammatory skin diseases are induced. In this process, different types of topical antigens can induce different types of cutaneous immune responses, and that the duration of antigen exposure modulates the cutaneous Th1/Th2 milieu dynamically. Since the recent immunological findings has lead to the development of new therapeutics, including biologics. To understand the skin immune responses is essential not only to basic scientists, including immunologists but also clinicians, such as allergologists and dermatologists.

Keywords Skin • Immunology • SALT • Dendritic cells • T cells • Photoconversion • Langerhans cells

1.1 Skin as an Immune Organ

The skin is a barrier between the living organism and its environment; as such, it defends against external stimuli, including physical and chemical stresses, dryness, ultraviolet light exposure, bacteria, fungi, viruses, parasites, haptens, and protein antigens. Some of this defensive activity occurs through the immune system. In the skin, Langerhans cells (LCs), dermal dendritic cells (DCs), endothelial cells, keratinocytes, mast cells, basophils, and other cells all participate under certain circumstances (Fig. 1.1).

It was recently demonstrated that there are about 20 billion T cells in the skin of an adult human, bearing markers that identify them as skin homing memory T cells (CD45RO/CLA/CCR4) [1]. Not only are there twice as many T cells in skin as in

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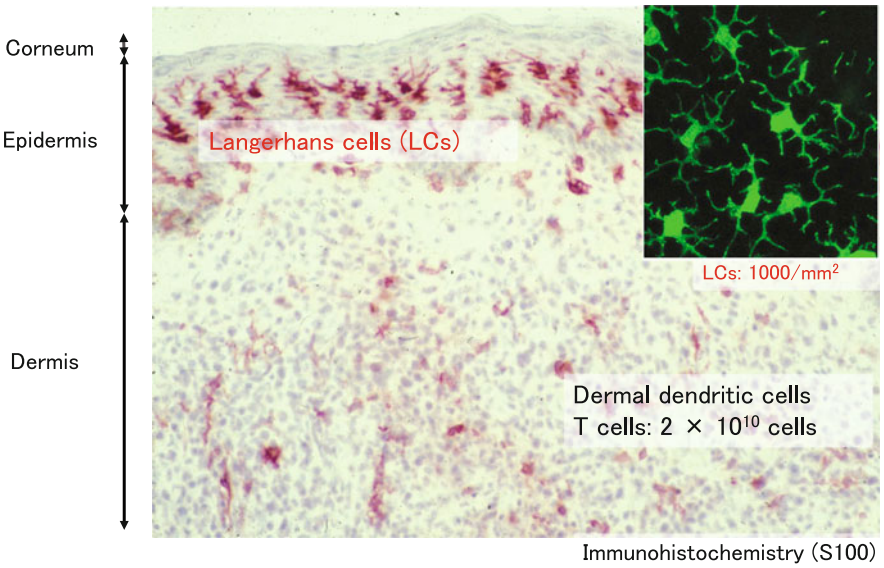

Fig. 1.1 Immunohistochemistry of the skin with S100. The skin consists of the corneum, epidermis, and dermis. The epidermis contains Langerhans cells, and the dermis contains dermal dendritic cells, which serve as antigen presenting cells

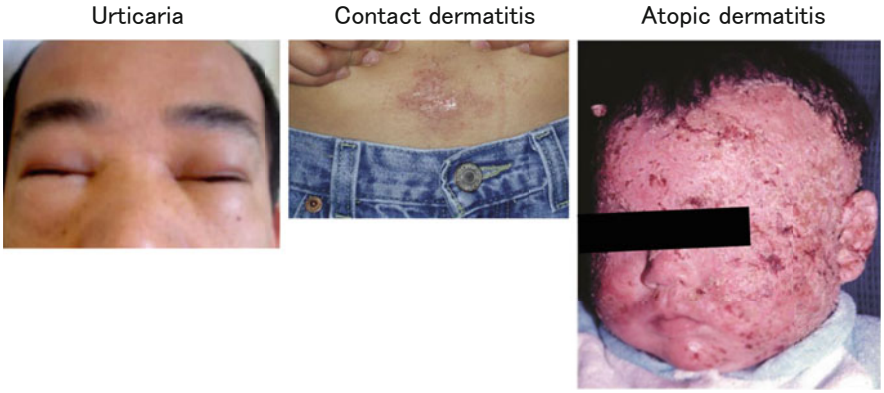

Fig. 1.2 Clinical manifestations of inflammatory skin diseases

blood, but the number of memory T cells with a skin homing phenotype is more than 20 times the number of those in the blood. In addition, LCs localize in the epidermis as antigen presenting cells at a density of 1000 per mm^2, suggesting that the skin is an important immune organ.

As a result of immune responses to external antigens, inflammatory skin diseases can be induced: urticaria by oral intake of allergens, including egg and fish; contact dermatitis by haptens, including metals and urushiol; and atopic dermatitis by proteins, including mites, house dust, and pollen (Fig. 1.2) [2–5].

1.2 Concept of SALT (Skin Associated Lymphoid Tissue)

Immune function is not limited to the skin. In submucosal areas, for example, specific sentinel lymphoid tissues called mucosa-associated lymphoid tissues (MALT) serve as peripheral antigen presentation sites [6]. By analogy, the concept of skin-associated lymphoid tissue (SALT) was proposed in the early 1980s based on the discovery that (1) the cutaneous microenvironment can accept, process, and present antigens, (2) the peripheral lymph nodes (LNs) can accept immunogenic signals derived from the skin, (3) subsets of T cells exhibit a differential affinity for skin, and (4) the acquisition of this affinity by T cells is determined by resident cutaneous cells [7].

On the other hand, there are distinct functional differences between MALT and SALT. MALT contains significant numbers of B cells and forms lymphoid follicles, whereas virtually all lymphocytes within the skin are T cells. MALT lymphoid follicles are surrounded by T-cell–rich areas in which high endothelial venules (HEVs) are embedded and serve as entry points for naïve T cells. Therefore, MALT provides a field for antigen presentation to naïve T cells as well as other secondary lymphoid organs. SALT, in contrast, contains no HEVs, and the T cells in skin are memory T cells rather than naïve T cells. Therefore, skin-draining LNs are necessary for the priming of naïve T cells to foreign antigens that have invaded through the skin.

The T-cell homing system is tightly regulated by the expression of adhesion molecules and the chemokine receptors called addressins. Certain T cell subsets have a high affinity for the skin and the gut as well as for secondary lymphoid organs (Table 1.1, Fig. 1.3). Thus it is clear that defending the outermost membranes, namely, the skin and the gut, is a high priority for the immune system.

Table 1.1 Receptors involved in tissue-specific homing

	Cell type	Receptor	Ligand
Peripheral LNs	Naive T, T_{CM}	CCR7	CCL19, CCL21
	Naive T, T_{CM}	CD62L	sLex
Gut	T_{EM}	CCR9	CCL25
	T_{EM}	α4β7-Integrin	MAdCAM-1
Skin	T_{EM}	CLA	E-selectin
	T_{EM} (Th1)	CXCR3	CXCL9, CXCL10
	T_{EM} (Th2)	CCR4	CCL17, CCL21
	T_{EM} (Th2)	CCR10	CCL27, CCL28
	T_{EM} (Th2)	CCR8	CCL8

Notes: *CLA* cutaneous lymphocyte-associated antigen, *LNs* lymph nodes, *MAdCAM-1* mucosal vascular addressin cell adhesion molecule-1, *sLex* sialylated Lewis x, T_{CM} central memory T cell, T_{EM} effector memory T cell, *Th1* T helper 1, *Th2* T helper 2, *Th17* T helper 17

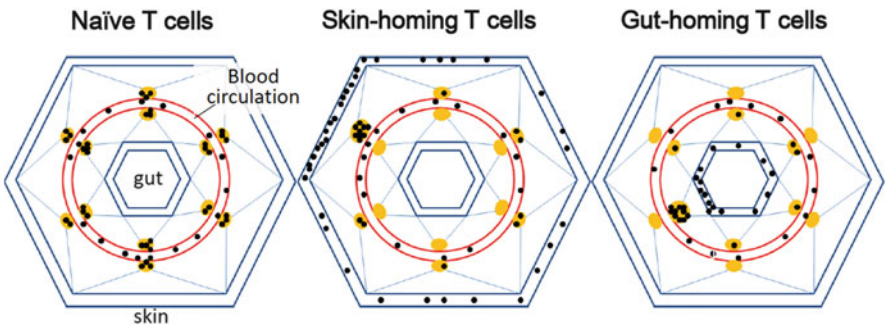

Fig. 1.3 Tissue-specific homing ability of T cells. Three distinct homing systems to the secondary lymphoid organs, skin, and gut. *Solid black* and *brown circles* denote T cells and lymph nodes, respectively

1.3 Immune Reactions to Foreign Antigens

Antigen presentation to T cells is essential for the induction of adaptive immunity. This event takes place not solely in the LNs where naïve T cells are primed but also in the skin where memory T cells are activated.

Upon protein antigen exposure, DCs acquire antigens and stimulate the proliferation of T cells to induce distinct T helper cell responses to external pathogens [3]. In mouse skin, there are at least three subsets of DCs [8–10]: LCs in the epidermis and Langerin-positive and Langerin-negative DCs in the dermis (Langerin$^+$ dermal DCs and Langerin$^-$ dermal DCs, respectively).

It has been reported that, when epicutaneously applied, large molecules such as protein antigens are above the size-selective barrier known as the tight junction (Fig. 1.4), and that activated LCs extend their dendrites through the tight junction to take up antigens [11]. Topically applied haptens, on the other hand, penetrate into the dermis.

1.3.1 Immune Reactions to Haptens

Haptens are external antigens that easily penetrate into the dermis (Fig. 1.4). As is well known, a single hapten application induces a classic delayed-type hypersensitivity called the contact hypersensitivity (CHS) response, which is mediated by IFN-γ–producing CD8$^+$ (Tc1) and CD4$^+$ T (Th1) cells (Fig. 1.5) [3].

LCs have long been regarded as essential antigen-presenting cells for the establishment of sensitization in hapten-induced CHS, but this concept is now being challenged by recent analyses using LC ablation murine models [12]. In the development of CHS to haptens, Langerin-negative dermal DCs play a major role, whereas LCs and Langerin-positive dermal DCs play a compensatory role [13].

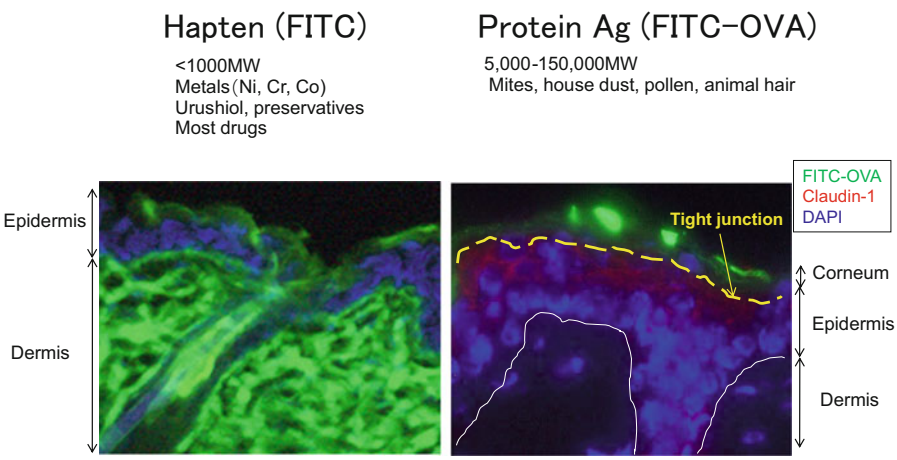

Fig. 1.4 Distribution of haptens and protein antigens upon epicutaneous application. After the epicutaneous application of green-fluorescent hapten (FITC) as a hapten and FITC-ovalbumin (FITC-OVA) as a protein antigen, FITC penetrates into the dermis whereas FITC-OVA is retained at the corneum

On the other hand, repeated applications of haptens (2,4,6-trinitrochlorobenzene; TNCB) induce atopic dermatitis-like skin lesions [14] by causing a shift from Th1- to Th2-mediated cutaneous inflammation with elevated IL-4 expression, eosinophil infiltration in the skin, and elevated hapten-specific serum IgE levels [14]. At present, which class of cells mediates the shift from Th1 to Th2 remains a topic of debate. One of the candidate classes appears to be basophils, which express MHC class II and IL-4 in the draining LNs [15] (Fig. 1.6).

It has also been reported that regulatory T cells (Treg) accumulate in the skin during CHS [16] and that Treg suppress both the sensitization and the elicitation of the CHS response [17–19]. In addition, IL-10 is induced in the repeat hapten application-induced chronic CHS model [20]. These findings demonstrate that chronic antigen exposure induces Treg accumulation in the skin (Fig. 1.6). Clinically, topical immunotherapy with squaric acid dibutylester (SADBE) is effective for the treatment of alopecia areata [21]. It remains unclear how SADBE controls this autoimmune disease, but the accumulation of Treg in chronically hapten-exposed skin may play an important role (Fig. 1.5).

1.3.2 Immune Reactions to Protein Antigens

Unlike haptens, the conventional allergens responsible for atopic dermatitis are rather large (Fig. 1.4). Therefore, LCs are thought to be the subset of DCs that is responsible for acquiring cutaneous allergens, such as house dust mites, in the development of atopic dermatitis [22].

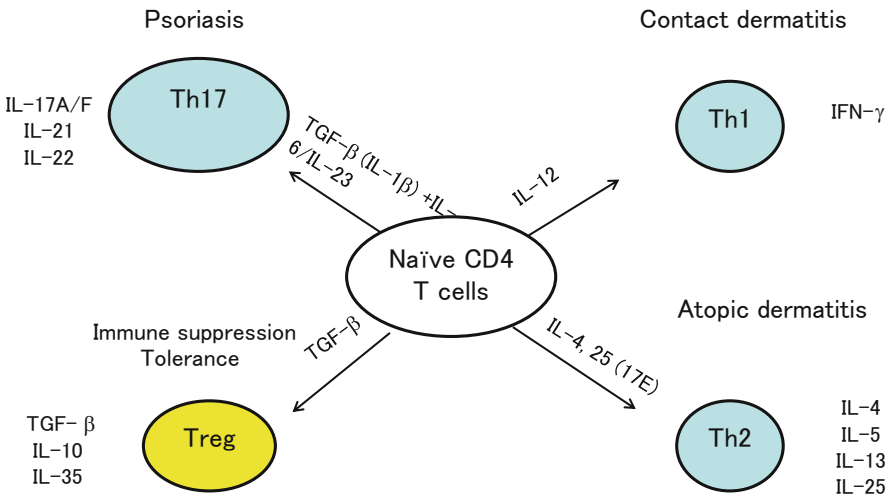

Fig. 1.5 T cell subsets. $CD4^+$ helper T (Th) cells have at least three subtypes, Th1, Th2, and Th17, that are involved in the pathogenesis of contact dermatitis, AD/urticaria, and psoriasis, respectively. These Th subtypes are induced by specific cytokine conditions. Regulatory T cells (Treg), on the other hand, are localized in the skin where they play an important role in maintaining homeostasis and terminating a variety of skin immune responses

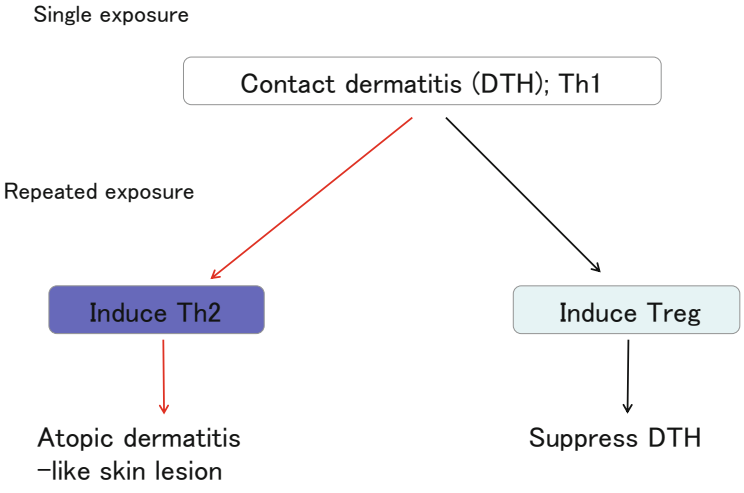

Fig. 1.6 Dynamics of cutaneous immune responses. A single hapten elicitation induces Th1-mediated delayed-type hypersensitivity (DTH), also known as contact dermatitis/contact hypersensitivity. Even during the contact dermatitis response, Treg accumulate in the skin and suppress DTH responses. Repeated hapten elicitation induces Th2 conditions in the skin, which are characteristic of atopic dermatitis

It is known that the epicutaneous application of the protein antigen ovalbumin (OVA) induces a rise in OVA-specific serum IgE and IgG1, both of which are induced in a Th2-dependent manner, as well as the development of dermatitis characterized by the infiltration of $CD3^+$ T cells, eosinophils, and neutrophils and the local expression of mRNA for the cytokines IL-4, IL-5, and, intriguingly, IFN-γ [23]. Consistently, chronic exposure to protein antigens, especially those with protease activities (e.g., house dust mite allergens), induces TSLP expression in the epidermis. The above findings suggest that different types of topical antigens can induce different types of cutaneous immune responses, and that the duration of antigen exposure modulates the cutaneous Th1/Th2 milieu dynamically (Fig. 1.6).

1.4 Interplay Between Skin Barrier Functions and Skin Immunology

It has been suggested that acute removal of the stratum corneum modulates the production of cytokines and chemokines by epidermal cells. Tape stripping upregulates TSLP levels in the skin, which polarizes skin DCs to elicit a Th2 response [24]. Therefore, barrier disruption seems to bias the skin environment towards Th2. In addition, Th2 chemokine (CCL17 and CCL22) and eosinophil chemoattractant (CCL5) mRNA levels were markedly elevated in mice as a result of barrier disruption, more markedly by tape stripping than by acetone rubbing [25]. In addition, tape stripping induced dermal infiltration of eosinophils in mice [25]. These findings suggest that acute barrier removal induces a Th2 milieu and the production of eosinophil chemokines by epidermal cells and easily evokes the late-phase reaction in response to an antigen challenge. Thus barrier dysfunction predisposes the skin environment to Th2 skewing conditions and makes exposure of the internal skin to antigens more feasible.

In an intriguing contrast, human keratinocytes differentiated in the presence of IL-4 and IL-13 exhibited significantly reduced *FLG* gene expression [26]. In addition, IL-17A downregulates the expression of filaggrin and genes that are important for cellular adhesion, which leads to impairment of epidermal barrier formation [27]. Consistently, the level of FLG expression in atopic dermatitis patients even without *FLG* mutations was decreased. These findings indicate that the Th2-type skin immune responses induce an acquired barrier defect and create a positive feedback loop through a highly complex interplay.

1.5 Communication Between the Skin and Draining LNs

The fate of skin-directed memory T cells is, at this point in time, largely unknown, and the majority of these cells progress to apoptosis after termination of skin inflammation. Recently, the trafficking of memory T cells between the skin and draining LNs has been examined in vivo using Kaede protein. Kaede protein is a newly developed photoconvertible fluorescent protein that can change emission spectra in response to light exposure (Fig. 1.7) [16]. In Kaede-transgenic (Tg) mice, all cell types constitutively exhibit Kaede-green fluorescent signals. Immediately after the skin is exposed to violet light, however, cells in the exposed area begin to emit Kaede-red fluorescent signals. Thus skin T cells can be easily identified and labeled under physiological conditions in vivo (Fig. 1.7).

In Kaede-Tg mice, it has been reported that approximately 5 % of $CD11c^+$ cells and 0.5–1 % of $CD4^+$ T cells in the skin-draining LNs are skin-derived cells, suggesting that memory T cells as well as cutaneous DCs can constantly migrate from the skin to draining LNs, even under steady-state conditions [16]. It is important to note that all skin-derived Kaede-red T cells express not only CD44,

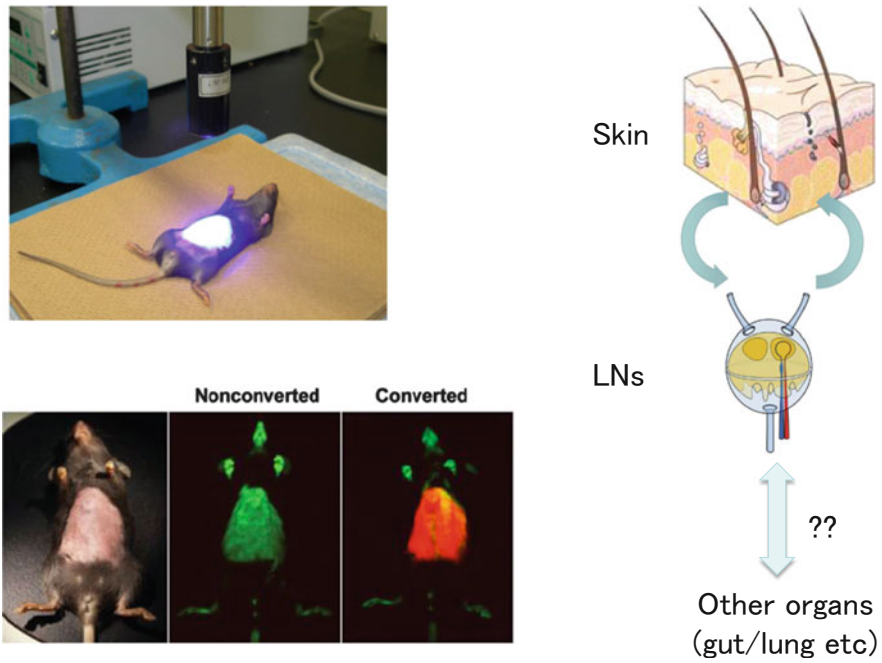

Fig. 1.7 Kaede transgenic mice and interplay among the skin, skin-draining LNs, and other organs. Kaede transgenic mice were photoconverted on the clipped abdominal skin (*upper* and *lower left panels*) and observed with a fluorescence stereoscopic microscope (*upper panel*). Nonphotoconverted clipped skin is shown as a control (*lower middle*) (Note: The nonclipped area shows up as *black* because the light cannot reach it)

a marker of memory T cells in mice, but also CCR7 and CD62L, suggesting that they exhibit a unique homing receptor expression profile which resembles that of central memory T cells (T_{CM}).

The trafficking of skin-associated memory T cells was also evaluated in the inflammatory state. Kaede-Tg mice were sensitized with hapten on a dorsal area of skin and challenged with the same hapten on abdominal skin. The antigen-challenged site was then exposed to violet light. After the photoconversion, the number of Kaede-red cells in the draining LNs increased to approximately ten times the number present in steady state, reflecting the accumulation of memory T cells into the abdominal skin. Intriguingly, when another site (the ear skin) was rechallenged, Kaede-red $CD4^+$ T cells were detected both in the blood and in the ear skin. These findings suggest that a portion of skin-directed effector memory T cells (T_{EM}) recover LN homing ability, CCR7 and CD62L expression, and return to skin-draining LNs, especially in the inflammatory state. Moreover, these cells re-enter the blood circulation system, and recover skin-homing addressins or produce skin-homing T_{CM} upon antigen rechallenge. The above findings provide evidence that T cells migrate between the skin and draining LNs efficiently (Fig. 1.7).

Patients with AD often have other allergic diseases, including food allergies, asthma, and allergic rhinitis [28]; these often begin early in life and progress in a typical fashion; this is called the allergic (or atopic) march [29]. The skin is an active immune system organ that influences systemic immunity [30]. The next question is whether skin-derived immune cells can circulate into other organs, such as the lungs and the gut; this question can be addressed in future studies using Kaede-Tg mice (Fig. 1.7).

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Part I
Components of Skin Immune Cells

Chapter 2
Stratum Corneum

Yoshikazu Uchida and Kyungho Park

Abstract The stratum corneum, consisting of denucleated keratinocytes, corneocytes that are eventually shed from skin, is a highly-functional outer layer of skin tissue. The structure of the stratum corneum is well-organized, and its formation is tightly regulated to insure its ability to perform competent epidermal barrier functions. An incompetent barrier cannot prevent harmful external microbes and stress (perturbation) from affecting internal tissues, leading to deleterious effects in cutaneous and extracutaneous cells/tissues. An abnormal permeability barrier increases the ingress of allergens that trigger inflammatory responses. These inflammatory responses then affect normal keratinocyte proliferation, differentiation, and barrier formation, keeping the formation of an incompetent barrier that sustains inflammatory responses. The stratum corneum is also responsible for innate immunity and modulation of adaptive immunity responses.

Keywords Barrier • Lamellar membrane • Stratum corneum • Corneocyte • Ceramide • Cornified envelope • Corneocyte lipid envelope • Antimicrobial peptide

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2.1 Introduction

The outer skin (the epidermis) consists of four layers: the stratum basale, stratum spinosum, stratum granulosum, and stratum corneum. Inasmuch as it was understood to be constructed of denucleated dead tissues with no significant function, very little attention was paid to the stratum corneum until fairly recently (Fig. 2.1). Yet the elucidation of unique structures, requirements for terrestrial mammalian survival, and association of the stratum corneum's structural and functional alterations with several cutaneous diseases have helped foster a great interest in this epidermal layer in the last decade. In particular, the finding of gene mutations in a constitutional protein, filaggrin, in the stratum corneum, and filaggrin's deficiency occurring in ichthyosis vulgaris and atopic dermatitis, has further stimulated research into the stratum corneum. The stratum corneum directly faces the external environment; therefore it functions as a barrier against this external environment, protecting internal cells and tissues from external insults while maintaining normal cellular functions. In addition to having protective barrier functions, the stratum corneum serves as a sensor of external conditions [1]. However, this function has not yet been well-characterized.

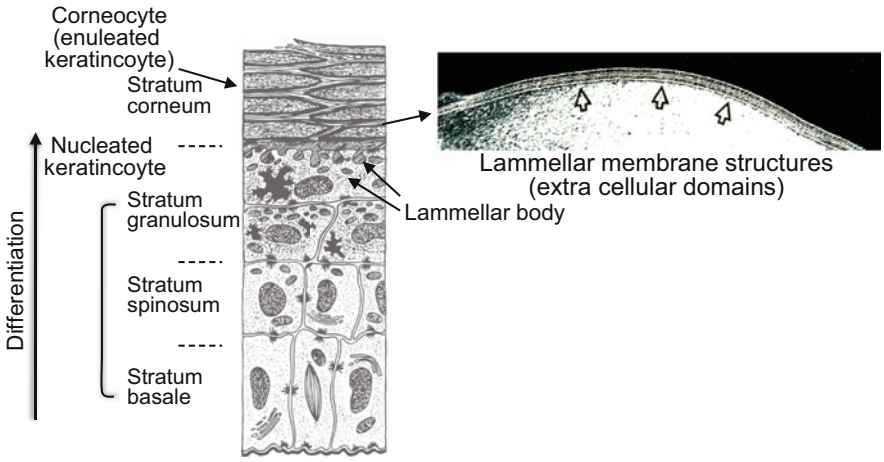

Fig. 2.1 Epidermal structures. Insert, electron micrograph: Murine skin was fixed in Karnovsky's fixative overnight, and postfixed with 0.25 % ruthenium tetroxide. Ultrathin sections were examined using an electron microscope

2.2 Stratum Corneum Structure

The stratum corneum is composed of two compartments, corneocytes and lipid-dominant extracellular lamellar membrane structures (Fig. 2.1). The architecture of the stratum corneum is referred to as "brick (corneocyte) and mortar (lamellar membranes)." Approximately 15 layers (20 μm) of corneocytes, denucleated forms of keratinocytes, are present in the normal human stratum corneum (with ≈ 200 layers on the sole and palm). In corneocytes, the plasma membranes are replaced by protein cross-linked cornified envelopes, which differs from nucleated cells surrounded by plasma membranes that comprise a lipid bilayer. Although de novo syntheses of proteins, lipids, and nucleotides do not occur in the stratum corneum, these existing cellular components and likely exogenous compounds are catabolized in the stratum corneum.

2.2.1 Corneocyte

During the transition from granular layers to stratum corneum, nuclei of keratinocytes are degraded. Denucleated keratinocytes, i.e., corneocytes, exhibit a flat shape and are filled with keratin fibers and degraded products of proteins, lipids, and nuclei. Endogenous humectant's natural moisturizing factor (NMF) is generated in the stratum corneum by degradation of histidine-rich proteins, primarily filaggrin [2]. Filaggrin deficiencies are due to mutations of the filaggrin gene associated with ichthyosis vulgaris and atopic dermatitis [3]. The filaggrin deficiencies cause decreased NMF and therefore declining hydration in the stratum corneum [4].

Corneodesmosomes comprised of desmoglein-1 and desmocollin-1 attach to other corneocytes [5]. Corneodesmosomes are degraded by an acidic pH optimum aspartyl protease, cathepsin D, in the stratum corneum [6] and neutral pH optimum chymotriptic- and tryptic-serine proteases [7–9], including kallikrein-related peptidases, which results in shedding corneocytes from the epidermis, i.e., desquamation. The kazal-type family (SPINK) is endogenous trypsin-like and chymotrypsin-like serine protease inhibitors that are present in the stratum corneum and are involved in the regulation of desquamation [10–12]. Netherton syndrome, a severe autosomal recessive ichthyosis, showing abnormal desquamation, is caused by a loss-of-function mutation in SPINK5, which encodes the lymphoepithelial kazal-type trypsin inhibitor (LEKT1) [13].

2.2.2 Cornified Envelope

Nucleated cells are surrounded by a plasma membrane consisting of lipid bilayer structures, and plasma membranes are replaced by a cornified envelope that is formed by cross-linked proteins [14]. The synthesis of constituted proteins of the cornified envelope, i.e., loricrin, involucrin, small proline-rich proteins (SPRs), envoplakin, and other minor protein components, increase at late stages of epidermal differentiation. Protein cross-linkages are due mainly to the ε-(γ-glutamyl) lysine isopeptide bond generated by transglutaminases (TG) [15]. Seven isoforms of transglutaminases are characterized in mammals. Five isoforms, TG1, TG2, TG3, TG5, and TG6 are expressed in keratinocytes [15–17]. In particular, TG1, which is a major isoform in keratinocytes [15], is critical for cornified envelope formation [18]. The cornified envelope exhibits a stable rigid property to resist mechanical barrier stress [19]. Mutations of the TG1 gene have been shown in lamellar ichthyosis [20], bathing suit ichthyosis [21], and congenital ichthyosiform erythroderma [22], and a TGM5 mutation is associated with peeling skin syndrome [23].

2.2.3 Corneocyte Lipid Envelope

The outer leaflet of the cornified envelope is covered by a monolayer of corneocyte lipid envelope (CLE), which consists of omega (ω)-hydroxy-ceramides and its catabolites, ω-hydroxy free fatty acid generated by ceramidase [24–26] (Fig. 2.2). CLE is formed as follows: (1) ω-O-acyl residue is released from ω-O-acyl (predominantly an essential fatty acid, linoleate) glucosylceramides; (2) ω-hydroxyl residue of ω-hydroxy-glucosylceramides is covalently bound to cornified envelope proteins (primarily to glutamate residues in cornified envelope protein [mainly involucrin]) [27]; (3) ω-hydroxy-glucosylceramides are deglucosylated by ß-glucocerbrosidase to cornified envelope-ω-hydroxy-ceramides; (4) some CE-ω-hydroxy-ceramides are hydrolyzed to cornified envelope-ω-hydroxy free fatty acid by ceramidase(s) [26, 28]. Releasing of linolate residue of ω-O-linoleoyl glucosylceramides is required by 12R lipoxygenase (12R-LOX) or epidermal lipoxygenase 3 enzymes, which oxidize the linoleate moiety of ω-O-linoleoyl glucosylceramides, generating an oxidized species that subsequently attach to cornified envelope proteins [29]. Mutation of these lipoxygenases is associated with nonbullous congenital ichthyosiform erythroderma [30]. TG1 is involved in lipid and protein binding. Yet, CLE is evident in the stratum corneum of lamellar ichthyosis patients that show trace levels of TG1 activities [25]. Hence, other transglutaminase(s), other enzyme(s), or nonenzymatic transterification might also serve in CLE formation [25]. A role for CLE has been proposed as a scaffold to form lamellar membrane structures (see below, Sect. 2.4) [24, 25]. It is also

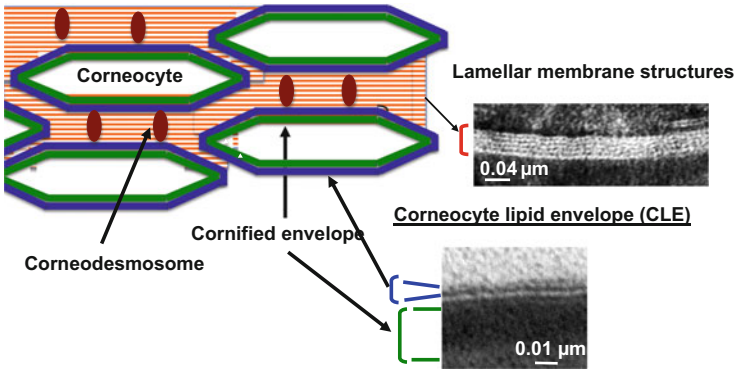

Fig. 2.2 Stratum corneum structure. Cornified envelope, *Green*; Corneocyte lipid envelope (CLE), *Blue*; Corneodesmosom, *Brown*. Insert, electron micrograph: Murine skin was fixed in Karnovsky's fixative, and post-fixed with 1 % aqueous osmium tetroxide, containing 1.5 % potassium ferrocyanide

possible that CLE regulates egress of hydrophilic substances from corneocytes. Yet, the roles of CLE in the stratum corneum are still largely unknown.

2.2.4 Lamellar Membrane Structure

In contrast to dermis in which extracellular matrix is filled with collagen, elastin, glycosaminoglycans, and glycoproteins, lipid-enriched lamellar membrane structures fill the extracellular domain in the stratum corneum. Lamellar membranes extend in a horizontal direction (Figs. 2.1 and 2.2). Corneodesmosomes disengage from the lamellar membrane structure to bind to other corneocytes. Corneodesmosomes increase integrity and also regulate desquamation by their degradation.

Ceramide, cholesterol, and free fatty acid are major constituents (95>% of total lipid) [31] of lamellar membrane structures. Ceramide metabolites, sphingosine [31], and ceramide-1-phosphate [32] are present as minor components. Ceramides in the stratum corneum comprise at least 12 molecular groups of heterogeneous molecular species, including epidermal unique ultralong chain (up to 34 carbon chain lengths) fatty acid, ultralong chain omega-hydroxylated fatty acid, and ultralong chain omega-O-acylated molecular species [33, 34]. These ceramides are produced from their immediate precursors, glucosylceramides and sphingomyelins, by ß-glucoceramidase and sphingomyelinase, respectively, at the transition from granular layer to stratum corneum [35, 36] (Fig. 2.1). Most of these precursor lipids are sequestrated in the epidermal lamellar body in the stratum granulosum [37, 38]. Incorporation of glucosylaceramide into lamellar bodies requires ABCA12 (ATP binding cassette transporter, family 12). A devastating ichthyosis, Harlequin ichthyosis, due to *ABCA12* mutations, leads to abnormalities

in lamellar body formation, lamellar membrane structures in the stratum corneum, and in permeability barrier function [39].

Similar to ceramide, most free fatty acids are generated from their immediate precursor lipids, i.e., glycerophospholipid and triacylglycerol, by phospholipase (s) and triglyceride lipase, respectively. In addition, a pool of cholesterol is also produced from cholesterol sulfate and cholesterol esters by cholesterol sulfatase and cholesterol esterase, respectively. Cholesterol sulfatase deficiency is the pathogenesis for X-linked ichthyosis [40].

Electron microscopic analysis characterizes lamellar membrane structures, whereas X-ray diffraction, neutron diffraction, FT-IR (Fourier transform infrared spectroscopy), and DSC (Differential scanning calorimetry) analyses reveal further details of lamellar membrane structures in the stratum corneum. Two lamellar phases with periodicities of approximately 6 nm (short-periodicity phase) and 13 nm (long-periodicity phase) are present [41]. Intracorneocyte soft keratin is a major reservoir of water in the stratum corneum [42]. In addition, a small angle neutron scattering analysis demonstrated the swallowing of the lipid lamellar structures following increased humidity, indicating the presence of water in lamellar membrane structures [43], and a recent small-angle diffraction study elucidated that the short-periodicity phase is a slightly altered periodicity phase following incorporation of water [42]. In addition to two-dimensional structures of lamellar membrane structures, three-dimensional structures representing lamellar packing (hydrocarbon packing) have been characterized. Both hexagonal and tightly packed orthorhombic structures are present in the stratum corneum. Moreover, a recent study using low flux electron diffraction analysis indicated the presence of a different type of orthorhombic structure showing a different distance of packing space, in the lamellar membrane structures in the stratum corneum [44]. The alteration of the lamellar organization has been shown in skin diseases associated with compromised permeability barrier function (e.g. atopic dermatitis) [45].

2.3 pH in the Stratum Corneum

The pH of nucleated cellular layers is neutral, whereas the stratum corneum is acidified.

The acidification is due to four major groups of acidic components derived from keratinocytes. First, free fatty acid generated from both de novo synthesis and hydrolysis of esterified lipids such as triacylglycerol, glycerophospholipids, and cholesterol esters. Second, cholesterol sulfate and sulfate following hydrolysis by cholesterol sulfatase contribute to acidification [46]. Ceramides are hydrolyzed to free fatty acid and sphingoid base by ceramidases, which are present in the stratum corneum [47–49]. However, because sphingoid bases are alkaline, ceramide hydrolysis is unlikely to contribute to acidification. Third, urocanic acid is produced from histidine (mainly from NMF) by histidase (See below, Sect. 2.5.2). And finally, proton (H^+) is discharged from keratinocytes through Na+/H+ antiporter [50]. In

addition to keratinocyte-derived components, sweat-derived lactate, metabolites of microorganisms, and chemicals from air also contribute acidification to the stratum corneum. Acidification contributes to form the antimicrobial barrier and also regulates enzyme activity in the stratum corneum.

Most prior studies measured skin surface pH or a different layer following tape stripping using a flat pH electrode, although recent technologies, confocal fluorescence microscopy, and fluorescence life-time imaging (FLIM) with a development of pH sensitive florescence dye allow us to observe major pH distribution in the stratum corneum, and also in intracellular and extracellular domains [51]. The pH gradient appears to be present across the stratum corneum; i.e., decreasing pH toward skin surface [51, 52], and recent studies show an opposite trend of pH gradient [53]. Hence, the presence of pH gradient and whether continuous acidification occurs in the stratum corneum still remains to be resolved.

2.4 Barrier Function

Stratum corneum deploys multiple barrier functions to protect internal cells and tissues from external perturbations while maintaining the internal environment and normal cellular functions (Table 2.1).

Table 2.1 Barrier roles of stratum corneum in maintaining epidermal homeostasis

Barrier	Roles	Effectors
Permeability	Prevents excess water loss	Primarily extracellular lamellar membrane structures
	Maintains body temperature	
	Prevents ingress of xenotoxic chemicals and allergens	
	Prevents invasion of microbes	
Antimicrobial	Protects against diverse microbes (gram-positive and gram-negative bacteria, fungi, and viruses)	Antimicrobial peptides
		Acidic pH
		Sphingoid bases
Antioxidant	Protects epidermis from oxidative stress	α-/γ-tocophenol
		Ascorbic acid
		Glutathione
Mechanical	Protects epidermis from mechanical stress	Primarily cornified envelopes
UV	Protects epidermis from cell death, DNA damage, and oxidative stress	Urocanic acid
		Structural components (proteins, lipid, nucleotides)

2.4.1 Epidermal Permeability Barrier

The epidermal permeability barrier prevents both egress and ingress of substances. Blocking excess water evaporation from nucleated layers of epidermis is critical for not only dehydration, but also maintaining body temperatures. Prevention of ingress of xenotoxic chemicals, allergens, and microbial pathogens is also an essential function of the epidermal permeability barrier. Substances of larger than 500 kDa are unable to penetrate into the stratum corneum [54]. Recent studies using liposome and nanoparticles demonstrate the penetration of larger sizes of molecules into the nucleated layer of cells. Penetration of nanoparticles into the stratum corneum is not completely understood.

2.4.2 Antimicrobial Barrier

Acidification of the stratum corneum increases antimicrobial defense. For instance, with pH below 5.5, the growth of *Pseudomonas acne*, *Staphyrococcus epidermidis*, and a virulent microbial pathogen, *Staphylococcus aureus*, are suppressed [55]. In addition, sphingoid bases generated from ceramide by ceramidases show antimicrobial activities in vitro [56–58]. Moreover, an innate immune component, antimicrobial peptides, is present in the stratum corneum to combat broad ranges of microbes (see Sect. 2.5).

2.4.3 Antioxidant Barrier

Antioxidant chemicals, α- and γ-tocophenol, ascorbic acid, and glutathione are present in the stratum corneum [59]. These antioxidants maintain stratum corneum homeostasis, i.e., enzyme activity, protection proteins/lipids, from oxidation [59].

2.4.4 UV Barrier

Longer wavelengths of UV, i.e., UVA (315–340 nm), reach to the dermis, whereas most lower UV wavelengths, i.e., UVB (280–315 nm), are absorbed in the epidermis. Urocanic acid, which is generated from histidine, has been shown to be an epidermal major chromophor, i.e., a potent endogenous UV absorbent (See Table 2.1) [60]. In addition, because the bulk amounts of proteins, lipids, and nucleotides, which are not potent chromophors, are abundant in the stratum corneum, these components could contribute to forming the UV barrier.

2.5 Roles of Stratum Corneum in Immunity

2.5.1 Innate Immunity

Antimicrobial peptides, small, cationic (some of them are anionic, e.g., dermcidin), amphipathic molecules, are a part of the host innate immunity forming the antimicrobial barrier [61, 62]. Antimicrobial peptides display potent antimicrobial activities against a broad range of microbes, including gram-negative and gram-positive bacteria, fungi, and some viruses [63, 64]. Antimicrobial peptides are synthesized in nucleated layers of keratinocytes, infiltrate immune cells in the skin, and are retained in the stratum corneum.

2.5.1.1 Cathelicidin

Cathelicidin antimicrobial peptide (CAMP) is synthesized as the inactive precursor protein CAP18, followed by proteolytic digestion yielding an active antimicrobial peptide, 37-amino-acid peptide (LL-37), i.e., 37 amino sequences of C-terminal of CAMP [64]. CAMP/LL-37 is inducible with infection, injury or inflammatory response [64–66]. CAMP expression is regulated by 1,25 dihydroxy vitamin D_3-mediated vitamin D receptor (VDR) activation [65, 66]. In addition, subtoxic external perturbations such as UV-B irradiation and acute barrier disruption trigger endoplasmic reticulum (ER) stress to stimulate the production of a signal lipid, sphingosine-1-phosphate (S1P) that induces CAMP production via NF-κB-C/EBPα activation, independent of the 1,25 dihydroxy vitamin D_3-mediated mechanism [67]. Note that S1P→NF-κB-dependent mechanisms are primarily operated under stressed conditions, which suppress VDR-dependent transcriptional activity [68]. Hence, both S1P→NF-κB- and VDR-dependent pathways could complementarily regulate CAMP expression to maintain antimicrobial defense.

CAMP is a multifunctional AMP. CAMP modulates epidermal immune function, i.e., stimulating cytokine production/secretion including inflammatory and cellular migration [69–72]. Excess CAMP/LL-37 expression as well as hydrolytic peptides of LL-37 are involved in inflammatory responses in rosacea [73, 74].

2.5.1.2 Defensins

The defensins are categorized in three subfamilies, α-, β-, and θ-defensin [75, 76]. Four human β-defensins (hBD1, hBD2, hBD3, and hBD4) are expressed in KC, and β- and θ-defensins are mainly produced by neutrophils and bone marrow, respectively. hBD1 is constitutively expressed in epithelial cells, including KC, whereas hBD2, hBD3, and hBD4 are inducible peptides in epidermis in response to microbial infection, inflammation, and differentiation [75, 77, 78]. hBD2 expression is increased in inflamed skin and is induced by IL-1α and

IL-1β, whereas hBD3 is induced by IL-6 and epidermal growth factors. hBD4 expression is stimulated in response to phobol 12-myristate 13-acetate (PMA) or calcium [75, 78]. Activation of toll-like receptor (TLR) 4 also induces expression of hBD2, but not hBD3, in KC [79].

2.5.1.3 Other Epidermal Antimicrobial Peptides

In addition to major epidermal AMP, CAMP, and hBDs, other AMPs, such as dermcidin [80], RNase7 [81], and S100A7/psoriasin [82] are present in the stratum corneum.

2.5.2 Adaptive Immunity

The *trans* form of urocanic acid is produced from histidine (mainly from NMF) by histidase. The *trans* form converts to *cis* form by UV irradiation. Because urocanic acid is a potent chromophor, topical urocanic acid was used as a natural-occurring, apparently safe UV absorbent in skin care products. However, immunosuppression effects of *cis* urocanic acid were found [83], and topical *cis* urocanic acid was found to increase skin cancer risk in murine skin [84]. Thus, urocanic acid is no longer formulated in skin care products. *Cis* urocanic acid binds to the serotonin [5-hydroxytryptamine (5-HT)] receptor to suppress immune function [85]. Moreover, recent studies show that *cis* urocanic acid activates T-regulatory cells [86, 87]. However, it is unclear if urocanic acid generated in the stratum corneum is transferred to the nucleated cellular layer of epidermis to suppress immunity.

Increased DNA damage following UV irradiation was evident in histidase-deficient mice compared with wild-type mice [88], and it has been proposed that *trans*-urocanic acid decreases DNA damage by thymidine dimer formation. However, it is unclear what the different roles of both *trans*- and *cis*-urocanic acid in skin are.

2.6 Conclusion and Perspective

The stratum corneum is located at the interface of the external and internal environment, two environments that have different features, e.g., humidity, temperature, and osmolarity. The stratum corneum deploys protective barrier mechanisms to minimize the impact of the external environment on internal cell/tissue, so their normal functions can be maintained. Most stratum corneum structures and their constituents are unique in forming this competent barrier. Necessary redundancies of constituents in the stratum corneum contribute to the maintenance of cutaneous and extracutaneous homeostasis. Most cellular components in the

stratum corneum are well-designed and their syntheses are well-regulated. The denucleation of keratinocytes to become corneocytes should be a strategy to prevent DNA damage, inasmuch as the repairing of DNA damage attenuates efforts devoted to high-priority tasks, i.e., the barrier function, in the stratum corneum. Utilization of saturated fatty acyl species to synthesize ceramide in the stratum corneum should also be a strategy to increase the stability of lamellar membrane structure against oxidative stress. The stratum corneum likely has another function: to act as a sensor of the external environment. This function needs to be further characterized. Compromised barriers influence living layers of epidermis leading to pathogenic effects, such as cell death and inflammatory responses. Inflammation alters normal keratinocyte proliferation and differentiation, resulting in attenuation of barrier formation to further decrease barrier functions. This spiral leads to chronic inflammation, delayed wound healing, infections, xerosis, and accelerated skin aging. Intervention in this spiral provides a therapeutic approach to these conditions. A barrier repair approach has been used for treatment of atopic dermatitis, in combination with anti-inflammatory medication. Characterization of the structures and their constituents in the stratum corneum, as well as their regulatory system, is a basis for developing therapeutic approaches. The importance of three stratum corneum lipids (ceramide, cholesterol, and free fatty acid) has been widely acknowledged. Yet, a recent study illuminates a minor ceramide catabolite, sphingoid base, which also contributes to and influences lamellar membrane structures in the stratum corneum, suggesting that further studies of previously uncharacterized/undefined structures, constituents, and their metabolism and roles in the stratum corneum will allow us to develop more potent therapeutic approaches for cutaneous diseases.

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Chapter 3
Keratinocytes

Koji Sayama

Abstract Keratinocytes form a multilayered epidermis that separates the inner body from the outer environment. The outermost epidermal layer of the body is constantly exposed to external pathogens, and keratinocytes are the first line of defense against invading pathogens. Keratinocytes sense pathogens through innate immune receptors and produce various cytokines, chemokines, and antimicrobial proteins, which have antimicrobial activity against diverse pathogens including gram-positive and -negative bacteria, fungi, and viruses. The epidermal barrier function is disrupted in atopic dermatitis or can be disrupted by environmental proteases. Barrier disruption increases the accessibility of the allergens to the keratinocytes, facilitating keratinocyte activation by pathogens or allergens. Among the environmental allergens, house dust mite allergens are important for the development of allergic diseases and activate the NLRP3 inflammasome of keratinocytes. Activated keratinocytes produce cytokines that can promote a cascade of antigen recognition and allergic inflammation. Thus, in addition to their role in innate immunity, epidermal keratinocytes initiate the onset or exacerbation of allergic reactions.

Keywords Keratinocyte • Innate immunity • Toll-like receptor • NLRP3 inflammasome • House dust mite allergen • Atopic dermatitis

3.1 Pathogen Recognition by Keratinocytes

The innate immune system is the first line of defense against microbial pathogens and is essential for efficient activation of adaptive immunity. Evolutionarily conserved pattern recognition receptors (PRRs) recognize pathogens by detecting pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharides (LPS) or peptideglycans [1]. The Toll-like receptor (TLR), nucleotide-binding oligomerization domain (NOD)-like receptor (NLR), and C-type lectin receptor (CLR) serve as PRRs that recognize different PAMPs. TLR1, 2, 4, 5, 6, and 11 are

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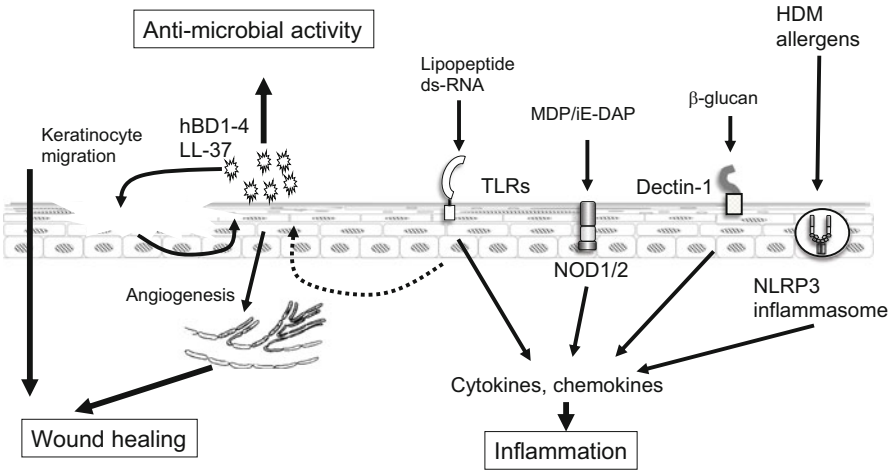

Fig. 3.1 Overview of epidermal keratinocytes in innate immunity. The keratinocyte is the first cell to come in contact with and sense pathogens. Keratinocytes detect gram-positive bacteria and virus-associated double-stranded RNA (ds-RNA) through Toll-like receptor (TLR) 2 and TLR3, respectively. Keratinocytes express the intracellular sensors NOD1/2 and recognize g-D-glutamyl-meso-dianopimelic acid (iE-DAP) and muramyl dipeptide (MDP). β-glucan can be detected by dectin-1. After pathogen recognition, keratinocytes produce various cytokines, chemokines, and antimicrobial proteins (AMPs). Group 1 house dust mite (HDM) allergens activate the NLRP3 inflammasome of keratinocytes

expressed on the cell surface where they detect mainly membrane components, such as LPS or peptideglycans (PGN). On the other hand, TLR3, 7, 8, and 9 are expressed on the intracellular vesicles (such as endosomes) where they sense nucleic acids. NLR family members detect PAMPs in the cytosol. Because pathogens express many PAMPs, they can be detected by several PRRs.

On the body surface, the epidermal keratinocyte is the first cell type that contacts and detects pathogens (Fig. 3.1). TLR2 forms a heterodimer with TLR1 or TLR6, and this heterodimer recognizes various PAMPs including PGN and lipopeptides [1]. Keratinocytes detect Gram-positive bacteria through TLR2 [2–4]. NOD1/2 are intracellular sensors that are members of the NLR family. Keratinocytes express NOD1/2 and recognize distinct motifs of PGN: g-D-glutamyl-meso-dianopimelic acid (iE-DAP) and muramyl dipeptide (MDP) [5, 6]. During viral infection, the keratinocytes recognize double-stranded RNA via TLR3 and produce INF-β [7, 8], which is essential for the antivirus immune reaction. β-glucan on pathogens such as fungi can be detected by a member of CLR dectin-1 on keratinocytes [9]. After pathogen recognition, keratinocytes produce antimicrobial proteins (AMPs), cytokines, and chemokines to initiate the primary immune response against the pathogens.

Although the epidermal keratinocytes are in constant contact with the pathogens on the skin surface, they do not induce inflammation. This lack of inflammation can be explained partially by the presence of *Staphylococcus*