Kenji Kabashima *Editor*

Immunology of the Skin

Basic and Clinical Sciences in Skin Immune Responses

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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

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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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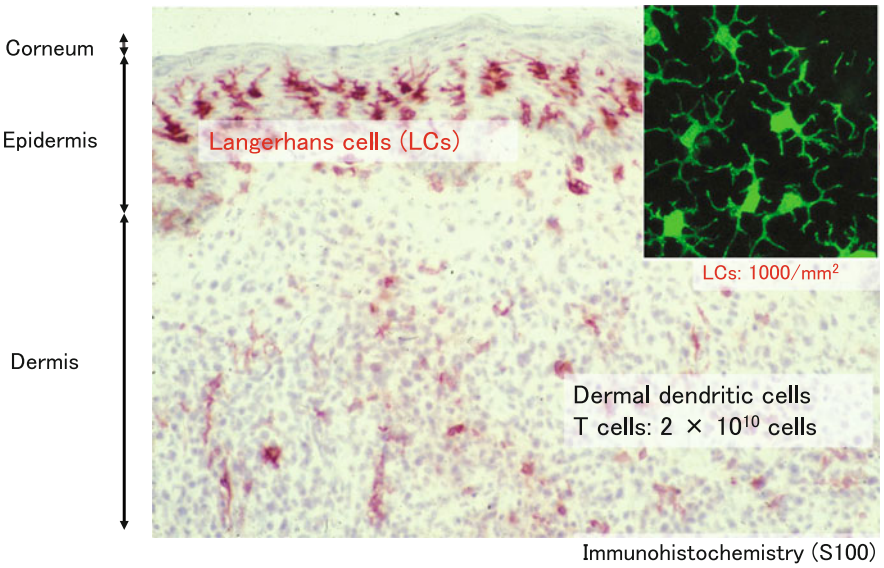

Fig. 1.1 Immunohistochemistry of the skin with S100. The skin consists of the corneum, epidermis, and dermis. The epidermis contains Langerhans cells, and the dermis contains dermal dendritic cells, which serve as antigen presenting cells

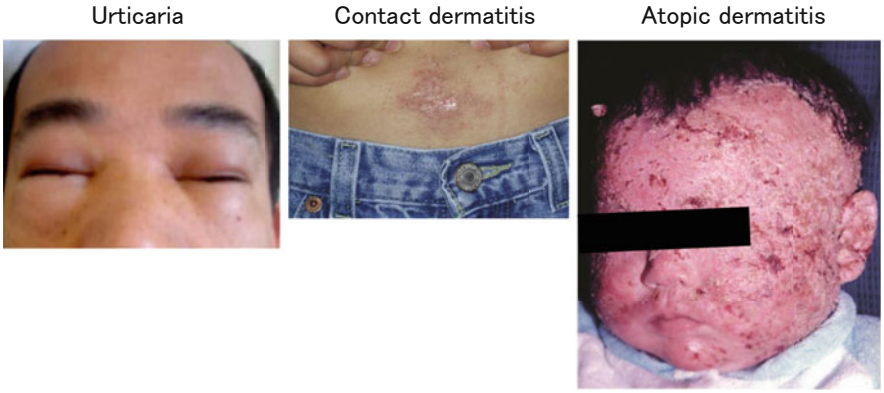

Fig. 1.2 Clinical manifestations of inflammatory skin diseases

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

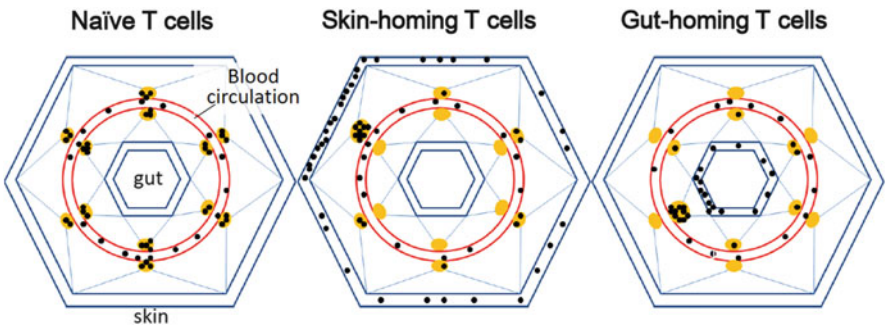

Fig. 1.3 Tissue-specific homing ability of T cells. Three distinct homing systems to the secondary lymphoid organs, skin, and gut. *Solid black* and *brown circles* denote T cells and lymph nodes, respectively

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].

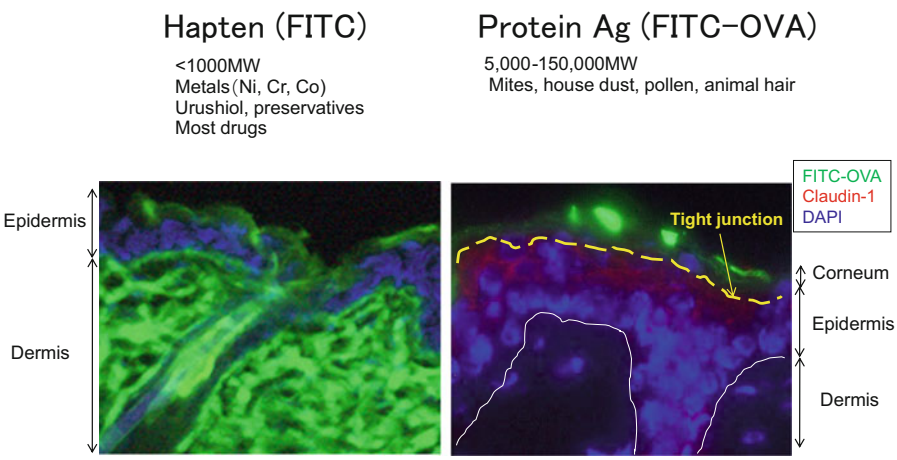

Fig. 1.4 Distribution of haptens and protein antigens upon epicutaneous application. After the epicutaneous application of green-fluorescent hapten (FITC) as a hapten and FITC-ovalbumin (FITC-OVA) as a protein antigen, FITC penetrates into the dermis whereas FITC-OVA is retained at the corneum

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].

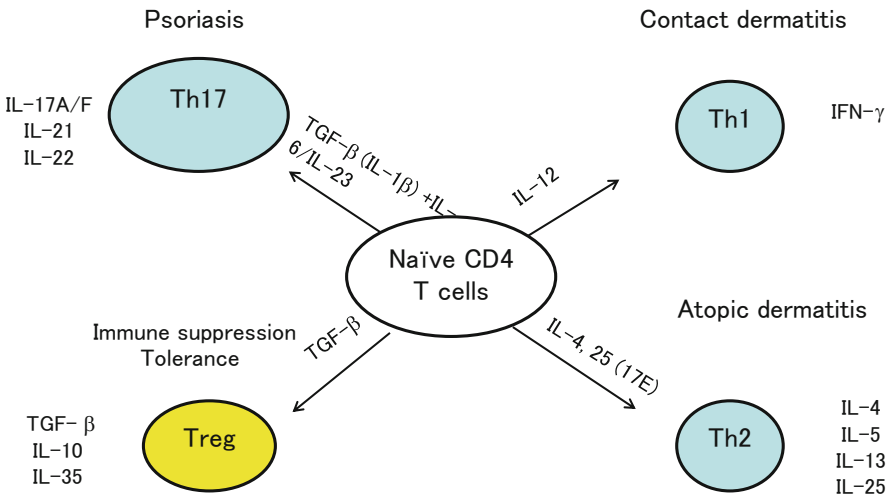

Fig. 1.5 T cell subsets. $CD4^+$ helper T (Th) cells have at least three subtypes, Th1, Th2, and Th17, that are involved in the pathogenesis of contact dermatitis, AD/urticaria, and psoriasis, respectively. These Th subtypes are induced by specific cytokine conditions. Regulatory T cells (Treg), on the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

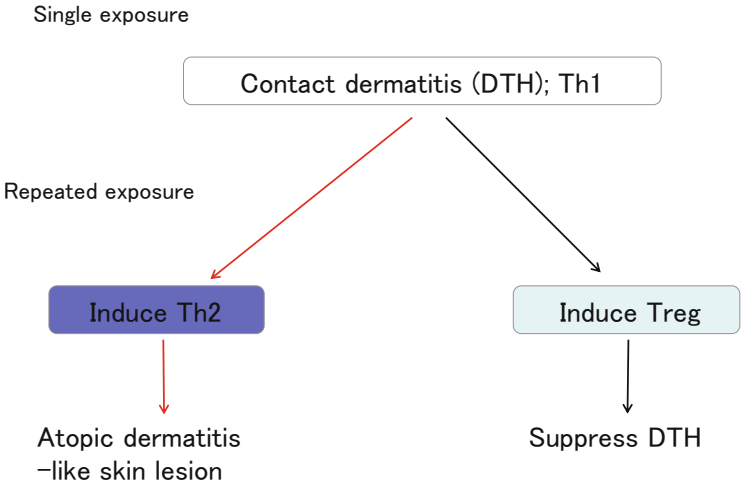

Fig. 1.6 Dynamics of cutaneous immune responses. A single hapten elicitation induces Th1-mediated delayed-type hypersensitivity (DTH), also known as contact dermatitis/contact hypersensitivity. Even during the contact dermatitis response, Treg accumulate in the skin and suppress DTH responses. Repeated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

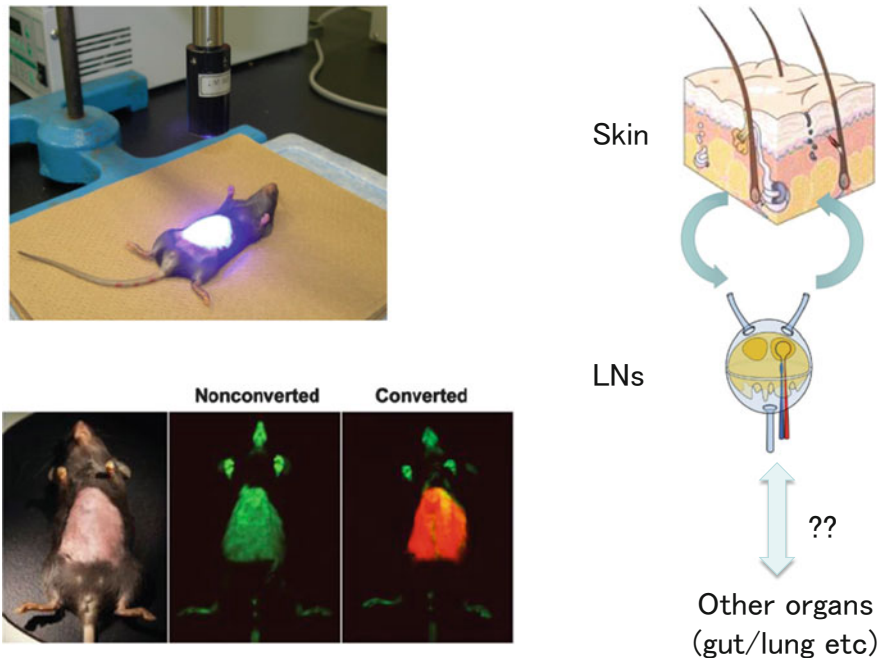

Fig. 1.7 Kaede transgenic mice and interplay among the skin, skin-draining LNs, and other organs. Kaede transgenic mice were photoconverted on the clipped abdominal skin (*upper* and *lower left panels*) and observed with a fluorescence stereoscopic microscope (*upper panel*). Nonphotoconverted clipped 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.

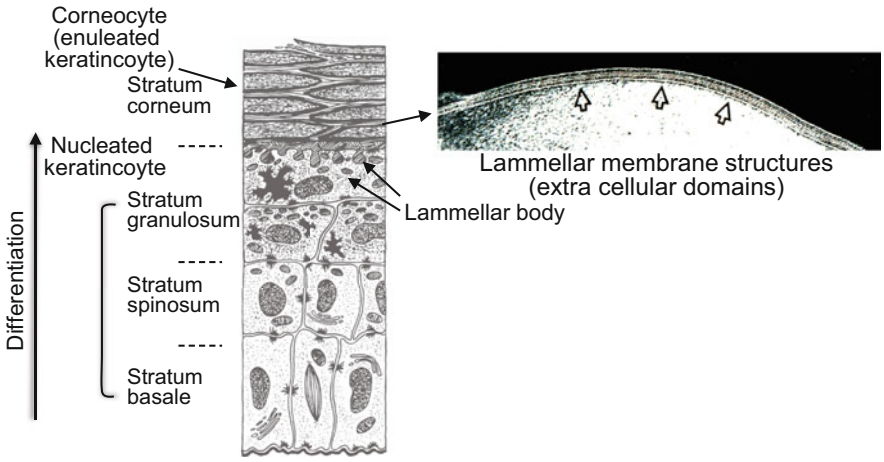

Fig. 2.1 Epidermal structures. Insert, electron micrograph: Murine skin was fixed in Karnovsky's fixative overnight, and postfixed with 0.25 % ruthenium tetroxide. Ultrathin sections were examined using an electron microscope

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

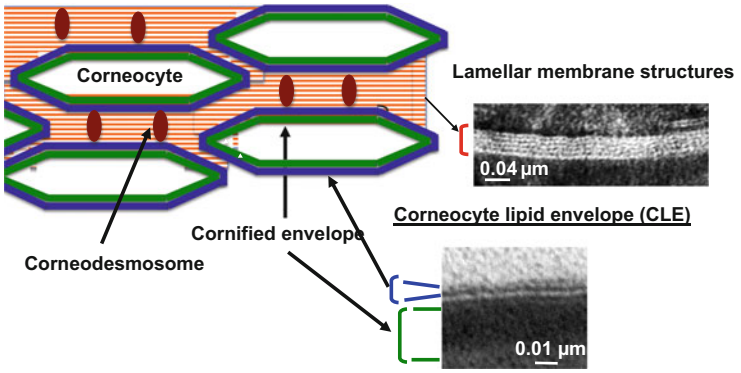

Fig. 2.2 Stratum corneum structure. Cornified envelope, *Green*; Corneocyte lipid envelope (CLE), *Blue*; Corneodesmosom, *Brown*. Insert, electron micrograph: Murine skin was fixed in Karnovsky's fixative, and post-fixed with 1 % aqueous osmium tetroxide, containing 1.5 % potassium ferrocyanide

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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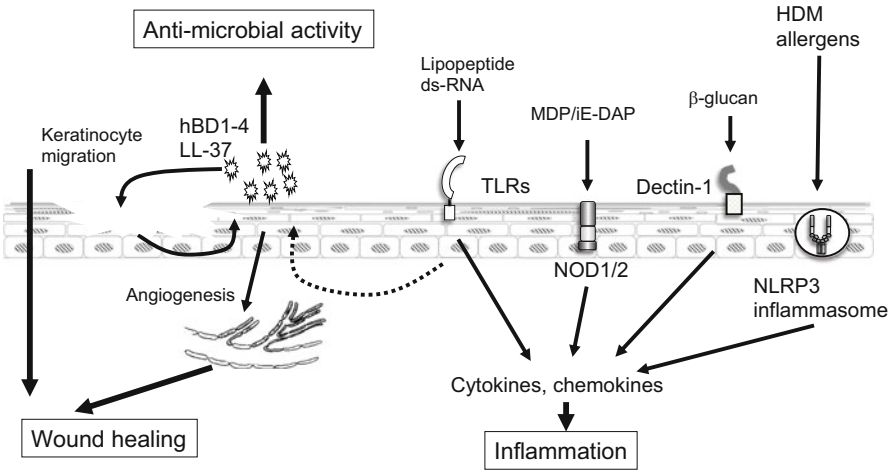

Fig. 3.1 Overview of epidermal keratinocytes in innate immunity. The keratinocyte is the first cell to come in contact with and sense pathogens. Keratinocytes detect gram-positive bacteria and virus-associated double-stranded RNA (ds-RNA) through Toll-like receptor (TLR) 2 and TLR3, respectively. Keratinocytes 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*

epidermidis on the epidermal surface because lipoteichoic acid derived from *S. epidermidis* reduces the TLR3-induced skin inflammation through TLR2 on epidermal keratinocytes [10].

3.2 AMPs

Various epithelial cells produce AMPs to protect against pathogens [11]. AMPs are members of diverse protein families including defensins, cathelicidins, C-type lectins, ribonucleases, and S100 proteins. Cathelicidin is cleaved to generate the active form, LL-37. Keratinocytes, the main source of AMPs in the epidermis, express human β-defensins (hBD) 1–4 and cathelicidin. Additionally, hBDs and cathelicidin are produced by the large intestine, respiratory tract, and urinary tract. The cationic hBDs and LL-37 interact with the negatively charged phospholipids in the pathogens, which is followed by insertion in the membrane of the pathogen and disruption of the cell wall (Fig. 3.2). Whereas expression of cathelicidin and hBDs remain low in the steady-state condition, expression increases markedly upon infection, inflammation, or presence of wounds [3, 11]. These peptides have antimicrobial activity against diverse pathogens including gram-positive and -negative bacteria, fungi, and viruses. However, methicillin-resistant *S. aureus* (MRSA) is resistant to hBD3 and LL-37 [12], which may explain the high susceptibility of MRSA in skin wounds. Although these peptides have been identified as antibacterial molecules, they have various biological activities. For example, LL-37 causes keratinocyte migration and angiogenesis (Fig. 3.1), which may accelerate wound closure [11, 13].

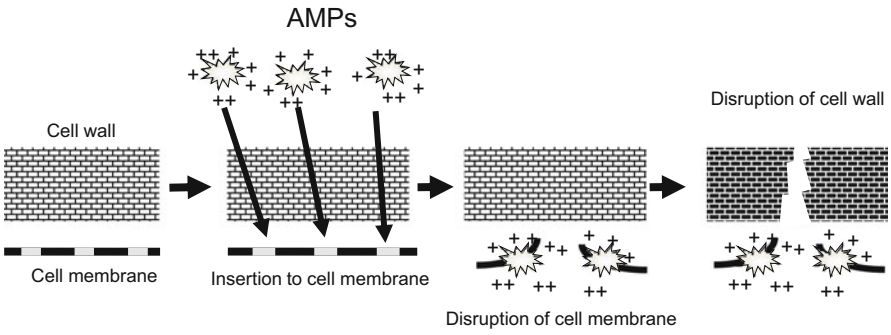

Fig. 3.2 AMP mechanism of action. Cationic human β-defensins (hBDs) and LL-37 (the active form of cathelicidin) interact with negatively charged phospholipids on the pathogens; this interaction leads to insertion into the membrane of the pathogen and disruption of the cell wall

3.3 Allergy Triggered by Keratinocytes in Innate Immunity

Epidermal keratinocytes function in innate immunity of the skin. On the other hand, the innate immunity function of epidermal keratinocytes is a key for triggering or exacerbating allergic diseases such as atopic dermatitis (AD).

3.3.1 Allergy Triggered by S. aureus

AMPs control the skin surface pathogens including *S. aureus*. However, AMP expression is decreased in AD [14], which causes colonization of *S. aureus* on the skin. Although *S. aureus* is not an allergen, this colonization on the skin is an exacerbating factor for AD (Fig. 3.3). Thymic stromal lymphopoietin (TSLP) is an epithelium-derived cytokine essential for generating skin allergic inflammation and the Th2 type response. TSLP expression is increased in the epidermal keratinocytes

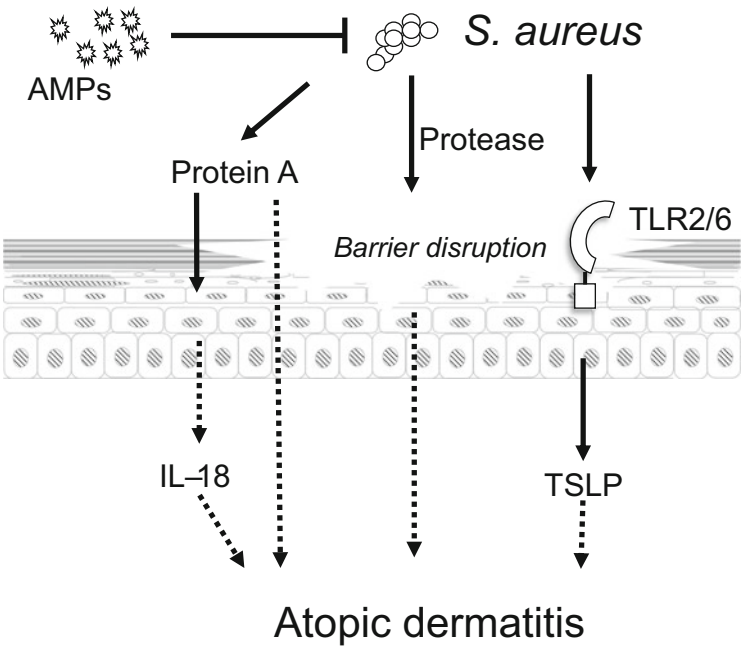

Fig. 3.3 *Staphylococcus aureus* is an exacerbating factor for atopic dermatitis (AD). AMP expression is decreased in AD, which causes colonization of *S. aureus* on the skin. *S. aureus* acts directly on keratinocytes through TLR2/6 to release thymic stromal lymphopoietin (TSLP). Protein A derived from *S. aureus* induces IL-18 release from keratinocytes. Furthermore, *S. aureus* protease disrupts the epidermal barrier function

affected by AD [15]. *S. aureus* acts directly on the keratinocytes through TLR2/6 to release TSLP [16]. Increased TSLP expression promotes allergen sensitization, which ultimately leads to AD.

Mice treated with repeated applications of *S. aureus* protein A develop AD-like skin inflammation [17], and protein A of *S. aureus* acts on keratinocytes to release IL-18 [18]. Furthermore, the extracellular protease of *S. aureus* causes epidermal barrier dysfunction [19], which allows allergens or pathogens to penetrate into the epidermis and stimulate keratinocytes.

Therefore, direct interaction of *S. aureus* with keratinocytes is a key event in the pathogenesis of AD.

3.3.2 AD and House Dust Mite (HDM) Allergen

The pathogenesis of AD involves the interactions of multiple factors including susceptibility genes, environmental factors, skin barrier defects, and immunological factors [20]. Among these, skin barrier dysfunction is one of the major manifestations of AD and a key contributor to its pathogenesis. Barrier disturbances resulting from genetic defects or abnormal expression of epidermal proteins caused by Th2-type cytokines may increase the risk of exposure to allergens and contribute to the development of AD. However, the mechanisms underlying the relationship between immunological and physical defects remain unclear. Among the environmental factors, HDM allergens are important for the development of AD, asthma, and rhinitis [21]. Barrier defects may allow epidermal penetration of HDM allergens and increase the likelihood of allergen exposure to epidermal keratinocytes. This direct contact between HDM allergens and keratinocytes is important for triggering inflammation because the innate immunity of keratinocytes is activated by the HDM allergens [22].

3.3.3 NLRP3 Inflammasome

NLR members form an inflammasome, a multiprotein complex that activates caspase-1, which ultimately leads to the processing and release of the proinflammatory cytokines IL-1β and IL-18. Growing evidence supports the idea that inflammasomes play important roles in skin inflammation, especially the NLRP3 inflammasome (also known as NALP3 or cryopyrin), which comprises oligomerized NLRP3, apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC), and caspase-1 (Fig. 3.4). Stimulated caspase-1, expressed initially as an inactive precursor, is activated to cleave IL-1β and IL-18 proforms. The inflammasome can be activated not only by pathogen-associated molecules but also by various other stimuli including the "danger-associated molecular pattern" molecules (such as ATP and urate crystals), environmental

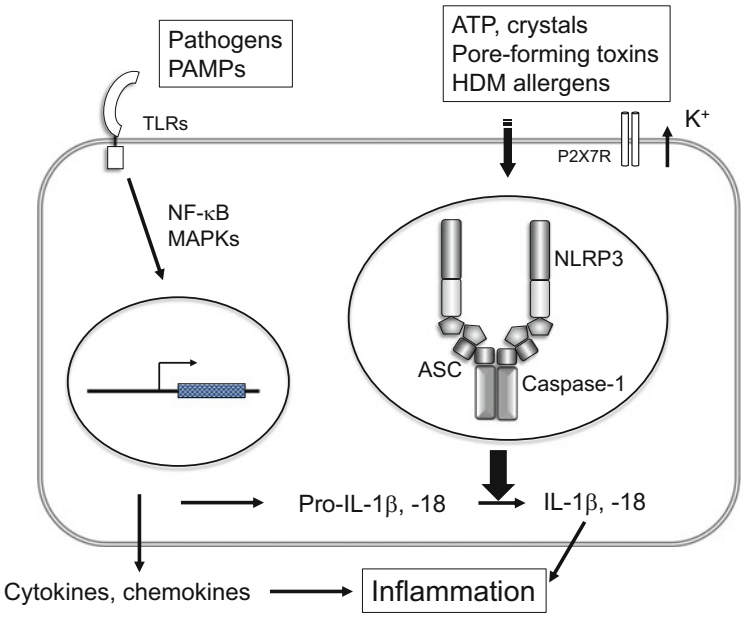

Fig. 3.4 NLRP3 inflammasome. Activation of the NLRP3 inflammasome requires two signals: (1) Pathogens or pathogen-associated molecular patterns (PAMPs) increase the expression of the IL-1β and IL-18 proforms through TLRs; and (2) ATP, crystals, bacterial pore-forming toxins, or HDM allergens activate the NLRP3 inflammasome. The NLRP3 inflammasome comprises oligomerized NLRP3, apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC) and caspase-1. Caspase-1 is expressed initially as an inactive precursor; following stimulation, activated caspase-1 cleaves IL-1β and IL-18 proforms

stimuli (such as silica crystals and aluminum salt) [23], and neurodegenerative stimuli (such as amyloid-β fibrils) [24].

3.3.4 Activation of the Keratinocyte Inflammasome by Group 1 HDM Allergens

Dermatophagoides pteronyssinus (Der p) and *D. farinae* (Der f) are the most common types of HDM in temperate climates. The group 1 allergens, which exhibit cysteine protease activity, activate caspase-1 and induce the caspase-1-dependent release of IL-1β and IL-18 from keratinocytes [22]. The group 1 allergens assemble inflammasomes by recruiting ASC, caspase-1, and NLRP3 to the perinuclear region (Fig. 3.5) [22]. The group 1 allergens are novel effective activators of the inflammasome in epidermal keratinocytes. Recent studies using mouse models

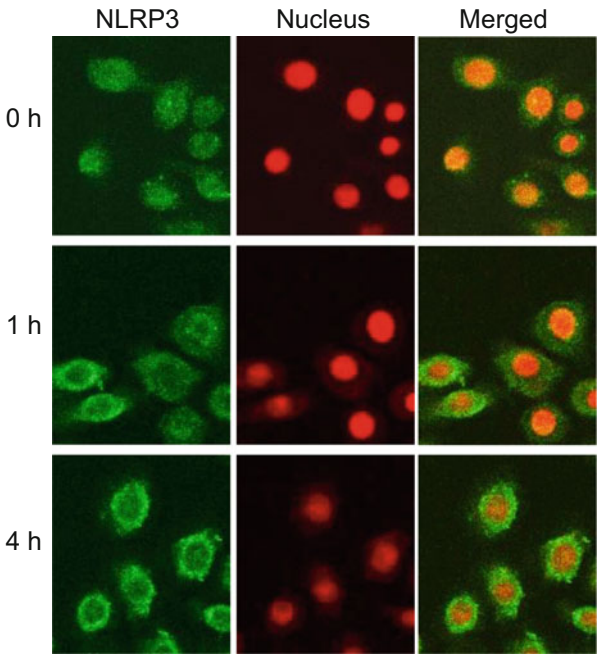

Fig. 3.5 Formation of NLRP3 inflammasomes in keratinocytes. Cultured keratinocytes were stimulated with house dust mite (HDM) allergens. Subcellular distribution of NLRP3 was assessed with immunofluorescence. Staining for NLRP3 revealed diffuse distribution throughout the resting cells (0 h). After 1 h of stimulation, NLRP3 translocated rapidly to the cytosol and accumulated in the perinuclear region. ASC and caspase-1 were also translocated to the perinuclear region [22], indicating that formation of the NLRP3 inflammasome was induced by the HDM allergen (Adopted from Ref. [22])

have indicated the importance of IL-18 for the development of AD [17, 25]. IL-18 regulates allergic inflammation by inducing Th2 cytokine production and eosinophilia; therefore, HDM-induced IL-18 release from keratinocytes might be important in the pathogenesis of AD.

3.3.5 Activation of Keratinocyte by Group 2, 3, and 9 HDM Allergens

As do class 1 HDM allergens, class 2, 3, and 9 HDM allergens also activate keratinocytes (Fig. 3.6). The class 2 allergens are major antigens for IgE, and lack protease activity [26]. Unlike class 1 HDM allergens, class 2 HDM allergens do not induce the release of IL-1β from keratinocytes, but stimulate IL-8 release via

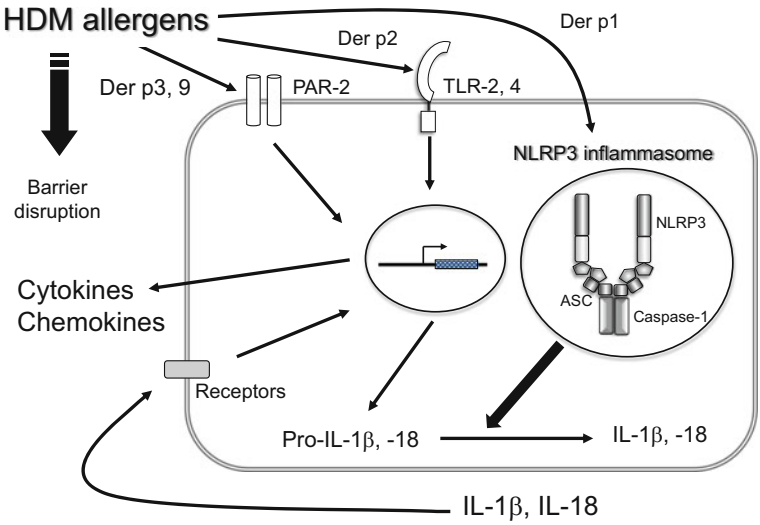

Fig. 3.6 Biological activity of HDM allergens. Each group of HDM allergens activates NLRP3 inflammasome, TLR-2,4 and PAR-2, respectively, resulting in the production of cytokines and chemokines. On the other hand, HDM protease disrupts the epithelial barrier

a nonproteolytic mechanism [22], which presumably involves the activation of NF-κB and MAPK signaling pathways [27, 28]. Class 2 HDM allergens activate airway smooth muscle cells in a TLR2/MyDD88-dependent manner [28]; therefore, class 2 HDM allergen-induced keratinocyte activation may involve the TLR2 pathway. Class 3 and 9 HDM allergens, which have serine protease activity, induce the release of IL-8 and GM-CSF from keratinocytes [29] and airway epithelial cells [30] by activating protease-activated receptor 2 (PAR-2) signaling. Thus, keratinocytes are able to detect HDM allergens directly and initiate local inflammatory responses, perhaps through the cooperation of the NLRP3 inflammasome, PAR2, and even the TLR pathways.

3.4 Allergen Activation of Epithelial Cells

The underlying mechanism by which particular molecules, such as HDM allergens, are recognized by hosts as “allergens” is largely unknown. Because epithelial cells are the first cells to come in contact with the allergens, the allergen-activation of the innate immunity of epithelial cells may be important for allergens to function as “allergens”.

The airway epithelial cells respond to HDM allergens as well as epidermal keratinocytes [30, 31]. In an allergic reaction in the airway, recognition of the HDM allergen by TLR4 on airway epithelial cells, rather than dendritic cells, is important [32]. Because Der p 2 has structural homology with the adaptor protein of TLR4 (MD-2), Der p 2 is involved in the LPS response by facilitating signaling through direct interactions with the TLR4 complex and reconstituting LPS-driven TLR4 signaling [33] (Fig. 3.6).

Ragweed pollen is one of the major allergens of seasonal rhinitis and conjunctivitis and is an exacerbating factor for AD. Similar to HDM allergens, ragweed pollen activates the keratinocyte NLRP3 inflammasome. Furthermore, in seasonal rhinitis, direct contact of ragweed pollen to nasal epithelial cells induces IL-33 release from the cells into the nasal fluid [34], which induces Th2 cytokine-mediated allergic inflammation.

All epidermal keratinocytes, airway epithelial cells, and nasal epithelial cells respond to allergens. Therefore, the direct contact and recognition of allergens by epithelial cells is important for allergens to function as "allergens" in the pathogenesis of allergic diseases such as AD, asthma, and seasonal rhinitis.

3.5 Protease Allergens and Epithelium

HDM allergens and ragweed pollen are protease allergens that increase the risk of sensitization to allergens. HDM allergens decrease the barrier function of the epidermis (Table 3.1) [35] and delay the recovery of the epidermal barrier [36]. Furthermore, group 1 HDM allergens disrupt intercellular tight junctions (TJs), which are the principal components of the epithelial permeability barrier [37]. Cleavage of TJs involves proteolysis of the TJ proteins, occludin, and ZO-1 [38]. Upon barrier disruption, HDM allergens gain increased access to the epithelial cells and can directly activate the epithelial cells. Similar barrier disruptions occur by other protease allergens such as pollen, aspergillus, and cockroach [36, 39]. Taken together, these protease allergens could function as "allergens" by disrupting the epithelial barrier to increase accessibility to the epithelial cells. This activation of epithelial cells may initiate a cascade of antigen recognition and allergic inflammation.

Table 3.1 Activity of allergen protease on epithelium

Antigen	Activity
HDM allergens	Disruption of tight junction, inflammasome activation, TLR-4 activation, and PAR2 activation
Aspergillus	Disruption of tight junction
Ragweed pollen	Disruption of tight junction, inflammasome activation, TLR-4 activation
Cockroach	Disruption of tight junction, PAR2 activation

3.6 Eccrine Sweat and Allergy

Eccrine sweat contains AMPs [40] and plays an important role in defending against pathogens. Sweat is secreted from eccrine glands onto the skin's surface. Although not harmful to normal skin, sweat acts as a common aggravating factor in the development of AD. Sweat secreted onto the skin surface has no contact with the epidermal keratinocytes in normal skin, so it is unlikely to have a physiological role in keratinocyte activation. However, in AD-affected skin with a defective epidermal barrier function, sweat components can penetrate into the epidermis, come into contact with keratinocytes and stimulate their activation as sensitizers. Sweat-activated keratinocytes produce IL-1, IL-8, and CCL2 [41], which initiate skin inflammation. The sensitizers in sweat have been identified as IL-1 and IL-31 [41]. Furthermore, sweat increases the expression of innate immune receptors NOD2 and RIG-I on keratinocytes, indicating the possible contribution of sweat to PAMP-mediated skin inflammation (Fig. 3.1).

Itching is a major manifestation of AD and other chronic skin diseases, which is exacerbated by sweating. IL-31 is associated with various pruritic skin diseases and was recently identified as a major pruritic factor in AD [42, 43]. Since sweat contains high levels of IL-31 [41], sweat-induced itching might be related to the IL-31 in the sweat.

3.7 Conclusion

Innate immunity of epidermal keratinocytes is the first line of defense against microbial pathogens. On the other hand, environmental stimuli such as protease allergens, *S. aureus*, or sweat can directly stimulate keratinocytes and trigger the allergic reaction cascade (Fig. 3.7).

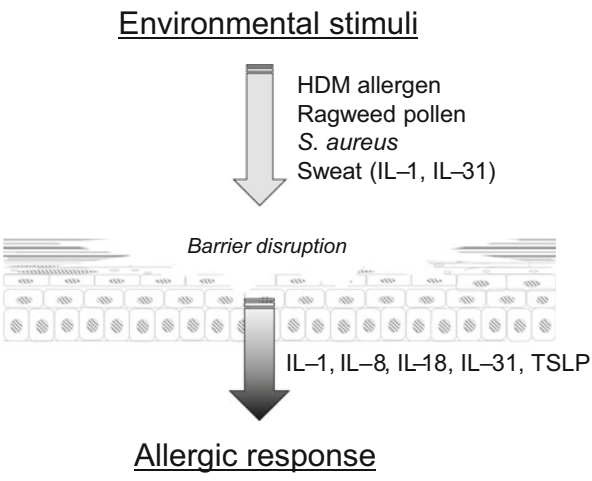

Fig. 3.7 Environmental stimuli trigger allergic response through keratinocytes. Environmental stimuli such as the HDM allergen, *S. aureus*, ragweed pollen, or sweat can directly stimulate the keratinocyte and trigger the allergic reaction cascade

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Chapter 4
Langerhans Cells and Dermal Dendritic Cells

Saeko Nakajima

Abstract Dendritic cells (DCs) form a heterogeneous group of antigen-presenting cells that play different roles in skin immunology. Recent studies have revealed the existence of distinct DC populations in the skin, highlighting the complexity of the cutaneous DC network in the steady state and in inflammatory conditions. This review focuses on defining the major DC subsets, such as Langerhans cells (LCs) and dermal DCs (dDCs), that populate the different layers of mouse and human skin, and summarizing the relative contribution of murine skin-resident DCs in the generation of cutaneous immune responses, such as contact hypersensitivity (CHS) and atopic dermatitis (AD).

Keywords Langerhans cell • Dendritic cell • Contact hypersensitivity • Atopic dermatitis

4.1 Introduction

The term "dendritic cell" (DC) was neoterized in 1973 when DCs in the lymph node were discovered by Ralph Steinman and Zanvil Cohn [1]. The main role of DCs is to induce specific immunity against invading pathogens while maintaining tolerance to self-antigens [2, 3].

Several subsets of DCs have been identified in mice and humans. Anatomically, DCs can be divided into two subsets: DCs that reside in lymphoid tissues and DCs that reside in nonlymphoid tissues. DCs in lymphoid tissues are further characterized into two subtypes: plasmacytoid DCs (pDCs) and classical myeloid DCs (mDCs). MDCs circulate in the blood or reside in the peripheral tissue and secondary lymphoid organs. Although pDCs are primarily present in the peripheral blood [4], they are capable of migrating to lymphoid organs or tissues during inflammation as well. DCs localized in the outer epidermal layer of stratified epithelia are called Langerhans cells (LCs), whereas DCs in connective tissues such as the dermis are called dermal DCs (dDCs). In the inflamed tissue itself, two

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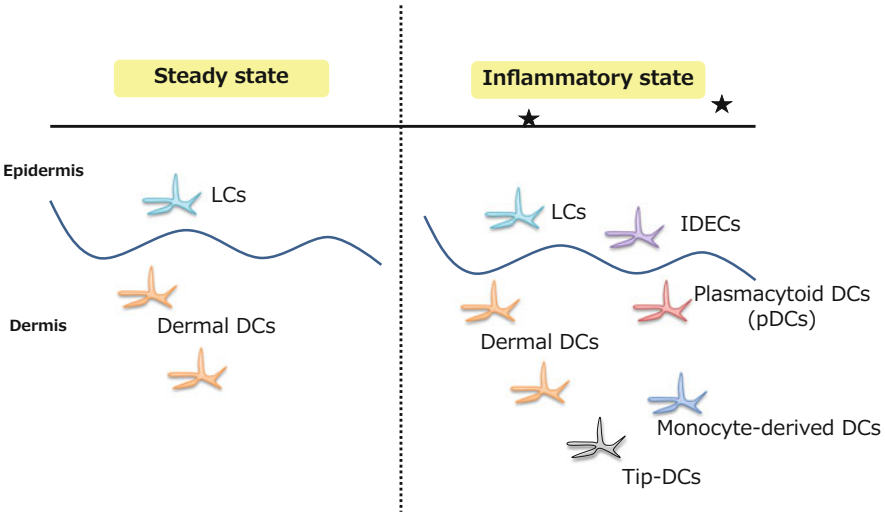

Fig. 4.1 Cutaneous dendritic cells in the steady state and in the inflammatory state. *LCs* Langerhans cells, *dermal DCs* dermal dendritic cells, *IDECs* inflammatory dendritic epidermal cells, *Tip-DCs* tumor necrosis factor, and inducible nitric oxide synthase-producing dendritic cells, *pDCs* plasmacytoid dendritic cells

additional subsets of DCs can be found: a DC population derived from blood monocytes [5] and pDCs [6, 7] (Fig. 4.1). Monocyte-derived DCs arise from inflammatory Ly6C^{hi} monocytes that are recruited from the blood to the inflamed dermis [5]. Although Ly6C^{hi} monocytes express Ly6C, once recruited and differentiated into DCs in the inflamed tissue, they will downregulate Ly6C.

Recently, several studies have revealed the complexity and functional specialization of the cutaneous DC network, showing that as in the lymphoid organs, multiple subsets of DCs coexist in the dermis, and that skin DC subsets exhibit specific immune functions.

This review defines the functions of each cutaneous DC compartment and discusses the potential implications of these functions in the development of skin immune responses.

4.2 Dendritic Cells (DCs) of the Skin

4.2.1 Langerhans Cells

4.2.1.1 Localization

The skin can be broadly classified into two distinct regions, the epidermis and the dermis. The epidermis is derived from ectoderm and presents as an epithelial layer

composed mainly of keratinocytes. Keratinocytes provide skin integrity and produce the stratum corneum, which is the water-impermeable outermost layer of the skin.

LCs are the only DCs that are found in the epidermis during the steady state. LCs are generally found in the basal and suprabasal layers of the epidermis, where they form a dense network of cells that account for approximately 2–4 % of the total epidermal cell population in the epidermis of mice and humans [8]. LCs are the only hematopoietic cells in the epidermis of human skin in the steady state, whereas mice have an additional population: epidermal γδ T cells [9].

LC precursors first populate the epidermis on embryonic day 18 and proliferate in situ to form a radio-resistant, self-renewing population [10, 11]. They are characterized by a unique cytoplasmic organelle known as a Birbeck granule. Although the precise function of Birbeck granules remains unclear, they are thought to play a role in receptor-mediated endocytosis and the transport of cellular materials into the extracellular space [12].

4.2.1.2 Surface Marker

Human and murine LCs are easily identified in the epidermis by the expression of CD45 and major histocompatibility complex (MHC) II molecules. They also constitutively express the lection receptor, namely, langerin/CD207, which is capable of binding sugar moieties commonly found on a variety of microorganisms [13]. Human and murine LCs express the adhesion molecules E-cadherin and epithelial-cell adhesion molecule (EpCAM), which anchor LCs to keratinocytes [14, 15], and CD205 that is implicated in antigen capture and antigen processing [16, 17]. Human LCs highly express CD1a, a member of the group 1 CD1 protein, which is capable of presenting microbial lipid antigens to T cells [18] (Table 4.1). Although the majority of LCs are thought to be derived from bone marrow precursors, recent studies have identified a novel pathway by which $CD14^+$ cells resident in the dermis acquire LC features following treatment with TGF-β [19, 20]. In humans, $CD34^+$ hematopoietic progenitor cells [21, 22], monocytes [23], and dermal $CD14^+$ DCs [19] were shown to give rise to LC-like cells in vitro. TGF-β is a critical factor for LC development both in vitro and in vivo, as LCs are absent in the epidermis of TGF-β-deficient mice [14]. LC differentiation may thus be somewhat dependent on the cytokine microenvironment of the skin at any given point in time.

Upon encountering pathogens, LCs can capture antigens and undergo a maturation that involves upregulation of MHC class I and class II molecules—costimulatory molecules including CD40, CD80, and CD86—and chemokine receptors such as CCR7, as well as downregulation of E-cadherin that allows them to migrate out of the skin to draining LNs, where they present antigens to T cells [24]. However, despite intensive studies to reveal the functional role of LCs

Table 4.1 Dendritic cell population in the skin of humans and mice

	Localization	Cell type	Cellular markers
Human	Epidermis	LC	CD45, MHC classII, CD1a, CD207 (Langerin), E-cadherin, EpCAM
		IDEC	CD1a, CD1b, CD1c, CD11c, FcεRI, CD23, HLA-DR, CD11b, CD206, CD36
	Dermis	$CD1c^+$ dDC	CD1c, $CD1a^{+/-}$, CD45, CD11b, CD11c, MHC classII
		$CD14^+$ dDC	CD1c, CD45, CD11b, CD11c, CD14, MHC classII, CD209 (DC-SIGN)
		$CD141^+$ dDC	CD1c, CD45, CD11c, CD141
		pDC	CD303 (CLEC4C), CD304 (neuropillin), CD123 (IL-3R)
		Tip-DC	CD11c, TNF-α, iNOS
Mouse	Epidermis	LC	CD45, CD11b, CD11c, CD24, MHC classII, CD205, CD207, E-cadherin, EpCAM
	Dermis	$Langerin^-$ dDC	CD45, $CD11b^{hi}$, CD11c, CD24, MHC classII, CD205, SIRP-1α
		$Langerin^+$ dDC	CD45, $CD11b^{dim}$, CD11c, CD24, CD103, MHC classII, CD207
		pDC	B220, SiglecH, PDCA-1

over the past several decades, the in vivo function of LCs remains not fully understood, as discussed later.

4.2.1.3 Homeostasis of LCs

Homeostasis of LCs in the inflamed skin depends on the type and the strength of inflammation. In severe inflammatory conditions, such as ultraviolet (UV) light exposure or cutaneous graft-versus-host disease, LCs are replaced by circulating blood precursors [11, 25]. In contrast, in less severe inflammatory conditions that lead to moderate LC loss, where the epidermal–dermal barrier integrity is preserved and is not accompanied by the release of inflammatory chemokines that recruit blood monocytes, the remaining LCs have the potential to repopulate themselves locally and achieve complete recovery in 1 to 4 weeks after tissue injury. These findings suggest that inflammatory signals may control the dynamics of LC proliferation.

4.2.2 Dermal Dendritic Cell

The dermis is a relatively cell-sparse tissue that is derived from mesoderm, which forms a stromal layer immediately below the epidermis. It is populated by fibroblasts that secrete components of the complex extracellular matrix. In the steady

state, a variety of immune cells can be found in the dermis including memory T cells, mast cells, and DCs [26, 27].

4.2.2.1 Components and Surface Markers of Dermal DCs

Dermal DCs (dDCs) consist of dermal-resident DCs and migratory LCs on their way to the LNs [28]. Until recently, human and murine dermal-resident DCs were thought to form a homogeneous population that is easily distinguishable from migratory LCs based on the absence of langerin/CD207 expression [13]. However, recent studies have shown that murine langerin-positive dendritic cells in dermis are composed of both LCs and a novel population of dDCs (langerin$^+$ dDCs) [29–31]. The langerin$^+$ dDC population represents 10–20 % of the total dDCs pool. In contrast to LCs, langerin$^+$ dDCs express the integrin αEβ7 (also called CD103) [32], lack the adhesion molecules E-cadherin and EpCAM, and express low levels of the integrin CD11b. Langerin$^+$ dDCs arise from bone marrow precursors in an FMS-like tyrosine kinase 3 (FLT3)-dependent manner [29, 30, 33]. Langerin$^+$ dDCs express the same high level of CD24 as LCs, but do not express CX3CR1, F4/80, or signal-regulatory protein alpha (SIRPα), and express low levels of CD11b and EpCAM [33] (Table 4.1).

The classical langerin$^-$ dDCs represent the majority (up to 70 %) of the dDC pool and express high levels of the integrin CD11b and several macrophage markers such as F4/80, CX3CR1, and SIRPα [33]. Interestingly, their expression of CD24 is heterogeneous, suggesting that dermal langerin$^-$ dDCs might not represent a homogeneous subset of dDCs, either in origin or in maturation status [34].

DCs were thought to be terminally differentiated cells, with no proliferative capacity. However, recent studies have revealed that in lymphoid organs DCs actively proliferate in the steady state [35, 36]. All dermal DC subsets proliferate, although CD103$^+$ dDCs do so at a higher rate than the CD11b$^+$ subset [33, 34].

The chemokine receptor CCR7 regulates the constitutive migration of all cutaneous DCs to the skin-draining LNs [37, 38]. In the inflammatory state, tissue-resident DC migration to the draining LNs increases markedly [39]. Interestingly, cutaneous DC subsets migrate differently from the skin-draining LNs in response to the sensitization with hapten. Migration of CD11b$^+$ and CD103$^+$ dDCs reaches a peak 1 day later, followed by LCs at 4 days postsensitization [40, 41]. It is also important to note that cutaneous DCs undergo phenotypic changes upon migration from the skin to the LNs [34].

4.2.3 Plasmacytoid Dendritic Cells

Plasmacytoid DCs account for 0.2–0.8 % of all peripheral blood mononuclear cells [42]. Under steady-state conditions, pDCs are absent from the skin and other

peripheral nonlymphoid tissues, but they have been shown to infiltrate into the dermis in viral infection [43] and chronic inflammation as seen in psoriasis [44–46], lupus erythematosus [47], and AD [44, 48, 49]. Plasmacytoid DCs lack myeloid antigens CD11b, CD11c, CD13, and CD33, but express CD45RA, variable CD2 and CD7, and may harbor T-cell receptor and immunoglobulin rearrangements. They are now separable from mDCs by positive markers CD123 (IL-3R), CD303 (CLEC4C; BDCA-2), and CD304 (neuropilin; BDCA-4) [50] (Table 4.1).

The propensity of pDCs to release type I interferon in response to viruses is one of the first specialized pDC functions to be described [51–53]. They express very high levels of Toll-like receptors (TLR) 7 and TLR9, which transduce signals from viral and self-nucleic acids [42, 54]. After stimulation by viral or other microbial products, pDCs produce large quantities of type I interferons, triggering pDC maturation and migration to the LNs. PDCs become activated by a regulated expression of antimicrobial peptides (AMPs) in damaged skin. However, overexpression of AMPs leads to the initiation of an autoimmune reaction, through an excessive sensing of self-nucleic acids and uncontrolled secretion of type I IFNs by pDCs, as seen in psoriasis.

4.2.4 Inflammatory Dendritic Cells (Tip-DCs and IDECs)

4.2.4.1 Tip-DCs

A newly identified DC subpopulation referred to as TNF-α and inducible NO synthase (iNOS)-producing DC (Tip-DC) has been reported to be critical in bacterial infections, as demonstrated in a model with *Listeria monocytogenes* [55]. - Tip-DCs express CD11c, TNF-α, and iNOS (Table 4.1). Evidence that TNF-α inhibitors were able to reverse disease activity in psoriasis suggests that Tip-DC may represent major inflammatory effector cells in psoriasis [56]. Pathogenicity of Tip-DCs in psoriasis is suggested by the rapid downmodulation of Tip-DC products TNF-α, iNOS, IL-20, and IL-23 during treatment with effective therapies [57, 58]. They have the capacity to skew T cells into Th1 and Th17 T cells, which are a new set of T cells associated with autoimmune inflammation in many disease models [59].

4.2.4.2 IDECs (Inflammatory Dendritic Epidermal Cells)

Inflammatory dendritic epidermal cells (IDECs) were found in the epidermis of atopic dermatitis (AD) skin lesions. Compared to LCs, they lack Birbeck granules and langerin expression, but show expression of the mannose receptor (MMR)/CD206, providing IDECs with the ability to take up mannosylated protein antigens [60]. They also express CD1a, CD1b, CD1c, CD11c, CD23, HLA-DR, CD11b, CD206, and the low-affinity receptor for IgE (FcεRII/CD23; Table 4.1). In lesional

skin of AD, both IDECs and LCs show expression of the high-affinity receptor for IgE (FcεRI) and CD36; however, expression of both receptors is stronger on IDECs. Peripheral blood monocytes as well as dermal DCs are regarded as potential precursor cells of IDECs [61].

4.3 Roles of Cutaneous DCs in Skin Immune Responses

4.3.1 The Role of Each Cutaneous DCs Subset in the Murine CHS Model

CHS is a murine model for allergic contact dermatitis, and has become a widely used technique to analyze adaptive skin immune responses. Mice are sensitized by application of hapten such as 2-dinitrofluorobenzene (DNFB) onto shaved abdominal skin. Five to six days later, the same hapten is applied to the ear. The extent of ear swelling that develops after 24 h correlates with the magnitude of the effector response [62].

Until recently, LCs have been considered to play central roles for antigen presentation in the sensitization phase of CHS. Because they have strong antigen-presenting ability in vitro, they reside abundantly in the basal layer of the epidermis, and their artificial removal by drugs, such as gliotoxin application to the skin, results in impaired CHS response [63]. However, novel depletion systems of LCs (langerin–diphtheria toxin receptor knock-in mice) have revealed that langerin$^+$ dDCs, but not LCs, may have a crucial role during the sensitization phase [29, 40, 64] (Table 4.2). Other groups have recently reported that these two populations work in a compensatory manner during the initiation of the sensitization phase [65, 66]. In line with this result, Batf3-deficient mice which lack langerin$^+$ dDCs exhibited a normal CHS response [67]. These findings indicate that the function of langerin$^+$ dDCs in the sensitization phase of CHS is compensated by other DC subsets (Table 4.2). On the other hand, the depletion of skin DCs in hapten-sensitized mice enhanced the effector phase of CHS [68]. This suggests that there exist other DC subsets that regulate the elicitation phase of CHS.

It has also been reported that LCs have a regulatory role, rather than a stimulatory role, during the sensitization phase of CHS. Research on another LC depletion system that uses the human langerin promoter combined with diphtheria toxin A (the active subunit of diphtheria toxin [69]) revealed that congenitally LC-depleted mice exhibited an exacerbated CHS response, suggesting that LCs may have a suppressive function in CHS [70] (Table 4.2). When the CHS response was induced with a hapten through full-thickness grafted skin, the development of CHS was suppressed. A high expression of receptor activators of nuclear factor κ-B ligand (RANKL) was observed in the grafted skin, and recombinant RANKL stimulated

Table 4.2 Dendritic cell ablation systems

	Mouse lines	LCs	Langerin$^+$ dDCs	Langerin$^-$ dDCs	CD8$^+$ DCs
Constitutive systems	Langerin-DTA	Absent	Normal	Normal	Normal
	Batf3$^{-/-}$	Normal	Absent	Normal	Absent
Inducible systems	Langerin-DTR (1 day after DT injection)	Absent	Absent	Normal	25 %
	Langerin-DTR (7–13 days after DT injection)	Absent	20–50 %	Normal	Normal

LCs to produce IL-10, suggesting that the LCs play important roles for regulating the peripheral inflammation in CHS [71].

Thus, although the function of LCs in the sensitization phase of CHS remains controversial, langerin$^+$ dDCs probably affect stimulatory effects during sensitization. The function of langerin$^-$ dDCs in sensitization has not yet been fully investigated; however, langerin$^-$ dDCs also appear to have stimulatory functions in the sensitization phase, because ablation of both LCs and langerin$^+$ dDCs before sensitization impairs CHS response but is unable to abrogate it completely [29, 64, 66].

4.3.2 The Role of Cutaneous DCs in Atopic Dermatitis (AD)

AD is a pruritic chronic retractable inflammatory skin disease that is induced by the complex interaction between susceptibility genes encoding skin barrier components and stimulation by protein antigens [72, 73]. Upon protein antigen exposure, DCs acquire antigens and stimulate the proliferation of T cells to induce distinct T helper cell responses to external pathogens [2]. Therefore, it has been suggested that DCs initiate AD in humans [74]; however, it remains unclear which cutaneous DC subset initiates epicutaneous sensitization to protein antigens. The application of large molecules is localized above the size-selective barrier, tight junction (TJ), and activated LCs extend their dendrites through the TJ to take up antigens [75]. Therefore, it can be hypothesized that it is not dermal DCs but rather LCs that initiate epicutaneous sensitization with protein antigens, as in the development of AD.

In keeping with this, skin DCs elicit a Th2 response in the presence of mechanical injury by inducing cutaneous thymic stromal lymphopoietin (TSLP) [76]. TSLP is a cytokine that is produced mainly by nonhematopoietic cells such as fibroblasts and epithelial cells, including epidermal keratinocytes. TSLP plays an important role in the induction of Th2 responses through the activation of DCs via

the expression of OX40L [77]. Previous reports have shown that TSLP is highly expressed in the lesional skin of AD patients and that it activates human epidermal LCs and DCs in vitro [78–80].

In the murine AD model, which is induced by epicutaneous sensitization with protein antigen, LCs are the essential cutaneous DC subset in the induction of IgE upon epicutaneous sensitization with protein antigens. Moreover, TSLP receptor (TSLPR) expression on LCs is enhanced upon protein antigen exposure to the skin and TSLPR signaling on LCs plays an important role in this process. TSLP stimulation causes LCs to express OX40L and BMDCs to induce Th2 chemokines while suppressing Th1 chemokines, which may shift the immune environment to a Th2 milieu [81]. These findings suggest that TSLPRs on LCs can be a therapeutic target of skin inflammatory reactions induced by epicutaneous sensitization with protein antigens, such as in the development of AD (Fig. 4.2).

The skin of AD patients is characterized by the presence of LCs and IDECs, showing high surface expression of FcεRI, which enables specific allergen uptake [82]. IDECs are regarded as important amplifiers of the allergic inflammatory reaction of DCs. Compared to LCs, IDECs show higher expression of costimulatory molecules, thus supporting the role of IDECs as the antigen-presenting DC population in inflamed skin [83].

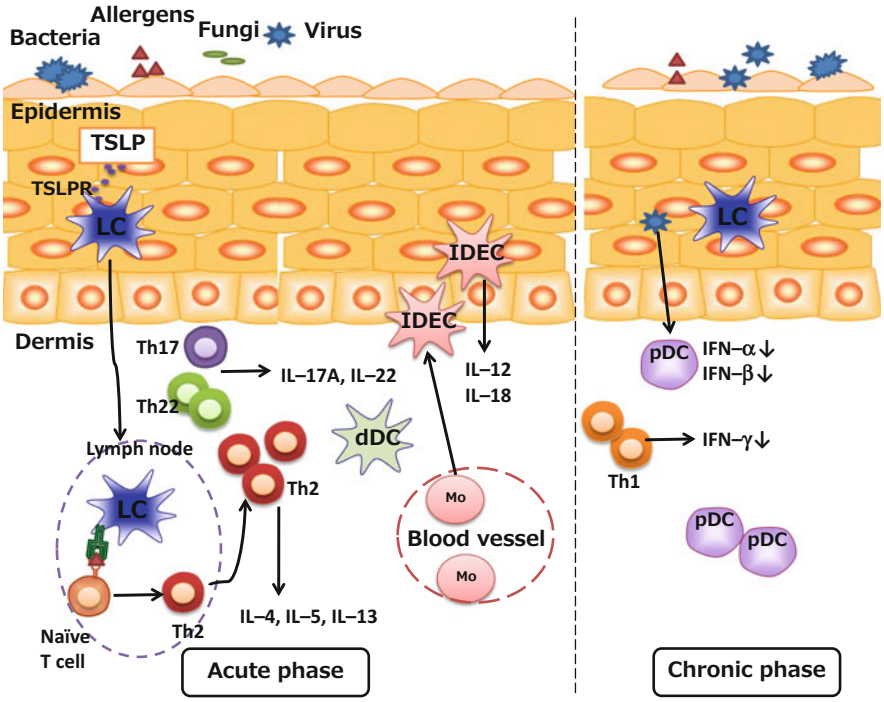

Fig. 4.2 Role of cutaneous DC subsets in atopic dermatitis. *LC* Langerhans cell, *IDEC* inflammatory dendritic epidermal cell, *pDC* plasmacytoid dendritic cell, *dDC* dermal dendritic cell, *Mo* monocyte, *IL* interleukin, *IFN* interferon

Compared to other inflammatory skin diseases, few pDCs are detected in the lesional skin of AD patients [44]. However, compared to healthy skin, a marked number of pDCs are accumulated within the dermal compartment, showing an activated phenotype [49]. The expression level of inflammatory chemotactic factor chemerin, the ligand to CMKLKR1, in AD skin lesions is weak, and this might explain the reduced infiltration of pDCs into inflamed tissue.

4.4 Concluding Remarks

DCs in the skin, with their constitutional plasticity, play crucial roles in initiating and modulating immune responses. Recent advances in the knowledge of skin DCs' network and pathology have opened avenues for the development of therapies that trigger skin DCs with specialized properties to control immunity.

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Chapter 5
T Cells

Takashi Nomura and Aya Shinohara

Abstract Skin protects the body from continual attack by microbial pathogens and environmental factors. The barrier function of skin is achieved by multiple components including the immune system. Thymus-derived lymphocytes (T cells) and B lymphocytes (B cells) are the major players in the adaptive immune system. T cells expressing T-cell receptor (TCR) α and β chains (αβT cells) control the strength and type of immune responses through multifaceted function. CD4 T cells are multifunctional lymphocytes that are divided into helper T (Th) cell subsets and immune suppressive regulatory T (Treg) cells. Th1 cells produce IFN-γ and protect against intracellular pathogens. Th2 cells produce IL-4 family cytokines and participate in the development of allergic skin diseases, including atopic dermatitis (AD). Th17 cells secrete IL-17, recruit granulocytes to fight against extracellular bacteria and fungi, and play a role in psoriasis. IL-22 produced by Th22 cells activates epithelial cells and mediates acanthosis in psoriasis and AD. On the other hand, Foxp3+ Treg cells mute immune responses partly via TGF-β or IL-10. Tissue resident memory T (Trm) cells—essentially all of which are epidermal CD8 T cells—constitute the first line of defense against repeated infections. CD8 T cells are also engaged in psoriasis, lichen planus, and drug eruptions. Skin harbors innate-like T cells such as natural killer T (NKT) cells as well, whose function is not fully understood. This chapter reviews these αβT cell-subsets.

Keywords Th1 • Th2 • Th17 • Th22 • Th9 • Tfh • Regulatory T cells • T-bet • GATA3 • RORγt • Foxp3 • TGF-β • Tissue resident CD8 memory T cells

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5.1 Introduction

There are three checkpoints in the defense system of skin. The first is the normal flora-rejecting microbial intruders. The second is the physical barrier made of corneum. The third is the immune system, which consists of the innate and adaptive immune system participated by lymphocytes, keratinocytes, and endothelial cells. Streilein proposed an autonomous operational integration of immune system in the skin, termed skin-associated lymphoid tissue (SALT) [136]. Although the definition of SALT is still under discussion, skin and its draining lymph nodes form a durable unit that protects the body surface from continual attack of microorganisms. The immune system is built of distinct types of cells including the lymphocytes—T lymphocytes (T cells), B lymphocytes (B cells), and natural killer (NK) cells—that determine the specificity of immunity. T cells, in particular, contribute to development of specific types of immune responses, production of antibody by B cells, activation of macrophages, and recruitment of granulocytes to the inflammatory sites. Moreover, T cells maintain unresponsiveness to particular antigens, i.e., immunological tolerance. This chapter reviews characteristics of T cells that express T cell receptor (TCR) α and β chains (αβT cells). The reader is directed to other chapters for details about γδT cells that express TCR γ and δ chains and have a complementary role in the skin immunity.

5.2 Characteristics of αβT Cells in the Skin

There are 1×10^6 T cells/cm^2 of normal skin and 20 billion in the entire skin surface, estimated to be twice the number of T cells in the circulation [21, 22]. Most of them are skin-homing memory αβT cells expressing CD45RO, CCR4, and cutaneous lymphocyte antigen (CLA). CD4 memory T cells, constituting the majority of skin T cells, reside in the dermis at the perivascular regions. In contrast, the epidermis contains only a few αβT cells, which are CD8 tissue-resident memory T (Trm) cells.

Protection against skin infection mainly relies on the skin Trm cells as demonstrated in patients with cutaneous T cell lymphoma treated with alemtuzumab (antihuman CD52), which spares nonmalignant skin resident effector memory T (Tem) cells [25]. Tropism of skin T cells is determined by the homing receptors expressed on the cell surface. For example, CLA—a modified version of P-selectin glycoprotein ligand-1 (PSGL or CD162)—binds to E-selectin (CD62E) on the endothelium of cutaneous postcapillary venules, thereby initiating tethering of T cells at the endothelium of the skin vessels.

Another feature of skin T cells is that 20–25% of dermal CD4 T cells are Foxp3+ Treg cells in steady state, and this percentage can reach 60% under inflammatory conditions in mice ([119] and personal observation).

5.3 Generation of Skin-Homing T Cells

T cells inexperienced with antigen are called naive T cells, which are characterized by the expression of CD62L (L-selectin), C-C chemokine receptor 7 (CCR7), and lymphocyte function associated antigen-1 (LFA-1). They roll over the endothelium of the high endothelial venules (HEV) in the lymph node. This rolling helps binding of CD62L to GlyCAM-1 and CD34, both of which are expressed by HEV. Binding of chemokine CCL21 (expressed by HEV) to CCR7 (on the naive T cells) activates the integrin LFA-1, which then tightly binds to ICAM-1 on the endothelium. This tight binding allows the naive T cells to squeeze between endothelial cells to enter the lymph node. Thus, CD62L acts as a homing receptor to enter secondary lymphoid tissues via HEV.

Dendritic cells (DCs) are indispensable for activation of T cells. Pathogen-associated or damage-associated inflammatory signals activate DCs, which reside in the nonlymphoid tissues including skin. Activated DCs express CCR7 and migrate to lymph nodes in a CCL21-dependent manner. Within the lymph nodes, these DCs activate naive T cells through direct cell–cell interaction in an antigen-specific manner. To fully activate naive T cells, multiple signals are required through ligation of TCR and CD28 by antigen/MHC complex and CD80/CD86, respectively, through the interaction between T cells and DCs. Langerhans cells (LCs) are a kind of DCs that lodge in epidermis. Roles of LCs in T cell-activation and tolerance induction are not fully clarified.

Once activated, T cells lose CD62L and CCR7 followed by acquisition of different homing receptors such as PSGL-1, CD44, CCR5, and CXCR3 [88] as well as integrins such as VLA-4 and VLA-5, which enable T cell retention in extravascular sites by binding to fibronectin. Skin-homing T cells additionally express CLA and CCR4, which recognize CCL27 (CTACK, cutaneous T cell-attracting chemokine) and CCL17 (TARC, thymus and activation regulated chemokine), respectively, both of which are expressed by keratinocytes.

How activated T cells acquire skin tropism and develop into skin-homing T cells is a fundamental question yet to be solved. Stromal cells of lymph nodes and constituting cells of peripheral nonlymphoid tissue may determine the migratory pattern of the T cells. In the skin, dermal DCs metabolize sunlight-induced epidermal vitamin D3 into 1,25(OH)(2)D3, which imprints T cells to express CCR10, another receptor for a skin-associated chemokine CCL27 that preferentially attracts skin-homing T cells [130]. Conversely, opposite imprinting can occur. For example, CD8 T cells, which are activated in the skin by cutaneous vaccinia virus infection, can acquire a gut-homing phenotype once they enter the mesenteric lymph node [79]. Such flexibility of tissue-tropism might contribute to protection against systemic infection. It is speculated that not only tissue-derived DCs but also undetermined signals from other cell types in the tissue environment affect the pattern of homing receptors on T cells [36, 37, 39, 87].

After the clearance of pathogens, effector T cells undergo apoptosis, leaving long-lived memory T cells composed of central memory T (Tcm), effector memory

Table 5.1 Features of memory T cells

	Naïve	Central memory	Effector memory	Resident memory
CD44	−/low	+	+	+
CD62L[a] (L-selectin)	+	+	−	−
CCR7[a]	+	+	−	−
CD127 (IL-7Rα)	Varied	+	+	Varied
CD69	−	−	−	+
CD103 (integrin αE)	−	−	−	+
CD11a	Varied	Varied	Varied	+
Distribution	LN, Sp, PB	LN, Sp, PB, BM	Sp, PB, peripheral tissue	Peripheral tissue
Migration	Yes	Yes	Yes	No

Resident memory CD8 T cells distribute in the skin, gut, vagina, salivary glands, lung, and brain. Resident memory CD4 T cells are fewer and difficult to prove in peripheral tissue except the lung. Resident memory T cells are suggested to be supported by the peripheral tissues in the absence of antigen stimulation although they can be enhanced by repeated immunization.
LN lymph nodes, *Sp* spleen, *PB* peripheral blood, *BM* bone marrow.
[a]Lymphoid homing molecules.

T (Tem), and tissue-resident memory T (Trm) cells (Table 5.1) [16, 59, 91, 128]. Tcm cells express CCR7 and CD62L and recirculate secondary lymphoid organs (lymph nodes and spleen). A feature of Tcm cells is their considerable ability to multiply on antigen stimulation. In contrast, Tem cells are less proliferative but promptly produce cytokines. Tem cells express neither CCR7 nor CD62L and are distributed in peripheral tissues to defense against reinfections. Trm cells do not circulate but reside in the peripheral tissues for a long time. Expression of CD103 (α-chain of αEβ7 integrin, which recognizes E-cadherin) is a feature of Trm cells. In general, skin T cells of healthy individuals are either CD4 Tem or presumably some CD4 Trm cells that reside in the dermis or CD8 Trm cells that are sessile in the epidermis [59]. The relationship between these memory T cell subsets is not fully understood.

5.4 Subsets of αβT Cells

The αβ T cells comprise multiple subsets (Fig. 5.1). Two important lineages of the αβT cells are CD4 T cells and CD8 T cells. These cells differ in how they recognize antigen and mediate effector functions. The CD4 T cell population consists of diverse helper T cells including Th1, Th2, Th17, Th22, Th9, Tfh, and Treg cells (immune suppressive T cells). Another subset of αβT cells is natural killer T (NKT) cells, which express invariant TCRs and share many functions with natural killer (NK) cells, a subset of innate type lymphocytes.

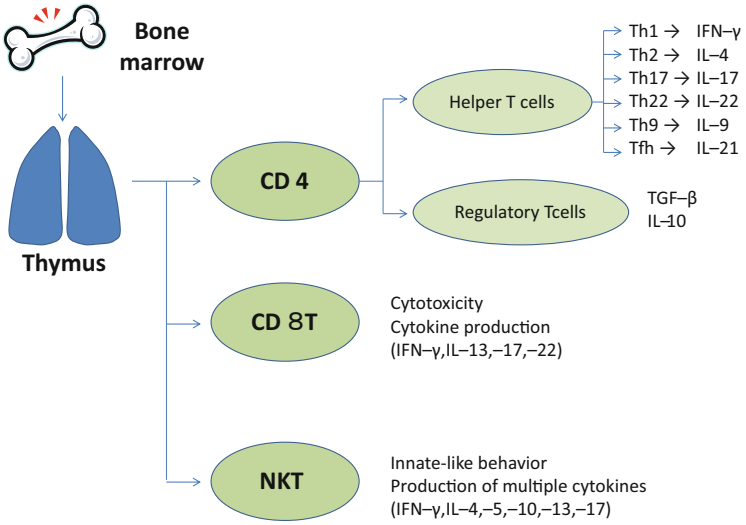

Fig. 5.1 Development of αβT cells. Bone-marrow–derived precursor cells enter the thymus to become thymocytes. Thymocytes complete their developmental steps to exit the thymus as CD4 T cells, CD8 T cells, or NKT cells expressing αβ T-cell receptors. The cytokine milieu induces differentiation of CD4 T cells into a variety of helper T (Th) cell-subsets such as Th1, Th2, Th17, Th22, Th9, and Tfh cells. CD4 T cells also differentiate to regulatory T (Treg) cells that downmodulate immune responses. Although CD8 T cells possess cytotoxicity, they can produce various cytokines depending on the inflammatory circumstances. Natural killer T (NKT) cells are a subset of αβT cells that share functions of T cells and NK cells. NKT cells behave like innate lymphocytes and promptly produce various cytokines on encounter with pathogens

5.5 CD4 T Cells

Host responses vary with infections. Whereas cutaneous exposure to *Mycobacterium tuberculosis* induces massive macrophage infiltration, helminthic infection promotes IgE production and activation of eosinophils. The discovery that CD4 T cells mediate these different responses led to the identification of heterogeneous CD4 T cell subsets (Table 5.2). The first two subsets identified were type 1 (Th1) and type 2 helper T (Th2) cells [89]. Later, IL-17 producing Th17 cells were identified to mediate some autoimmune diseases that had been attributed to Th1 cells. Currently, IL-9 producing Th9 cells, IL-22 producing Th22 cells, and germinal center-forming follicular helper T (Tfh) cells are recognized. Furthermore, CD4 T cells harbor immune-suppressive subsets such as Foxp3+ Treg cells, Tr-1, and Th3 cells. It is important to note, however, that each CD4 T cell can express cytokines or transcription factors that are not specific for its subset. CD8 T cells can produce a series of cytokines that are classified into the CD4 T cell subsets. Such CD8 T cells are called Tc1, Tc2, Tc17, or Tc22. Therefore, it seems that each T cell possesses functional plasticity or flexibility. The question whether the CD4 or

Table 5.2 Subsets of CD4 T Cells

	Inducing cytokines	Master regulator	Effector cytokines	Inhibitors	Function	Host defense	Pathology
Th1	IFN-γ	T-bet	INF-γ	IL-4	Activation of macrophages, Ig class switch to IgG1/G3 (human) or IgG2a/G3 (mouse)	Intracellular pathogens	Granulomatous diseases, inflammatory bowel disease
	IL-12						
Th2	IL4	GATA3	IL-4	IFN-γ	Activation of mast cells, basophils, eosinophils, alternative macrophages, barrier function, class switching to IgE	Helminths	Atopic dermatitis, allergic asthma
			IL-5				
			IL-10				
			IL-13				
Th17(β)	IL-6	RORγt	IL-17A/F	IL-2	Recruitment of neutrophils	Extracellular bacteria and fungi	Undefined
	TGF-β		IL-10	IFN-γ			
				IL-4			
				Foxp3			
				T-bet			
Th17 (23)	IL-6	RORγt	IL-17A/F	Ditto	Ditto	Ditto	Psoriasis
	IL-23	T-bet	IL-22	TGF-β1			RA
	IL-1β		IFN-γ				MS
	TGF-β3		GM-CSF				
Th22	IL-6	AhR (?)	IL-22	TGF-β1	Induction of defensins	*Klebsiella pneumoniae*	Psoriasis
	TNF-α	RORγt (?)					
		Notch (?)					
Th9	IL-4	PU.1	IL-9	Undefined	Production of mucus	Undefined	Undefined
	TGF-β	IRF-4					
Tfh	IL-6	Bcl6	IL-21	IL-2	B cell maturation, Ig class switching, memory B cell development	B-cell–mediated protection	Undefined
				Blimp1			

Foxp3+ Treg	IL-2	Foxp3	TGF-β	IL-6	Maintenance of peripheral tolerance, attenuation of immune response	Tuning of inflammation	Impaired host defense, cancer
	TGF-β		IL-10	RORγt			
			IL-35	HIF1a			
Tr1	IL-27	c-Maf	IL-10	Undefined	Ditto	Ditto	Undefined
	TGF-β						
Th3	Undefined	Undefined	TGF-β	Undefined	Ditto	Ditto	Undefined
LAG3 Treg	Undefined	Undefined	IL-10	Undefined	Ditto	Ditto	Undefined

RA rheumatoid arthritis, *MS* multiple sclerosis, *Ditto* same as above.

CD8 T cell subsets represent a terminally differentiated state or they just reflect certain aspects of their transient states remains to be solved.

5.5.1 Th1 Cells

(Summary) Th1 cells produce IFN-γ and protect against intracellular pathogens such as *Mycobacteria*, *Listeria*, *Toxoplasma*, and viruses. Th1 cells dominate during the early phase of contact dermatitis.

5.5.1.1 Characteristics of Th1 Cells

Th1 cells are defined by secretion of IFN-γ. Th1 cells express IL-12 receptor β1 and β2, IL-18 receptor, E-selectin, P-selectin, CXCR3, and CCR5. IFN-γ activates macrophages, dendritic cells, CD8 T cells, NK cells, and B cell class-switching to IgG1 and IgG3 in humans (IgG2a and IgG3 in mice). Th1 cells suit the immune response to the protection against intracellular pathogens, such as *Mycobacteria*, *Listeria*, *Toxoplasma*, and viruses.

5.5.1.2 Differentiation of Th1 Cells

IFN-γ promotes differentiation of Th1 cells in synergy with IL-12, IL-18 and type I IFN (IFN-α/β) (Fig. 5.2). IL-12 activates the signal transducer and activator of transcription (STAT) 4, a modifier for over 4,000 genes encoding factors related to Th1 cells, such as IFN-γ and receptors for IL-12 and IL-18 [150]. IFN-γ activates STAT1, which reinforces differentiation to Th1 cells. Activated STAT4 and STAT1 induce expression of T-bet (the master regulator for Th1 cells) encoded by *TBX21*, which regulates genes such as *Ifng* that affect Th1 cell-commitment [75, 83, 139]. Th1 cells can differentiate without STAT4 and T-bet via Hlx, Runx3, and Ets. It is not fully understood how such alternatively differentiated Th1 cells function.

5.5.2 Th2 Cells

(Summary) The signature cytokine of Th2 cells is IL-4. Th2 cells promote humoral immune response, contribute to host defense against extracellular pathogens such as parasitic worms, and play a role in atopic diseases. Epithelial cell-derived cytokines (IL-25, IL-33, and TSLP) initiate Th2 cell-differentiation.

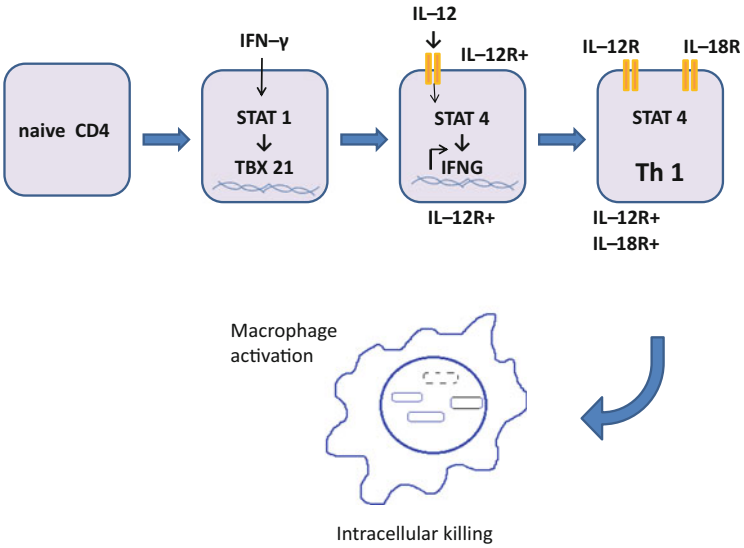

Fig. 5.2 Differentiation of Th1 cells. Binding of IFN-γ to its receptor on naive CD4 T cells induces the expression of *TBX21* encoding T-bet, in a STAT1-dependent manner. T-bet induces the expression of IL-12Rβ2, which combines with IL-12Rβ1 (expressed on naive CD4 T cells) to form the receptor for IL-12 (IL-12R). In concert with T-bet, binding of IL-12 activates STAT4 and induces the expression of *IFNG* gene encoding IFN-γ, by which the receptor for IL-18 (IL-18R) is expressed. Fully committed Th1 cells produce a large amount of IFN-γ and activate macrophages

5.5.2.1 Characteristics of Th2 Cells

Th2 cells are defined by secretion of IL-4, IL-5, and IL-13. IL-24, IL-25 (IL-17E), and IL-31 are produced by Th2 cells as well. Receptors for IL-25, IL-33 (an IL-1 family alarmin), TSLP (thymic stromal lymphopoietin), CRTH2 (chemoattractant receptor-homologous molecule on Th2 cells), and chemokine receptors such as CCR3, CCR4, and CCR8 are expressed on Th2 cells. IL-4, IL-5, and IL-13 help B cells to produce immunoglobulin (Ig). IL-4 initiates Th2 cell differentiation and promotes Ig class-switching to IgG4 and IgE in humans (IgG1 and IgE in mice). IL-4 and IL-13 induce alternatively activated macrophages while suppressing IFN-γ-induced classically activated macrophages. IL-4, IL-5, and IL-13 mobilize eosinophils, basophils, and mast cells, and increase mucous secretion. IL-31 (an IL-6 family cytokine) promotes dermatitis in mice [34]. Eosinophils and Th2 cells express CRTH2, which is a receptor for prostaglandin (PG) D2 and induces chemotaxis of these cells to the inflammatory sites. PGD2 is produced by mast cells, which reside in epithelial tissues including the skin. Th2 cells cause allergic responses including AD, pollen rhinitis, and asthma.

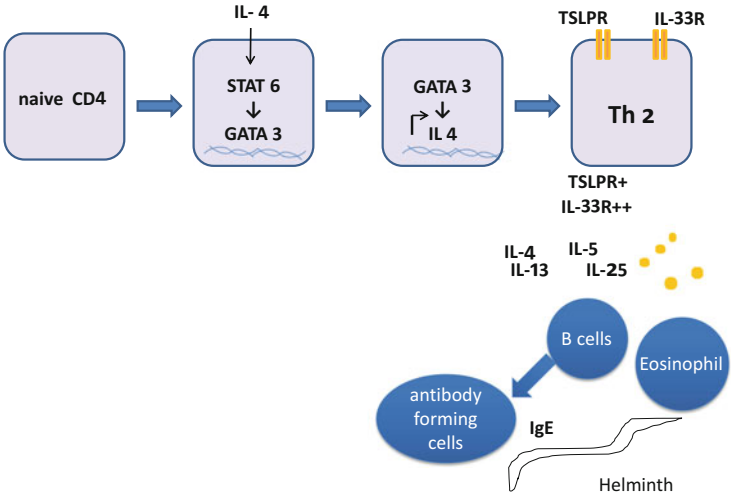

Fig. 5.3 Differentiation of Th2 cells. Binding of IL-4 to its receptor on naive CD4 T cells suppresses the expression of IL-12Rβ1 and induces expression of *GATA3* encoding GATA3, in a STAT6-dependent manner. GATA3 transactivates a set of genes required for Th2 cell differentiation. Fully committed Th2 cells express receptors for TSLP (TSLPR) and IL-33 (IL-33R). IL-4, IL-5, IL-13, and IL-25 produced by Th2 cells activate basophils and B cells. B cells further differentiate to antibody forming cells (AFCs, or plasma cells) to produce immunoglobulins such as IgE to protect the body against helminth infection

5.5.2.2 Differentiation of Th2 Cells

In contrast to other Th subsets, activation of antigen-presenting cells (APCs) is not sufficient to provide IL-4. Instead three Th2-promoting cytokines (IL-25, IL-33, and TSLP) are necessary for Th2 cell differentiation (Fig. 5.3). Epithelial cells produce these cytokines by antigenic or environmental stimuli. IL-25 and IL-33 recruit and activate innate immune cells such as basophils, eosinophils, mast cells, NK cells, and recently identified natural helper (NH) cells. These innate cells produce IL-4 in addition to IL-5 and IL-13. TSLP is an IL-7-like cytokine that activates B cells and DCs. TLSP primes DCs to express CD40 ligand (CD40L) and to decrease the production of p40 subunit of IL-12 [100]. CD40L thus induced on DCs triggers naive CD4 T cells to produce IL-4, IL-5, and IL-13 [64]. IL-4 activates STAT6 followed by expression of GATA3, the master regulator of Th2 cells encoded by *Gata3*. GATA3 transactivates genes such as *Il4*, *Il5*, *Il13*, and *Gata3*, all of which reinforce commitment to Th2 cells [149].

5.5.2.3 Th2 Cells and Skin Pathology

Th2 cells are engaged in the allergic skin diseases such as AD. AD is a chronic and relapsing inflammatory skin disease with severe pruritus. AD presents early in life,

in general, and is associated with sensitivity to food or inhalant allergens, indicating a complex interaction between the skin and systemic immune system. Dry skin is a feature of AD, which suggests breakage of the skin barrier with these patients. Indeed, certain AD patients harbor mutations in the *FLG* gene, which encodes filaggrin (filament-aggregating protein), a key protein in facilitating epidermal differentiation and the skin barrier [102, 131, 151]. Breakage of the skin barrier opens the entry sites for exogenous antigens leading to stimulation of keratinocytes that produce Th2-promoting cytokines, i.e., IL-25, IL-33, and TSLP, which stimulate group 2 innate lymphoid (ILC2) cells including NH cells to produce IL-5 and IL-13 [134]. TSLP stimulates DCs to express CD40L and reduce IL-12 p40, thereby skewing activated T cells toward Th2 cell-differentiation. TSLP-primed DCs secrete CCL17 (TARC) and CCL22 (MDC), both of which attract Th2 cells to the site of inflammation [109, 133]. Therefore, barrier dysfunction and production of IL-25, IL-33, and TSLP play a central role in the exacerbation of Th2 cell-mediated inflammation.

Advancing biologics provide clues to understand Th2 cell-mediated immunity in skin diseases although evidence is conflicting now as follows [42]. Anti-IL-5 mepolizumab did not ameliorate AD whereas the number of blood eosinophils decreased. Efficacy of anti-IgE omalizumab onto AD differs among the studies, reflecting heterogeneity of the disease. Clinical studies are underway for anti-IL-31 antibody, CRTH2 inhibitor, TSLP receptor binding protein, and IL-4Rα binding protein antagonizing IL-4 and IL-13. These studies should clarify the role of Th2 cells in skin diseases.

5.5.3 Th17 Cells

(Summary) IL-17 production defines Th17 cells that are divided in Th17(β) or Th17 (23), which are induced by the combination of IL-6 and TGF-β or of IL-6, IL-23, and IL-1β, respectively. Th17 cells are supposed to cause autoimmune diseases such as psoriasis, rheumatoid arthritis, and multiple sclerosis. IL-23 is critical for activation of Th17 cells.

5.5.3.1 Characterization of Th17 Cells

Self-reactive Th1 cells had been postulated to cause autoimmune diseases [70]. Indeed, loss of T-bet prevented experimental autoimmune encephalomyelitis (EAE), a mouse model for multiple sclerosis in humans [12]. However, EAE can occur in the absence of IFN-γ or its receptor [44, 153]. Furthermore, studies of gene-targeted mice for IL-12 (a p70 heterodimer composed of a p35 subunit and a common p40 subunit shared by other cytokines) came to a seemingly contradictory conclusion: p40−/− mice were resistant to EAE, whereas p35−/− showed even more susceptibility than wild-type. This finding indicated that a molecule other than

IL-12 (p35/p40)—identified later as IL-23 (p19/p40) that activates Th17 cells—fulfills the function previously attributed to IL-12 [9, 55]. Such difficulties in explaining the autoimmune diseases simply on the basis of the Th1/Th2 paradigm led to the identification of CD4 T cells that produce IL-17, termed Th17 cells [29, 53, 73, 148]. Signature cytokines of Th17 cells are IL-17A and IL-17F, both of which recruit and activate neutrophils. Th17 cells express CCR6 and its ligand CCL20 [2], and produce IL21 and IL-22 as well. Th17 cell subset seems to include at least two subsets; Th17(β) and Th17(23) [71].

The natural role of Th17 cells is protection against bacterial and fungal species. IL-17 recruits and generates neutrophils through the production of chemokines and GM-CSF (granulocyte macrophage colony stimulating factor), respectively. Recruited neutrophils then besiege extracellular bacteria and fungi. Defense by Th17 cells is oriented to mucocutaneous surface rather than to the whole body, and its main targets are *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Candida albicans* [6, 26, 68]. Autosomal dominant hyper IgE syndrome (HIES or "Job's syndrome") is explained by dysfunctional Th17 cells due to the mutation in STAT3 that is activated by IL-6 and IL-23. These patients are susceptible to candidiasis and staphylococcal infections [84]. This impairment is due to lack of IL-17, as autosomal recessive IL-17RA deficiency or autosomal dominant IL-17F deficiency leads to chronic mucocutaneous candidiasis (CMC) with *Staphylococcus aureus* dermatitis as well [106]. Likewise, autoantibodies against IL-17A, IL-17F, and IL-22 cause CMC [67, 107].

5.5.3.2 Differentiation and Activation of Th17 Cells

Differentiation of Th17 cells requires STAT3 and RORγt, both of which are regulated by key cytokines including IL-1β, IL-6, IL-21, IL-23, and transforming growth factor-beta1 (TGF-β1 or TGF-β otherwise indicated; Fig. 5.4). Combinations of these cytokines induce two subsets in Th17 cells, namely Th17(β) and Th17(23), and another relative Th22 (described later) [71]. Th17(β) cells express IL-17A, IL-17F, IL-10, CCL20, and CXCR6, and are differentiated by the combination of IL-6 and TGF-β. On the other hand, Th17(23) cells, which are more pathogenic and express IL-22, CCL9, and CXCR3 in addition to IL-17A and IL-17F, are differentiated by the combination of IL-1β, IL-6, and IL-23 [52]. TGF-β1 rather inhibits IL-22 expression of Th17 cells. IL-21 and IL-1β expand and promote Th17(β) and Th17(23) in vitro [56, 69, 125, 138, 159].

The most important cytokines that regulate Th17 cell-differentiation are IL-6 and IL-23. They activate STAT3 to regulate genes such as *Il17a/f*, *Il23r*, *Il21*, and *Rorc*. *Rorc* encodes RORγt (also referred as RORc), which is the master regulator for Th17 cells. TGF-β is a pleiotropic cytokine produced by various kinds of cells. It works as an anti-inflammatory cytokine and induces Foxp3+ Treg cells. However, in the presence of IL-6, TGF-β induces RORγt [63, 167] in a fashion other than the Smad2 and Smad3 pathway [141].

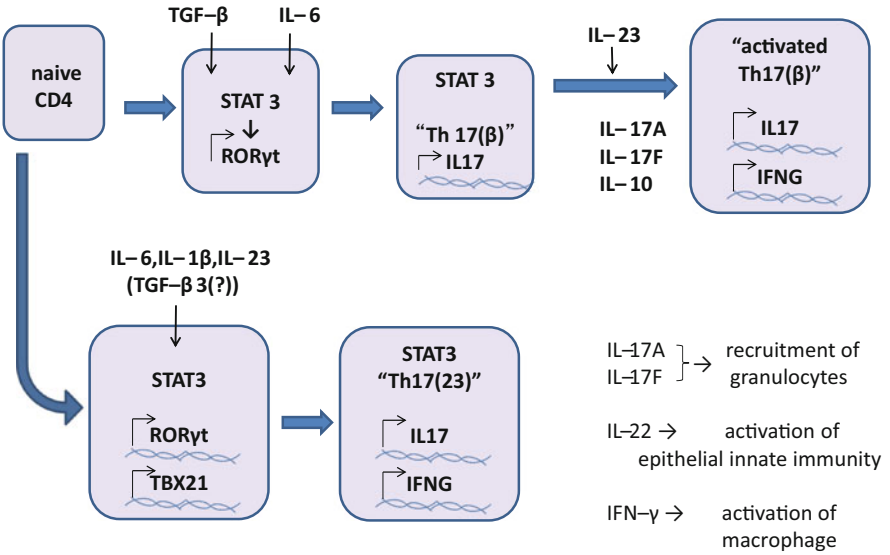

Fig. 5.4 Differentiation of Th17 cells. Recent studies revealed there are two pathways in differentiation of Th17 cells. In one pathway, combination of IL-6 and TGF-β induces *RORC* encoding RORγt in a STAT3-dependent manner. RORγt commits the cell to Th17(β), a Th17 lineage that produces IL-17A, IL-17 F, and IL-10. Th17(β) can be activated by IL-23 and produce IL-22, IFN-γ, and GM-CSF. GM-CSF is suggested to play a role in the inflammation of the central nervous system. In another pathway, combination of IL-6, IL-1β, and IL-23 induces both *RORC* and *TBX21* in a STAT3-dependent manner, by which Th17(23) are differentiated. Th17(23) cells produce IL-17A, IL-17 F, IL-22, and IFN-γ. IL-17A and IL-17 F recruit granulocytes. IL-22 activates epithelial innate immunity to reinforce the protective function of skin and mucosa. IFN-γ activates macrophages

RORγt is a T-cell–specific splice variant of RORγ, which is an orphan retinoid receptor expressed in various tissues and engaged in development of thymus, lymphoid tissue inducer cells, and IL-22-producing subset of NK cells as well [80, 120, 121, 140]. RORα, another member of the orphan retinoid receptor family, also induces Th17 cell-differentiation with RORγt [159]. Digoxin and its derivatives inhibit RORγt and suppress Th17 cell-differentiation [62].

With respect to Th17(23) cells, it is not fully understood how IL-6, IL-23, and IL-1β induce RORγt without TGF-β1. It is reported that a combination of TGF-β3 and IL-6 induces very pathogenic Th17 cells—distinct from Th17(β) cells—in mouse EAE, and that IL-23 is required for the production of TGF-β3 by the developing Th17 cells [78]. It is noteworthy that Th17(β) cells are not pathogenic unless they are exposed to IL-23, whereas Th17 cells induced by TGF-β3 and IL-6 are very pathogenic without IL-23. Whether the TGF-β3-induced Th17 cells are equivalent to Th17(23) is not confirmed yet. At least it is clear that IL-23 plays a central role in the generation of pathogenic Th17 cells.

Other transcription factors such as aryl hydrocarbon receptor (AhR), Batf, IκBζ, IRF-4, and Runx1 are known to promote Th17 differentiation, although the precise mechanism is ambiguous [71].

5.5.3.3 Metabolism and Other Factors Affecting Differentiation of Th17 Cells

Metabolic state affects development of Th17 cells. Indole-amine-pyrrole 2,3-dioxygenase (IDO) is an enzyme that catabolizes tryptophan and suppresses the activity of mTORC1 (the metabolic target of rapamycin complex 1) in T cells [74]. Lack of IDO increases production of IL-17 and Th17 cell infiltration [27]. Halofuginone inhibits Th17 cell differentiation through amino acid starvation response, which is correlated with mTOR (the metabolic target of rapamycin) [137]. Thus, mTOR promotes differentiation of Th17 cells. Hypoxia-inducible factor (HIF)-1 forms a complex with RORγt and recruits acetyl transferase p300 to the *Il17* locus [30, 126]. HIF-1α operates on a metabolic state of the cell and switches the oxidative phosphorylation state to an aerobic glycolysis state during the hypoxia [123]. Therefore, the hypoxic condition supports Th17 cell differentiation. Notably, the above regulations affect differentiation of memory T cells and Foxp3+ Treg cells in an opposite way. It remains unclear whether such mTOR-mediated metabolism affects Th17-mediated skin diseases such as psoriasis vulgaris.

Environmental pollutants affect Th17 cell differentiation through the aryl hydrocarbon receptor (AhR) [108, 144]. AhR is expressed selectively in Th17 cells; and its ligation accelerates onset of EAE in mice in a Th22 cell-dependent manner [144]. The fate of T cells depends on the ligands for AhR. For example, 2,3,7,8-tetrachlorodibenzo-p-dioxin activates AhR and induces Foxp3+ Treg cells, ameliorating EAE, whereas 6-formylindol[3,2-b]carbazole interferes with Treg cell-development and boosts Th17 cell differentiation, exacerbating EAE [108]. Thus, various aromatic molecules may regulate Th17 and Treg cell differentiation depending on the context. It is under investigation how AhR affects skin pathology.

5.5.3.4 Th17 (and Th22) Cells in Skin Diseases

Th17 cells (and Th22 cells) have been studied in the context of endodermal barriers such as intestinal and respiratory tracts. However, these cells occupy important roles in the skin as well [147]. Irritated keratinocytes produce IL-1β and IL-6 that stimulate LCs and dermal DCs to produce IL-23 and migrate to regional lymph nodes, where Th17 and Th22 cells are differentiated in response to antigens presented by these APCs. Skin-homing Th17 and Th22 cells produce IL-17A, IL-17F, IL-22, and TNF-α. These ligands affect keratinocytes to produce various cytokines (IL-32 and IL-36), chemokines (CXCL1 and CCL20), and antimicrobial peptides (HBD-2 and S100A7/proriasin, and S100A15/koebnerisin); all of which enhance local inflammatory responses.

Psoriasis is a prototype of dysregulated Th17 cell-mediated immune response in the skin. Psoriasis was thought to be a Th1/Tc1 autoimmune disease because IFN-γ-producing Th1 and Tc1 (CD8 T cells) cells were identified from the lesion.

Ustekinumab, an anti-IL-12p40 monoclonal antibody, was originally chosen for this reason. However, it is now appreciated that neutralization of IL-12p40—a shared subunit between IL-12 and IL-23—rather targets the IL-23/IL-17 axis and Th17 cell-mediated pathway. Genomewide association study (GWAS) reinforced the importance of the IL-23 signaling pathway through identification of *IL23R*, *IL23A*, and *IL12B* loci as psoriasis-susceptibility genes [17, 93]. Furthermore, an antihuman IL-17A monoclonal antibody was effective for psoriasis in a clinical study [61]. Thus, the onset of psoriasis may arise from dysregulated IL-17-dependent expansion of CD4 (Th17) and CD8 (Tc17) cells. Although there is no clear explanation for the role of IFN-γ-producing (Th1 and Tc1) cells in the psoriatic lesion, these cells may be derived through developmental plasticity between Th17 and Th1 cells. Another unsolved question is the role of TNF-α. As inhibition of TNF suppresses IL-17-pathway genes in responders, at least there is a link between these cytokines [160].

5.5.4 Th22 Cells

(Summary) Th22 cells produce IL-22 and TNF-α, but not IL-17. The role of IL-22 is to activate the innate immune response of the epithelium. Th22 cells mediate acanthosis and skin lesions of psoriasis vulgaris and AD. IL-22 producing CD8 T cells are called Tc22.

5.5.4.1 Function of IL-22

The IL-10 family cytokine IL-22 operates in both pro- and anti-inflammatory ways [115, 161]. Th1, Th17, Th22, and γδT cells produce IL-22 in humans [132]. Interaction with LCs via CD1a enhances IL-22 production in self-reactive T cells [32]. IL-22 activates epithelial innate responses, which can be protective or detrimental [43]. IL-22 suppresses inflammatory bowel disease and acute liver inflammation in mice whereas it mediates mucosal host defense against gram-negative bacterial pneumonia [6]. An example of detrimental effect is epithelial hyperplasia in psoriasis, which can be induced by IL-22 in human artificial skin cultures [13]. In mice, IL-22 produced by Th17 cells mediates IL-23-induced dermal inflammation and acanthosis [164]. Increased IL-22 production is correlated with the squamous cell carcinoma occurring in immunocompromised transplant recipients [163]. Therefore, IL-22 and Th22 cells are involved in various skin diseases such as psoriasis vulgaris, AD, contact dermatitis, scleroderma, and tumorigenesis [48, 163].

5.5.4.2 Characterization of Th22 Cells

Certain helper T cells, CCR10+ ones in particular, are called Th22 cells as they produce IL-22 but not IL-17 [38, 43, 116, 143, 164, 166]. Th22 cells mediate dermal inflammation and acanthosis in an IL-23-dependent manner [164]. Skin-homing memory CD4 T cells expressing CCR4, CCR6, and CCR10 are supposed to be Th22 cells (or T-22 cells when Tc22 cells are also considered) [38, 43, 95, 143]. Th22 cells may regulate epidermal immunity, remodeling of epidermis, dermal inflammation, and acanthosis.

5.5.4.3 Differentiation of Th22 Cells

Plasmacytoid DCs induce Th22 cells from naive T cells in a way dependent on both IL-6 and TNF [38] (Fig. 5.5). Specific transcription factor is not defined for Th22 cell differentiation. AhR and RORγt are at least important for IL-22 production [144, 145]. Notch induces IL-22 secretion in CD4 T cells by stimulating AhR [4]. TGF-β inhibits IL-22 secretion through expression of c-Maf [116]. In a transcriptome analysis of human Th22 cell clones, the presence of BNC-2 and FOXO4 expression was observed whereas RORC2, GATA3, and T-bet were reduced [43]. It remains unclear whether these transcription factors play roles in Th22 cell-differentiation.

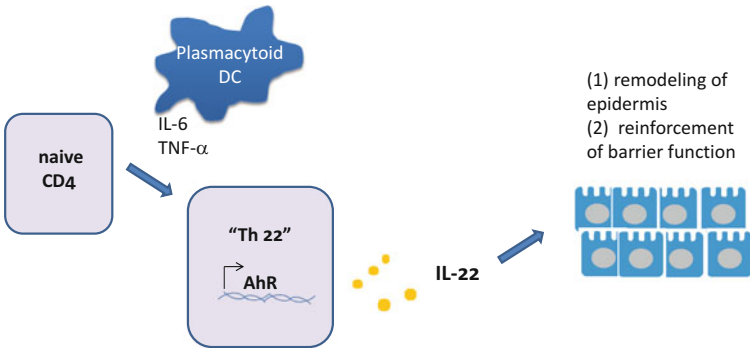

Fig. 5.5 Differentiation of Th22 cells. Th22 cells can be induced from naive CD4 T cells by plasmacytoid dendritic cells (pDCs) in a manner dependent on IL-6 and TNF-α. Aryl hydrocarbon receptor (AhR) encoded by the *AhR* gene is one of the candidates that regulate the differentiation of Th22 cells. IL-22 produced by Th22 cells (or by Tc22 cells, i.e., CD8 T cells producing IL-22) induces remodeling of the epidermis and mucous epithelium to reinforce the protective function of the surface barrier

5.5.4.4 Th22 Cells in Skin Diseases

Accumulation of Th22 and Tc22 cells indicate involvement of these cells in psoriasis and AD [43, 95, 156]. The majority of Th22 cells respond to CD1a [32]. Therefore, Th22 and Tc22 cells may proliferate in response to lipid antigens presented by the LC network in the epidermis and mediate acanthosis of AD. On the other hand, a dioxin-intoxicated patient showed an increased number of dermal Th22 cells suggesting a pathogenic role, however, how IL-22 contributes to the dioxin-induced skin lesions—chloracne—remains unresolved [14].

5.5.5 Th9 Cells

(Summary) Th9 cells produce IL-9, which promotes proliferation of mast cells and plays a role in allergy. Th9 cells are induced by IL-4 in the presence of TGF-β.

5.5.5.1 Function of IL-9

A subunit of the IL-9 receptor is the common cytokine receptor γ (cγ) chain, which is shared with the receptors for cytokines including IL-2, IL-4, IL-7, IL-13, IL-15, IL-21, and TSLP. IL-9 promotes proliferation of erythroid progenitor cells, B cells, B1 B cells, mast cells, and fetal thymocytes in mice. IL-9 is highly expressed in the lung of patients with asthma [41]. Neutralization of IL-9 reduces production of IL-5 and IL-13, indicating a link between IL-9 and Th2 type immune response [154]. Th17 and Treg cells also produce IL-9 [40].

5.5.5.2 Differentiation of Th9 Cells

IL-4 and TGF-β induce Th9 cells in a pathway dependent on interferon regulatory factor (IRF) 4 [135] (Fig. 5.6). IL-4 inhibits differentiation of TGF-β-induced Foxp3+ Treg cells and induces IL-9+IL-10+Foxp3- T cells instead [31]. Alternatively Th9 cells are induced from Th2 cells [145]. The transcription factor PU.1 is required for Th9 cell differentiation [18].

5.5.5.3 Role of Th9 Cells

Although IL-9 seems to exacerbate allergy [18], unclear issues remain. Blockade of IL-9 or IL-9Rα ameliorates EAE; whereas disruption of IL-9Rα gene worsens it [94]. Human *IL9* is located in the region encoding *IL3*, *IL4*, *IL5*, *IL13*, and *GMCSF* (5q31–5q35), whereas mouse *Il9* is on chromosome 13, not on chromosome

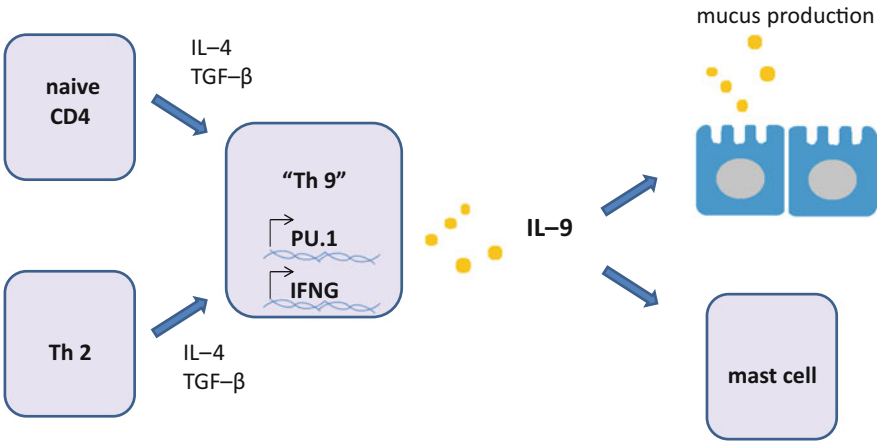

Fig. 5.6 Differentiation of Th9 cells. Combination of IL-4 and TGF-β induces Th9 cells from naive CD4 T cells or Th2 cells. It is claimed that differentiation of Th9 cells requires the transcription factor PU.1. IL-9 produced by Th9 cells activates mast cells and enhances the production of mucus

11 where *Il3*, *Il4*, *Il5*, *Il13*, and *Gmcsf* are clustered. This indicates the function of IL-9 in humans should not be extrapolated from the knowledge from mice.

5.5.6 Follicular Helper T (Tfh) Cells

(Summary) Tfh cells help B cells, supporting germinal center formation, affinity maturation, and generation of long-lived memory B cells.

Tfh cells reside in lymph nodes and help B cells [28]. Tfh cells produce IL-21, which promotes germinal center formation and generation of plasma cells. Surface markers for Tfh cells are PD-1, CXCR5, BTLA, PSGL-1, and ICOS. Tfh cells express BCL6, a transcription factor induced by IL-6 and IL-21. The role of Tfh cells in the skin is not known.

5.5.7 Regulatory T (Treg) Cells

(Summary) Gershon and Kondo showed that thymus-derived immune cells suppress antibody formation in an antigen-specific manner [51]. Through the pursuit of the suppressor T cells, several subsets were claimed to mediate suppression. Foxp3+CD4+ regulatory T (Treg) cells (Treg cells otherwise indicated) constitute the most characterized subset.

5.5.7.1 Foxp3+ Treg Cells

(Summary) Foxp3+ Treg cells maintain peripheral tolerance, preventing autoimmune diseases and limiting immune pathology, although they may deteriorate antitumor immunity and chronic infections [146]. The transcription factor Foxp3 is critical for Treg cell development in mice and humans [46, 60, 66]. Foxp3+ Treg cells contain thymus-derived (tTreg) and peripherally induced (pTreg) cells.

5.5.7.2 Characterization of Foxp3+ Treg Cells

Neonatally thymectomized (ntx) mice before day 3 of age spontaneously develop organ-specific autoimmune diseases later in life, such as gastritis, thyroiditis, and oophoritis. Alternatively, fractionated irradiation on the thymus induces similar autoimmune diseases in rats. These experiments indicated that thymus generates distinctive T cells that suppress immune responses. Through screenings of T cell subsets on the basis of surface markers, CD25+CD4+ T cells were shown to protect ntx mice from the autoimmune diseases [117]. Discovery of Foxp3, selectively expressed by the majority of CD25+CD4+ T cells, has enabled us to investigate the regulatory/suppressor T cells on a molecular basis.

Currently, Foxp3+CD4+ Treg cells are recognized as the major T cells with suppressive activity [114]. Expression of Foxp3 does not always correlate with suppression, particularly in humans [85]. Nonetheless, absence of Foxp3 results in a fatal autoimmunity in mice and humans, i.e., *Scurfy* phenotype and IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome, respectively [76]. IPEX syndrome is a fatal disease characterized by autoimmune enteropathy, psoriasiform dermatitis, eczematous dermatitis, cheilitis, nail dystrophy, and autoimmune endocrinopathies (early onset type I diabetes mellitus and thyroiditis) [57, 76]. The hypomorphic version of IPEX syndrome is often accompanied by serum hyper-IgE, eosinophilia, alopecia areata, chronic urticaria secondary to food allergies, and bullous pemphigoid, indicating engagement of Treg cells in these conditions [57].

Treg cells represent about 10 % of the peripheral CD4+ T cell subset. Most of them express CD25 (IL-2Rα), cytotoxic T lymphocyte-associated antigen 4 (CTLA4, a ligand for CD80/CD86), and glucocorticoid-induced TNF receptor (GITR). Although these markers indicate an activated state, Treg cells are anergic, nonproliferative to antigen stimulation, and hardly produce cytokines except anti-inflammatory IL-10 and TGF-β.

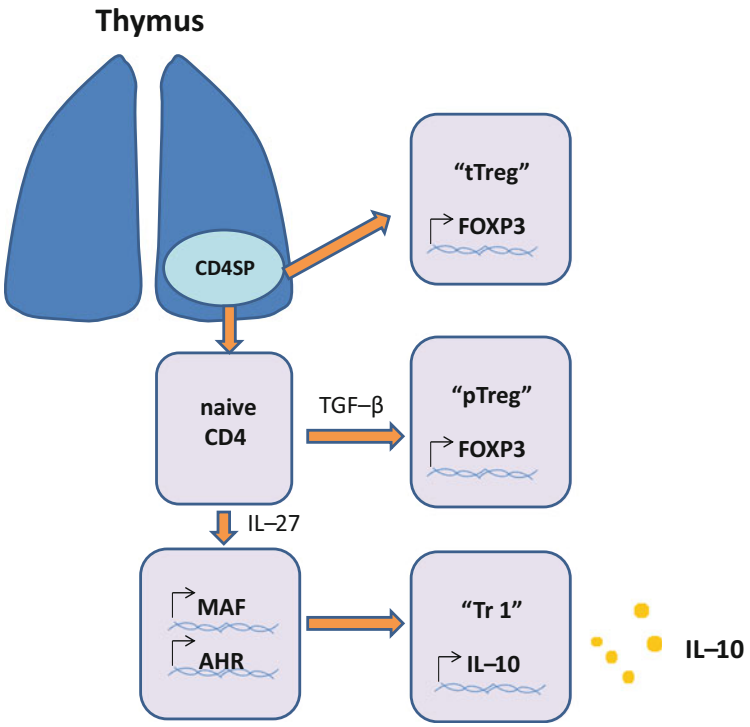

Fig. 5.7 Differentiation of Treg cells. Regulatory T (Treg) cells consist of Foxp3+ and Foxp3- Treg cells. Foxp3+ Tregs cells are further divided into thymically derived Treg (tTreg) cells and peripherally induced (pTreg) cells. Development of tTreg cells occurs in the thymus. Differentiation of pTreg cells occurs in the periphery and depends on TGF-β. Continuous exposure to antigen at a low dose seems to promote differentiation of pTreg cells. Tr1 cells are induced by antigen stimulation in the presence of IL-10, vitamin D3, type I interferon, IL-27, and ICOS in a manner dependent on c-Maf and AhR (aryl hydrocarbon receptor). Th3 cells, which are induced in oral tolerance models in animals, are suggested to be equivalent to pTreg cells. Differentiation of LAG3 Treg cells is obscure

5.5.7.3 Subsets of Treg Cells

Treg cells that arise in the thymus are called "thymus derived Treg (tTreg) cells." CD4+ T cells acquiring Foxp3 expression in the periphery are called "peripherally derived Treg (pTreg) cells" [1] (Fig. 5.7).

It is a fundamental question whether tTreg and pTreg cells are discernible. Helios, a member of the Ikaros family of transcription factors, is expressed at a high level in tTreg cells [142]. However, intravenously injected peptide can induce expression of Helios in pTreg cells [54]. Thus, Helios may be an activation marker rather than a differential for subsets [3]. An epigenetic approach identified a difference in degree of demethylation of the *Foxp3* locus between tTreg and pTreg cells: the *Foxp3* locus is fully demethylated in tTreg cells but not in pTreg.

It is speculated that unstable expression of *Foxp3* in pTreg cells associates with incomplete demethylation in *Foxp3* [45]. However, it is technically impossible to discriminate the methylation state in living cells. To date, no biomarkers are identified for tTreg and pTreg cells. However, it is critical to determine the contribution of these subsets in inflammatory diseases or tumor immunity before we develop Treg cell mediated immune therapy.

5.5.7.4 Differentiation of tTreg Cells

TCR signaling is required for Foxp3 expression and lineage commitment to tTreg cells in developing CD4+CD8+ double positive (DP) thymocytes [65]. Expression of CD25, CD5, and CTLA4 is the first indication of commitment to tTreg cells. CD5 attenuates TCR signal strength by recruiting tyrosine phosphatase SHP-1. TCR specificity is decisive for tTreg cell differentiation. Myelin basic protein-specific TCR transgenic mice on a Rag-deficient state succumb to fatal EAE because tTreg cells cannot develop with the transgenic TCR [72, 99]. Thymic selection of tTreg cells is instructed by TCRs recognizing self peptide–MHC ligands; and signals by IL-2, IL-7, and IL-15 are required for development [65]. TGF-β signaling is not required for commitment of tTreg cells, although it promotes survival of tTreg precursors [101].

5.5.7.5 Differentiation of pTreg Cells

TCR signaling and TGF-β induce expression of Foxp3 in CD4+ T cells in vitro and in vivo. Retinoic acid (RA) enhances the induction of Foxp3 in this context [90]. A high level of RA (1 nM) maintains the TGF-β-mediated pTreg cell-differentiation by abrogating effects of IL-6 and nitric oxide, which promotes differentiation of Th17 and Th1 cells, respectively, in vitro [77]. Retinoids are functional analogues of RA. They are used topically or systemically to treat acne vulgaris, photoaging, psoriasis, pityriasis rubra pilaris, cutaneous T cell lymphoma, and eczema. It remains obscure to what extent retinoids exert their effects through the modulation of pTreg cells.

IL-2 is another important factor for pTreg cell differentiation. It activates STAT5 to transactivate the *Foxp3* gene, which regulates differentiation of pTreg cells [165]. *Foxp3* forms a complex with RORγt and Runx1 and interferes with differentiation to Th17 cells [162, 167].

5.5.7.6 Treg Cells in the Skin

Human skin contains a resident population of Treg cells representing 5–10% of the total skin T cells [23, 24]. Treg cells are activated during skin inflammation and attenuate subsequent autoimmune reactions. Antigen-experienced Treg cells are

maintained in the same tissue after the resolution of inflammation, and are promptly activated on antigen re-exposures. Therefore Treg cells confer "regulatory memory" to the skin [113]. LCs can activate skin resident Treg cells in the absence of exogenous antigen in vitro. However, LCs can activate Tem cells to produce IFN-γ and IL-17 in the presence of pathogenic antigens such as *Candida albicans* [124]. These mechanisms may operate immune homeostasis in the skin.

5.5.7.7 Mechanism of Suppression

Treg cells have multiple modes of suppression that can be divided in three classes [118]: (a) humoral factor-mediated suppression, (b) cell-contact–dependent suppression, and (c) functional modification of APCs. Treg cells produce immunosuppressive cytokines such as TGF-β, IL-10, and IL-35 (heterodimer of EBI3 and p35 subunit of IL-12). Absorption of IL-2 (one of the T-cell growth factors) by CD25 (IL-2 receptor α) induces apoptosis of T cells nearby. Treg cells can induce apoptosis of neighboring T cells and APCs through cell-to-cell contact by granzyme- or perforin-dependent manner. CD39 and CD73 expressed by Treg cells generate peri-cellular adenosine, which is a strong immune suppressor. Moreover, Treg cells transmit cyclic AMP (cAMP) into the target T cell through a gap junction, by which proliferation and IL-2 production of the target are inhibited. Treg cells constitutively express CTLA4, which downmodulates CD80/CD86 on APC and induces indoleamine 2,3-dioxygenase (IDO), which catabolizes essential amino acids tryptophan to kynurenines that are toxic to T cells. LAG3 expressed by a subset of Treg cells mimics CD4 and binds to MHC-II, thereby attenuating the efficiency of antigen presentation by APCs.

Mechanism of suppression is more complicated in vivo. Treg cells coordinate their phenotype to match the type of immune response to be suppressed by sharing transcriptional regulation. A subtype of Treg cells suppresses Th1 cells, Th2 cells, or Th17 cells in a way dependent on T-bet, IRF4, or Stat3, respectively [15]. Furthermore, proper distribution of Treg cells in nonlymphoid tissues is important. For example, CCR4 expression is necessary for Treg cells to distribute in the skin to maintain the immune tolerance there [119].

Stability of a suppressive phenotype of Treg cells is another factor that affects the peripheral tolerance. Semaphorin-4a expressed on APCs ligates neuropilin-1 (Nrp1) on Treg cells to increase the nuclear fraction of Foxo3a resulting in stability of Treg cells [33].

5.5.7.8 Evidence for Presence of Treg Cells That Do Not Express Foxp3

(Summary) Tr1, Th3, and CD4+CD25-LAG3+ Treg cells are known to possess a suppressive function. These T cells do not express Foxp3.

Maintenance of T cell-tolerance depends on both central (thymic) and peripheral (nonthymic) tolerance. Absence of Foxp3+ Treg cells results in severe

inflammation of multiple organs (such as lungs, liver, and skin). However, an additional defect in the central tolerance by the loss of *Aire* (autoimmune regulator gene) does not extend the affected sites despite exacerbation [19]. This indicates a tolerance system that depends neither on central tolerance nor Foxp3+ Treg cell-mediated peripheral tolerance. Tr1, Th3, and CD4+CD25-LAG3+ Treg cells may contribute to such a tolerance system.

5.5.7.9 Tr1, Th3, and LAG3 Treg Cells

Tr1 or Th3 cells are CD4+ T cells that produce IL-10 or TGF-β, respectively [152]. These subsets do not necessarily express Foxp3.

Tr1 cells are induced by antigen stimulation in the presence of IL-10 and vitamin D3 in vitro. Type I interferon, IL-27, and ICOS are reported to induce IL-10 producing T cells in a transcription factor c-Maf–dependent manner [104] (Fig. 5.7). IL-27 induces AhR, which activates c-Maf [5, 105]. Nasal administration of anti-CD3 antibody induces such AhR-dependent Tr1 cells in mice [158]. The ligand for AhR includes halogenated aromatic hydrocarbons such as dioxin that are famous as environmental pollutants with pleiotropic effects such as teratogenesis and immune suppression.

Th3 are induced in animal models for oral tolerance. Th3 may be a subset of pTreg cells.

LAG3 Treg cells are CD4+CD25-LAG3+ T cells that express IL-10, but neither IL-2, nor IL-4, nor Foxp3 [97]. The suppressive function of LAG3 Treg cells is mediated by IL-10. LAG3 (lymphocyte activation gene-3) structurally resembles CD4, and binds to MHC-II with higher affinity than that of CD4, thereby attenuating activation of CD4+ T cells [20]. The cytoplasmic domain of LAG3 inhibits homeostatic expansion of T cells [157]. In humans and mice, expression of both LAG3 and CD49b is a marker for Tr1 cells [49]. Therefore, Tr1 cells—a subset originally induced in a test tube—may naturally exist in mice and humans as LAG3 Treg cells.

5.6 CD8 T Cells

(Summary) CD8 T cells are the principal effector cells that recognize antigens on MHC-I. CD8 T cells can produce inflammatory cytokines such as IL-17 and IL-22, and play a central role in contact dermatitis, drug eruption, graft versus host disease, and tumor immunity. Epidermal CD8 T cells constitute resident memory T (Trm) cells.

5.6.1 Characterization of CD8 T Cells in the Skin

CD8 T cells are called killer or cytotoxic T lymphocytes (CTLs). They recognize antigens expressed on MHC-I on the infected cells. CD8 is a cell surface molecule that binds a conserved region of the MHC-I molecule. Engagement of the TCR activates the cytolytic machinery of CD8 T cells. The cytolytic factors include perforin, granulysin, and Fas ligand, which induce apoptosis of target cells. Skin-homing CD8 T cells express P-selectin ligands, E-selectin ligands, PSGL-1 (P-selectin glycoprotein ligand-1, CD162, a ligand for P-, E-, and L-selectin), CLA, CD43 (a ligand for E-selectin), and CD44 (a memory marker, a receptor for hyaluronic acid) [7, 11, 47, 82]. CD8 T cells occupy a central role in skin immune responses such as contact dermatitis, drug eruptions, graft versus host disease-mediated skin inflammations, and tumor immunity against skin tumors such as melanoma and squamous cell carcinoma.

5.6.2 Tissue-Resident Memory T Cells in the Skin

In steady state, many, if not all, of the skin CD8 T cells are tissue-resident memory T (Trm) cells, which reside in the epidermis [59, 91, 127]. CD8 Trm cells are not only poised themselves to resume attack but are also capable of maximizing their protective function by summoning other memory T cells in the circulation to the site of virus reactivation or re-entry [122]. Parabiosis experiments and transfer experiments revealed that memory CD8 T cells do not enter the circulation. Disconnection of Trm cells from the circulating pool is also evident from the fact that transferred male T cells that migrate in the skin of a female recipient survive the immunological attack by the female immune system [50]. Development of skin Trm cells is not fully understood. The skin CD103+CD8+ Trm cells, which develop in the epidermis under the influence of IL-15 and TGF-β, show a unique transcriptional profile shared with that of Trm cells in other tissues, providing a piece of circumstantial evidence that Trm cells are distinct subsets different from Tcm or Tem cells. Moreover, the precursor of CD103+CD8+ Trm cells does not express KLRG1 and is suggested to be other than Tem cells that express KLRG1 [81]. In human herpes simplex virus 2 (HSV-2) reactivations, effector CD8αα T cells of Trm phenotype are shown to persist in the dermal–epidermal junction [169]. It is not clear whether expression of CD8αα isoforms is related to continuous activation of HSV-2 infections. To understand the mechanism of how skin Trm cells are generated will contribute to efficient vaccine designing.

5.6.3 CD8 T Cells and Skin Diseases

Effector CD8 T cells are involved in various skin diseases. IL-17 or IL-22 producing CD8 T (Tc17 or Tc22) cells play roles in psoriasis. In addition to the eradication of virally infected cells, CD8 T cells induce apoptosis of tumor cells, allogeneic graft cells, or keratinocytes in the lesion of drug eruption. Epidermal CD8 Trm cells, in particular, contribute to the pathogenesis of fixed drug eruption [129].

5.7 Innate-Like T Cells

(Summary) Innate-like T cells express semi-invariant or germline encoded TCRs specific for conserved microbial and self-antigens. They link innate and adaptive immune responses. Well-characterized innate-like T cells in the skin are natural killer T (NKT) cells, which clear pathogens such as herpes simplex virus (HSV), or induce immune suppression in an UV-dependent manner. Controversial functions of NKT cells may reflect heterogeneity of this subset.

Innate-like lymphocytes belong to a large family of lymphocytes that include B1 B cells, marginal zone B cells, intraepithelial γδT cell subsets, CD1d-restricted natural killer T (NKT) cells, the CD8αα αβT cells of the intestine (in mice), and MR1-restricted mucosal associated invariant T cells [10]. They release a wide range of cytokines and chemokines, and affect adaptive immunity through the maturation of dendritic cells. The function of innate-like T cells is to bridge the gap between the innate and adaptive immune systems.

NKT cells are one of the well-characterized innate-like T cells in the skin. They develop in the thymus, and express invariant TCRs and NK1.1, sharing characteristics of both T cells and natural killer cells. NKT lineage is defined by the expression of *Zbtn16*, which encodes promyelocytic leukemia zinc factor (PMLZ). NKT cells produce various cytokines such as IFN-γ, IL-4, and IL-10.

Invariant NKT (iNKT) cells are the most characterized subset of NKT cells, which is distinguished by the restricted αβTCR repertoire, Vα14/Vβ8 in mice and Vα24/Vβ11 in humans. iNKT cells recognize glycolipids presented on the nonclassical MHC molecule CD1d. The importance of iNKT cells in the clearance of herpes simplex virus (HSV) was studied using CD1 gene knockout (GKO) and Jα281 GKO mice, which lack iNKT cells. iNKT cells can be visualized by CD1d tetramers loaded with α-galactosylceramide (α-GalCer) regardless of the expression of NK1.1. RORγt+ CCR6+ CD103+ CD121a (IL-1R type I)+ NK1.1– cells reside in the skin and peripheral lymph nodes and produce IL-17 [35].

NKT cells are immune suppressive as well. UV was shown to modify immunological potential by generating suppressor cells in mice and promoting photocarcinogenesis. According to a study, such induced suppressor cells belong to NKT cells [86]. These controversial functions of NKT cells may reflect the multifariousness of this subset. How their function is regulated is not known.

5.8 Manipulation of T Cells to Treat Skin Inflammation and Cancer

T cells are the major focus of efforts to treat a variety of diseases because of their capacity to: selectively recognize peptide- and lipid-antigens by conventional and innate-like T cells, respectively; order proper immune responses by CD4 helper T cells and Treg cells; and directly recognize and kill target cells mainly by CD8 effector T cells.

There is an increasing interest in manipulating Treg cells to preserve and restore tolerance to self-antigens, alloantigens, or foreign antigens [112]. Adaptive transfer of Treg cells is proved to prevent not only autoimmune diseases but also skin graft rejection in animal models. Furthermore, immunosuppression by transferred Treg cells is more robust than in vivo-transfer of immunosuppressive cytokines as observed in an experiment where administration of antigen-specific Tr-1—but not exogenous IL-10—blocked allograft rejection of the islet [8]. However, there are several problems to solve before commencing Treg cell therapy. First, the effect of transferred cells must be sustained lifelong; otherwise there is a high risk of recurrence of the disease. Second, collecting an adequate number of Treg cells is difficult. Finally, safety of the infused Treg cells is not validated. For example, if infused Treg cells were converted to a pathogenic phenotype by losing their suppressive function, the result would be disastrous. Furthermore, it is not established to monitor, prevent, and revise uncontrolled cell proliferation, pan-immunosuppression, and consequent tumor development, all of which could occur by the infused cells.

On the other hand, it has been pursued to treat cancer by harnessing the immune response. Abrogation of inhibitory pathways of the immune system is becoming a promising approach to activating antitumor immunity [103, 111]. Clinical studies showed that blockade of CTLA4 or the programmed cell death 1 (PD-1) receptor resulted in regression of melanoma [110]. CTLA4 is expressed after T cell activation and initiates negative signaling on T cells by competing with CD28 for binding to CD80/CD86 on DCs. The inhibition by the CD28-CTLA4 system is suggested to occur in the priming phase of T cell response in lymph nodes. Additionally, constitutive expression of CTLA4 on Treg cells also seems to be engaged in Treg cell-mediated suppression [155]. PD-1 is expressed on T cells during long-term antigen exposure and results in negative regulation on T cells during ligation with PD-1 ligand (PD-L) 1 and PD-L2, which are expressed within inflamed tissues and the tumor microenvironment [98]. The role of PD-1 is not at the initial stage of T cell activation. And the natural role of the PD-1 axis is suggested to limit collateral tissue damage in response to microorganisms. Blocking of the CD28-CTLA4 system by anti-CTLA4 antibody (ipilimumab or tremelimumab) or PD-1 axis by anti-PD-1 (lambrolizumab or nivolumab) or anti-PD-L1 (BMS936559) provided evidence of improvement in survival for the treatment of patients with metastatic melanoma [58, 110].

5.9 Conclusion

T cells underlie the intricate regulation of adaptive immunity. Identification of cell surface markers, cytokines, and transcription factors has enabled us to discern T cell subsets and has advanced our understanding of the immune function. However, recent studies indicate that flexibility or plasticity in expression of master regulators and cytokines is common among the subsets of CD4 T cells [92, 96, 168]. This issue is also important for a pragmatic reason. Autoimmune diseases and allergic inflammatory disorders are associated with a variety of helper T cell subsets. If T cell subsets are flexible, pathologically skewed Th cells can be instructed to normal or benign subsets. Conversely, administration of Treg cells can be devastating if these cells were converted to Th17 cells under an inflammatory environment. Thus, investigation into the mechanism of differentiation and plasticity should be useful for prevention and treatment of various human diseases.

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Chapter 6
γδ T Cells

Kazuhiro Kawai

Abstract γδ T cells represent a minor population of T cells in the peripheral blood and lymphoid tissues in both mice and humans, but are abundant in certain epithelial tissues including the skin, especially in mice. In contrast to conventional αβ T cells with the diverse T-cell receptor (TCR) repertoire for adaptive immunity, epithelial γδ T cells express tissue-specific invariant or restricted TCRs and have innate-like properties. Dendritic epidermal T cells (DETCs) that reside in the murine epidermis and express an invariant γδ TCR are prototypic epithelial γδ T cells. DETCs mediate stress surveillance in the epidermis, and play key roles in immunoregulation, wound healing, epidermal homeostasis, hair cycle regulation, and tumor surveillance. Recently, a novel subset of γδ T cells residing in the murine dermis has been identified. Resident murine dermal γδ T cells preferentially produce IL-17, and are essential for defense against cutaneous infection and also for development of psoriasiform dermatitis. γδ T cells are infrequent in the human skin, but Vδ1 TCR-expressing T cells with functional similarities to murine epithelial γδ T cells are present in the normal human skin (mainly in the dermis). Vγ9Vδ2 T cells that are the major circulating γδ T cells in the human peripheral blood can migrate to the inflamed skin, and contribute to immunity against pathogens and tumors, and development of inflammatory skin diseases.

Keywords Dendritic epidermal T cell • Dermis • DETC • Epidermis • γδ T cell • IEL • Intraepithelial lymphocyte • Psoriasis • Skin • Wound healing

6.1 Introduction

Whereas the great majority of murine and human T cells in the peripheral blood and lymphoid tissues express αβ T-cell receptors (TCRs), only a small population of T cells (2–5%) expresses γδ TCRs in these locations. γδ T cells are enriched in the epithelial tissues, but there are significant differences in their number and

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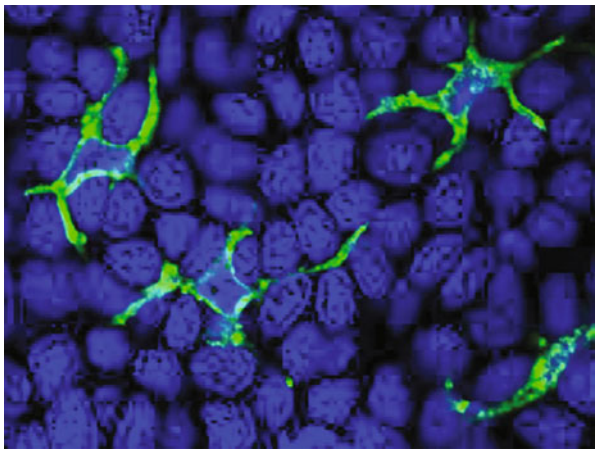

Fig. 6.1 Dendritic epidermal T cells (DETCs). Epidermal sheet from a normal adult mouse stained with an anti-γδ TCR monoclonal antibody (*green*, DETCs) and DAPI (*blue*, mainly keratinocytes)

composition between tissues and between species. Murine epidermis contains large numbers of γδ T cells (2–4% of epidermal cells), known as dendritic epidermal T cells (DETCs) [1, 2] (Fig. 6.1). γδ T cells constitute nearly all resident T cells (>99%) in the murine epidermis. T cells are less frequent in the murine dermis (0.5–1% of dermal cells), but 50% of the resident murine dermal T cells are γδ T cells [3, 4]. Human epidermis harbors no equivalent of murine DETCs, and only a few T cells are found in the normal human epidermis (0.5–1 % of epidermal cells) [5]. These epidermal T cells contain γδ T cells (<1–30%) [6–8], but most of the human skin-resident γδ T cells (>90%) are present in the dermis [6]. γδ T cells constitute 5–16% of T cells in the normal human dermis [6, 8, 9].

Unlike conventional αβ T cells that express diverse TCRs and play a central role in adaptive immunity, tissue-resident γδ T cells express tissue-specific invariant or restricted TCRs and mount rapid, innate-like responses to tissue stress [10, 11]. Nevertheless, γδ T cells are not a functionally homogeneous population. Instead, there exist heterogeneous γδ T-cell subsets exerting a diverse array of functions in each tissue.

6.2 Murine γδ T Cells

6.2.1 Murine γδ T-Cell Subsets and Their Development

Several subsets of murine γδ T cells have been identified (Table 6.1). Murine γδ T-cell subsets have been classified based on the TCR Vγ (variable region of γ chain) and/or Vδ gene usage [11, 13], because TCR-defined γδ T-cell subsets differentially localize in specific tissues. These γδ T-cell subsets also exert distinct functions, although functional γδ T-cell subsets do not exactly overlap with the TCR usage [14, 15].

Table 6.1 γδ T-cell subsets

Mouse	**TCR usage** (Nomenclature According to Garman et al. [12])	**Characteristics**
DETCs	Vγ3Vδ1	Development: early fetal thymus, Skint1-dependent, IL-15-dependent
		Localization: epidermis
		Phenotype: CD27− CD44+ CD62L− CD122+ SCART2−
		Functions: IFN-γ, IL-13, FGF7, FGF10, IGF1, cytotoxic, immunoregulatory
IL-17–producing γδ T cells	Vγ4Vδ1	Development: early fetal thymus (next to Vγ3Vδ1 T cells)
		Localization: vagina, uterus, tongue, peritoneal cavity
		Phenotype: CD27−CD44+ CD62L−CD122−CCR6+ SCART2+ RORγt+
		Functions: IL-17A
	Vγ2-biased	Development: late fetal/perinatal thymus, TGF-β–dependent, IL-7–dependent
		Localization: lymphoid tissues, dermis
		Phenotype: CD27−CD44+ CD62L−CD122−CCR6+ SCART2+ RORγt+
		Functions: IL-17A
NKT-like γδ T cells	Vγ1.1Vδ6.3/6.4	Development: late fetal thymus
		Localization: liver, spleen
		Phenotype: CD27+ CD44+ CD62L−CD122+ PLZF+
		Functions: both IFN-γ and IL-4
T10/T22-specific γδ T cells on the T10/T22-expressing background	Diverse (W. . .EGYEL motif in CDR3δ)	Development: mainly adult thymus
		Localization: spleen
		Phenotype: CD27+ CD44+ CD62L−CD122+ T-bet+
		Functions: IFN-γ
Naive γδ T cells	Mainly Vγ1.1 and Vγ2	Development: mainly adult thymus
		Localization: lymphoid tissues
		Phenotype: CD27+ CD44−CD62L+ CD122−
		Functions: IFN-γ

(continued)

Table 6.1 (continued)

Mouse	**TCR usage** (Nomenclature According to Garman et al. [12])	**Characteristics**
Intestinal γδ IELs	Mainly Vγ1.1 and Vγ5	Development: neonatal thymus (first 3 weeks after birth), cryptopatches
		Localization: intestinal epithelium
		Phenotype: CD27+ $CD122^{lo}$ CCR9+
		Functions: IFN-γ, FGF7, cytotoxic, immunoregulatory
Human	**Characteristics**	
Vδ1 T cells	Localization: skin (mainly dermis), intestinal epithelium, liver, lymphoid tissues	
	Antigen specificity: MICA/MICB, CD1c/CD1d-lipid	
	Functions: IFN-γ, IGF1, cytotoxic	
Vγ9Vδ2 (Vγ2Vδ2) T cells	Localization: peripheral blood	
	Antigen specificity: phosphoantigens	
	Functions: IFN-γ, IL-17A (CD161+ and CLA+ subsets), CTGF, FGF9, cytotoxic, APC function	

Table 6.2 Nomenclature for murine Vγ genes

Garman et al. [12]	Heilig and Tonegawa [16]
Vγ1.1	Vγ1
Vγ1.2	Vγ2
Vγ1.3	Vγ3
Vγ2	Vγ4
Vγ3	Vγ5
Vγ4	Vγ6
Vγ5	Vγ7

Murine γδ T-cell subsets expressing different TCRs develop in the thymus at different stages of ontogeny. T cells expressing an invariant (canonical) Vγ3Vδ1 TCR (nomenclature according to Garman et al. [12]; Table 6.2) are the first T cells to develop in the fetal thymus around day 14 of gestation [13]. Both Vγ3 and Vδ1 chains lack junctional diversity [13] due to the absence of terminal deoxynucleotidyl transferase (TdT) in fetal thymocytes. These Vγ3Vδ1 T cells migrate to the epidermis and become DETCs [17, 18]. Vγ3Vδ1 T cells are generated only in the early fetal thymus, and found only in the epidermis in adult mice [19, 20]. T cells expressing an invariant Vγ4Vδ1 TCR are the next to develop in the fetal thymus, and migrate to the vagina, uterus, tongue, and peritoneal cavity [21, 22]. Vγ2 T cells, which have diverse junctional sequences, develop in the thymus after these cells and reside mainly in the peripheral lymphoid tissues, but a subset also migrates to the dermis [3, 4, 23]. In the adult thymus, Vγ1.1 and Vγ2 T

cells are predominantly generated, and emigrate mainly to the peripheral lymphoid tissues. This sequential development of murine γδ T-cell subsets is programmed at the level of Vγ gene rearrangement. Ordered Vγ gene rearrangement is determined by the location of Vγ genes in the TCRγ locus and by the altered accessibility of Vγ genes in the adult thymus [24].

Unlike conventional αβ T cells that differentiate into distinct effector/regulatory subsets during activation in the periphery, functions of murine tissue-resident γδ T cells are programmed during thymic development [11, 15, 25–27]. Maturation of Vγ3Vδ1 DETC precursors in the fetal thymus requires "positive selection," which depends on TCR engagement with its ligand/agonist and interaction with the Skint1 molecule expressed on thymic epithelial cells [28–34]. Mature DETC precursors selected by the TCR ligand express skin-homing receptors and CD122 (IL-15 receptor β chain) [29, 34–38], which are crucial for their migration to and survival/expansion in the skin, respectively [34, 39–41]. Ligand-selected mature Vγ3Vδ1 thymocytes are CD27+ CD44+ CD62L−CD122+ and produce IFN-γ, although DETCs become CD27− [4, 42].

The CD27+ CD44+ CD62L− CD122+ phenotype is shared by natural killer T (NKT)-like γδ T cells that express restricted Vγ1Vδ6.3/6.4 TCRs and secrete both IFN-γ and IL-4 [43–45] and by T10/T22-specific γδ T cells that develop on the T10/T22-expressing background and produce IFN-γ [46]. Similar to DETCs, precursors of these γδ T-cell subsets receive strong TCR signals by recognizing the TCR ligands and are positively selected during thymic development [46–48].

IL-17-producing γδ T-cell subsets share a CD27−CD44+ CD62L−CD122−CCR6+ SCART2+ RORγt+ phenotype. IL-17-producing γδ T-cell subsets include Vγ4Vδ1 T cells residing in the vagina, uterus, tongue, and peritoneal cavity [22] and Vγ2-biased CD27−γδ T cells in the peripheral lymphoid tissues [49, 50] and in the dermis [3, 4, 23, 51]. Unlike ligand-selected IFN-γ-producing γδ T-cell subsets, precursors of IL-17–producing γδ T-cell subsets receive weak TCR signals by recognizing the TCR ligands or independently of the ligand recognition during thymic development [34, 46, 49, 52]. IL-17–producing γδ T cells expand in response to IL-7 [3, 53].

γδ T cells with a "naive" CD27+ CD44−CD62L+ CD122−phenotype residing in the peripheral lymphoid tissues can secrete IFN-γ upon activation [49]. They express diverse TCRs (mainly Vγ1.1 and Vγ2) [49]. Precursors of these γδ T cells may develop in the absence of TCR engagement and emigrate from the thymus as naive γδ T cells [49, 54, 55].

Intraepithelial lymphocytes (IELs) of the intestine contain γδ T cells expressing mainly Vγ1.1 and Vγ5 TCRs. The origin and development of intestinal γδ IELs are controversial [56]. Thymus exports γδ IEL precursors during the first 3 weeks after birth [57, 58], which may colonize cryptopatches in the intestinal lamina propria and develop into mature IELs extrathymically [59, 60]. Intestinal γδ IELs are CD27 + $CD122^{lo}$ CCR9+ [55], IFN-γ–producing, and cytotoxic T cells [56], but also have an immunoregulatory (suppressive) function and produce fibroblast growth factor 7 (FGF7), also called keratinocyte growth factor 1 (KGF1), to maintain tissue integrity [61].

6.2.2 DETCs

DETCs located in the basal epidermis have a dendritic morphology, and are in contact with neighboring keratinocytes and overlaying Langerhans cells through dendrites. DETCs uniformly express an invariant (canonical) Vγ3Vδ1 TCR [13] and recognize an as-yet-undetermined self ligand induced on stressed, damaged, or transformed keratinocytes through the TCR [62, 63]. In situ immunofluorescence staining with the soluble DETC TCR demonstrated that the DETC TCR ligand is undetectable in the normal epidermis, but upregulated on periwound keratinocytes rapidly and transiently after wounding [64, 65]. There is evidence suggesting that low levels of the DETC TCR ligand, which might not be detectable by immunofluorescence staining, are expressed in the normal epidermis [66–71]. A recent study showed that TCRs on DETCs are clustered and triggered at steady state in immunological synapse-like structures on the apical dendrites located near the keratinocyte tight junctions [72]. In response to tissue stress, these TCR-triggered proximal signals are relocated from the apical dendrites to the newly formed synapses [72].

DETCs also express several non-TCR stress receptors such as junctional adhesion molecule-like protein (JAML) [73], CD100 [74], and NKG2D [75, 76]. In addition to these coactivating/costimulatory receptors, DETCs constitutively express coinhibitory receptors including Ly49E, CD94-NKG2A [77], and E-cadherin [78]. Therefore, DETC activation is regulated by the balance between positive and negative signals provided through various coactivating, costimulatory, and coinhibitory receptors.

Upon activation, DETCs produce a variety of proinflammatory cytokines and chemokines, and may promote cutaneous inflammation [2, 79–81]. DETCs have been regarded as an IFN-γ-producing γδ T-cell subset, but can secrete IL-13 and trigger T helper 2 (Th2)-type responses [82]. DETCs also have an immunoregulatory function to downregulate cutaneous inflammation [83, 84].

Activated DETCs secrete FGF7/KGF1, FGF10/KGF2, and insulin-like growth factor 1 (IGF1), and promote wound healing [85, 86]. DETCs recognize wounded keratinocytes through the TCR [64, 70], JAML [73], CD100 [74], and NKG2D [87, 88]. DETCs constitutively produce low levels of IGF1 in the normal, unperturbed epidermis, which may mediate maintenance of epidermal homeostasis by preventing keratinocyte apoptosis [86]. DETC-deficient mice have defects in the epidermal barrier function and epidermal structure [89, 90]. FGF7, FGF10, and IGF1, which are secreted by DETCs, are also known as the major hair growth regulators. DETC-deficient mice have defects in depilation-induced hair cycling (delay in anagen completion and acceleration of subsequent hair cycling) [91].

DETCs are potent cytotoxic T cells against cutaneous tumor cells [92], and DETC-deficient mice are susceptible to cutaneous carcinogenesis [75, 93]. DETCs can recognize tumor cells through the TCR, NKG2D, or both [65, 75, 94, 95].

6.2.3 *Murine Dermal γδ T Cells*

Dermal γδ T cells are a newly discovered murine γδ T-cell subset [3, 4, 23, 51]. They are round or amoeboid in shape [3, 51], reside mainly in the superficial dermis, but are mobile [3, 51] and migrate to the lymph nodes at low rates [96]. Dermal γδ T cells express intermediate intensity of TCRs [3, 4, 51]. About 30–50% of dermal γδ T cells express Vγ2 TCRs [3, 4, 97]. Dermal γδ T cells express CCR6, SCART2, RORγt, and IL-23R [4, 23, 51] and secrete IL-17A upon IL-23 plus IL-1β stimulation or during cutaneous infection [3, 4, 51]. As do other IL-17–producing γδ T cells [14], dermal γδ T cells arise from the precursors that develop in the perinatal thymus [51]. Dermal γδ T cells express CD127 (IL-7 receptor α chain) [3, 51], and their development/maintenance depends on IL-7 but not IL-15 [3].

IL-17A secreted by dermal γδ T cells plays an important role in antipathogen responses during intradermal BCG infection [3]. IL-17–producing dermal γδ T cells may also mediate defense against cutaneous infection with *Staphylococcus aureus* [98], which has been attributed to DETCs [99, 100]. A recent study showed, however, that a subset of DETCs produces IL-17A upon TCR stimulation, which induces epidermal antimicrobial peptides and promotes wound healing [42].

Dermal γδ T cells can also produce IL-22 [4, 51] and are involved in the development of psoriasiform dermatitis in several different murine models [4, 96, 101–103].

Dermal γδ T cells induce hair follicle regeneration after wounding by producing FGF9, but the primary source of FGF9 was shown to be Vγ4Vδ1 T cells rather than Vγ2 T cells [97].

6.3 Human γδ T Cells

6.3.1 *Human γδ T-Cell Subsets*

Human γδ T cells are divided into two major subsets based on the TCR usage [26, 104] (Table 6.1). Vδ1 T cells are the predominant subset in the epithelial tissues. By contrast, most of the peripheral blood γδ T cells are Vγ9Vδ2 (also called Vγ2Vδ2) T cells. Although both subsets are implicated in anti-infection and antitumor immunity, they have distinct migratory capabilities and exert distinct functions according to the type of pathogens or tumors [105].

6.3.2 Human Vδ1 T Cells

Vδ1 T cells are the major human γδ T-cell subset preferentially residing in the epithelial tissues including the skin and intestine [8, 9, 106]. Human skin-resident Vδ1 T cells are present mainly in the dermis but also found in the epidermis [8]. They express skin-homing receptors CCR8 and cutaneous lymphocyte antigen (CLA) [8, 9].

The ligands for Vδ1 TCRs are largely unknown, but Vδ1 T cells were shown to recognize stress-induced self molecules such as MHC class I chain-related proteins MICA and MICB, which can also bind to NKG2D in addition to the TCR [107–109], and lipids presented by CD1c or CD1d [110–113].

Vδ1 T cells are IFN-γ-producing, cytotoxic T cells, and thought to mediate epithelial tumor surveillance [9, 114]. Indeed, Vδ1 T cells are frequent in the lymphocytes infiltrating solid tumors. Vδ1 T cells also accumulate in the skin lesions of leprosy patients [115] and expand in the peripheral blood during cytomegalovirus (CMV) infection [116, 117].

Similar to murine DETCs, human skin-resident Vδ1 T cells can produce IGF1 upon activation and promote wound healing [8]. Vδ1 T cells may also participate in pathological fibrosis, because they increase in the skin lesions and peripheral blood of systemic sclerosis patients [118].

6.3.3 Human Vγ9Vδ2 T Cells

The majority of γδ T cells in the adult human peripheral blood express Vγ9Vδ2 TCRs. Circulating Vγ9Vδ2 T cells dramatically expand during certain infections, such as tuberculosis [119]. An equivalent subset of human Vγ9Vδ2 cells is absent in mice.

Vγ9Vδ2 T cells recognize low-molecular–weight nonpeptide phosphorylated antigens, called phosphoantigens [104, 119]. Phosphoantigens include (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP), which is an intermediate metabolite in the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway of microbial isoprenoid biosynthesis, and isopentenyl pyrophosphate (IPP), which is an intermediate metabolite in the self mevalonate pathway, but IPP is only active at high concentrations. Infection with most bacteria and protozoa using the MEP pathway (staphylococci and streptococci do not use the MEP pathway) induces rapid expansion of Vγ9Vδ2 T cells through recognition of HMBPP. Vγ9Vδ2 T cells can also recognize high levels of IPP accumulated in stressed cells or tumor cells. Vγ9Vδ2 T cells are also indirectly activated by aminobisphosphonates and alkylamines that inhibit farnesyl pyrophosphate synthase, leading to accumulation of IPP. Therefore, Vγ9Vδ2 T cells can contribute to immunity against both pathogens (by recognizing microbial phosphoantigens) and tumors (by recognizing overproduced endogenous phosphoantigens). The mechanism by which

phosphoantigens activate Vγ9Vδ2 T cells remains unclear, but butyrophilin 3A1 has been identified as a candidate antigen presenting molecule for phosphoantigen-reactive Vγ9Vδ2 T cells [120, 121].

Like conventional αβ T cells, Vγ9Vδ2 T cells can be subdivided into four subsets based on the expression of CD27 and CD45RA: CD27+ CD45RA+ naive, CD27+ CD45RA−central memory (T_{CM}), CD27−CD45RA−effector memory (T_{EM}), and terminally differentiated cytotoxic CD27−CD45RA+ effector memory (T_{EMRA}) cells [122, 123]. Vγ9Vδ2 T cells expand significantly during the perinatal period [124] and most of the Vγ9Vδ2 T cells acquire a memory phenotype by 1 year of life [123]. In contrast, about 30–40% of Vδ1 T cells remain naive in young adults [123].

Vγ9Vδ2 T cells secrete IFN-γ upon TCR stimulation, but can be polarized to Th1-, Th2-, Th17-, T_{FH}-, or Treg-like cells [125, 126]. Vγ9Vδ2 T cells can produce connective tissue growth factor (CTGF) [127] and FGF9 [128], and may be involved in wound healing and pathological fibrosis.

IL-17-producing Vγ9Vδ2 T cells can be induced or expanded in vitro, especially from neonates [53, 129–131]. IL-17-producing Vγ9Vδ2 T cells are CD161(NK1.1) + CCR6+ and show the CD27−CD45RA+ T_{EMRA} phenotype [53, 131, 132]. They expand during bacterial infection [131]. A subset of circulating Vγ9Vδ2 T cells that express CLA and CCR6 can be rapidly recruited into perturbed skin [133]. These skin-homing Vγ9Vδ2 T cells produce proinflammatory cytokines including IL-17A, chemokines, and IGF1, and are implicated in the development of psoriasis [133]. Indeed, increased numbers of γδ T cells, which produce IL-17A upon IL-23 stimulation, are present in the skin lesions of psoriasis patients [4, 134].

Vγ9Vδ2 T cells are potent cytotoxic T cells and recognize infected cells or tumor cells through the TCR and/or NKG2D [135–138]. CD56 expression correlates with cytotoxicity in Vγ9Vδ2 T cells [139, 140]. Vγ9Vδ2 T cells have been tested for cellular immunotherapies in clinical trials for various cancers [141].

Upon activation, Vγ9Vδ2 T cells also act as professional antigen-presenting cells (APCs) that express MHC class II and costimulatory molecules and the immunoproteasome [142, 143]. Activated murine γδ T cells were also shown to function as APCs [144].

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Chapter 7
B Cells

Manabu Fujimoto

Abstract The B cell is the central player of the humoral immune response by its capability to differentiate into an antibody-secreting plasma cell. Thus, B cells provide an essential component to defend the host from a variety of infections, and autoantibodies play significant roles in autoimmune diseases. In addition to antibody production, B cells are capable of influencing immune responses through several other mechanisms such as presenting antigens and providing costimulatory signals to T cells, regulating lymphoid tissue neogenesis and structure, and secreting cytokines. B cells are considered to be a promising therapeutic target in various diseases. This chapter describes B cell development, subset, and activation, as well as the role in the diseases and therapeutic manipulation.

Keywords B cell • Immunoglobulin • Cytokine • Regulatory B cell • Autoimmune disease • Therapy

7.1 Introduction

B cells can be defined as a population of cells that express clonally diverse cell surface immunoglobulin (Ig) receptors recognizing specific antigenic epitopes. They differentiate into plasmablasts and plasma cells as well as memory B cells to produce antibodies. This role of B-lineage cells as a central player of humoral immunity has been well appreciated. Antibodies are essential for optimal host defense, and pathogenic autoantibodies to cutaneous self-antigen cause autoimmune blistering diseases including pemphigus and pemphigoid. In addition to antibody production, B cells are capable of influencing immune responses through several other mechanisms. B cells can function as antigen-presenting cells and provide costimulatory signals to T cells. B cells also regulate lymphoid tissue neogenesis and structure. Some B cells are potent cytokine-secreting cells. Therefore, the B cell is considered as a therapeutic target in a variety of diseases including rheumatic diseases and autoimmune blistering diseases, not to mention B-cell leukemia/lymphoma.

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7.2 B Cell Development

B cell development is a tightly regulated process [1–3]. B cell lineage subpopulations are identified by the expression of several cell-surface molecules and intracellular transcriptional factors, the status of Ig heavy (H) and light (L) chain rearrangement, and the cell-cycle status and expression of pre-B cell and B cell receptor (BCR) complex. In the bone marrow, the pluripotent hematopoietic stem cell first differentiates into a multipotent progenitor cell, and then into the common lymphocyte progenitor cell through interaction with bone marrow stromal cells. The B cell lineage cell starts as the pro-B cell, which is characterized by the cytoplasmic expression of the B cell specific molecule, CD22, which is later expressed on the cell surface when IgD becomes positive. Immunoglobulin rearrangement, in which recombinase activating gene-encoded enzymes, RAG1 and RAG2, play an essential role, begins in pro-B cells. Pre-B cells, which arise from pro-B cells that express neither the pre-BCR or surface Ig, are characterized by the pre-BCR on the cell surface as a consequence of completed rearrangement of the heavy μ chain paired with the surrogate light chain. The induction of CD19 expression is also a hallmark of the pre-B cell.

Immature B cells first express the BCR, complete IgM molecules on the cell surface. Immature B cells are subject to negative selection, and those with potential reactivity to self-antigens undergo clonal deletion, anergy, or receptor editing. Immature B cells that escape this process start expressing IgD, are allowed to leave the bone marrow, and emerge in the periphery as transitional B cells.

Transitional B cells are immature splenic B cells and are subject to selection before they develop into mature naïve B cells. Transitional B cells have mostly been studied in mice. Mouse transitional B cells are subdivided into two populations based on their phenotype: T1 (IgM^{hi} IgD^{low} $CD21^{low}$ $CD23^{-}$ $CD24^{hi}$) and T2 (IgM^{low} IgD^{hi} $CD21^{hi}$ $CD23^{+}$ $CD24^{dim/low}$) transitional B cells [4]. T1 transitional B cells are located at the periarteriolar lymphoid sheaths (PALS) of the spleen. T1 B cells that survive negative selection mature into T2 transitional B cells found in the primary follicles of the spleen. Another transitional B cell subset, T3 B cells, are currently considered anergic B cells, which are autoreactive and thus regress back [5]. Humans also have a similar developmental stage, and the subsets are defined by the expression levels of CD24, CD38, and CD10 [6, 7]. After passing the transitional stage, they become mature B cells.

7.3 B Cell Subset

Mature naïve B cells consist of several cell populations (Fig. 7.1). Mature B cells are classified into B-1 cells and B-2 cells. B-2 cells include follicular (FO) B cells and marginal zone (MZ) B cells. FO B cells, with typical phenotype of IgM^{low} IgD^{hi} $CD5^{-}$ $CD23^{+}$, constitute the majority of B cells in the spleen, tonsil, and lymph

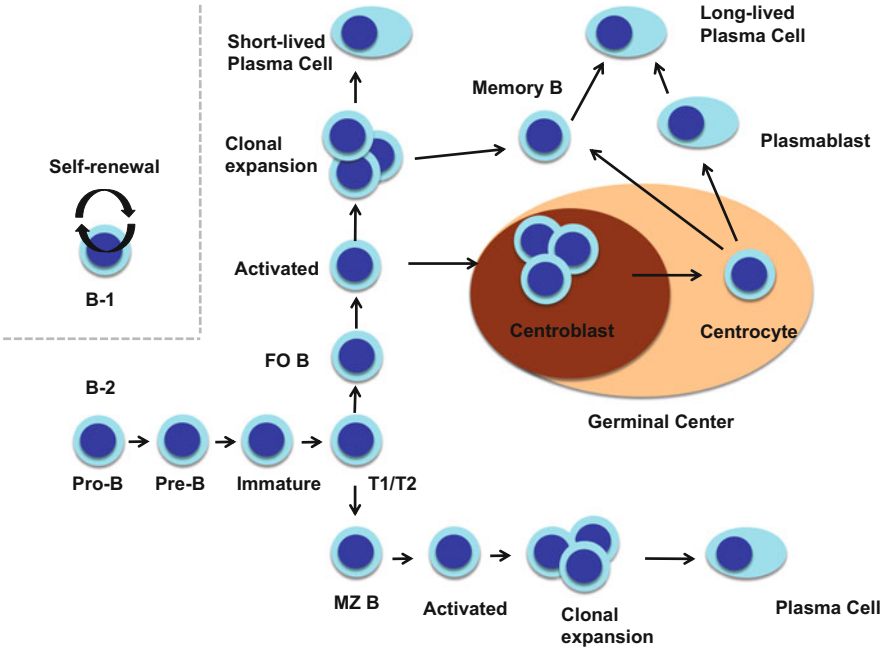

Fig. 7.1 B cell development, subset, and differentiation. In the bone marrow, the B cell lineage cell starts as the pro-B cell, which differentiates into the pre-B cell expressing the pre-BCR on the cell surface. Immature B cells that escape this process start expressing IgD and emerge in the periphery as transitional B cells (T1/T2). Mature B cells are classified into B-1 cells and B-2 cells. B-2 cells include follicular (FO) B cells and marginal zone (MZ) B cells. FO B cells are activated through the interaction with antigen-presenting cells and T cells. Some B cells directly differentiate into short-lived plasma cells by clonal expansion and produce IgM promptly. Other B cells migrate into the primary follicle, differentiate into centroblasts, and initiate the germinal center reaction. Somatic hypermutation occurs in centroblasts, and those that undergo somatic mutation resulting in high-affinity antibody production are positively selected, expand, and differentiate into centrocytes. Centrocytes undergo isotype switching, acquire different Ig isotypes, and differentiate into memory B cells or plasmablasts. Some become long-lived plasma cells in a niche in the bone marrow. MZ B cells also undergo clonal expansion and become plasma cells. B-1 cells have a distinct ontogeny in mice

nodes. FO B cells are recirculating cells that home and organize into the primary follicles of B cell zones in the white pulp of the spleen. FO B cells mediate the majority of T helper cell-dependent humoral immune responses that result in immunological memory. They are activated through the interaction with antigen-presenting cells and T cells. Some B cells directly differentiate into short-lived plasma cells by clonal expansion and produce IgM promptly, which plays an important role in the first-line defense of the humoral response against the antigen. Other B cells migrate into the primary follicle, differentiate into centroblasts, and initiate the germinal center (GC) reaction [8, 9]. Somatic hypermutation occurs in centroblasts, and those that undergo somatic mutation resulting in high-affinity

antibody production are positively selected, expand, and differentiate into centrocytes. Centrocytes interact with T cells and follicular DCs, and undergo isotype switching. These cells acquire different Ig isotypes, and eventually differentiate into memory B cells or plasmablasts. Memory B cells are marked by CD27 expression. They exit the germinal center, and reenter the circulation. Some find a niche in the bone marrow and survive as long-lived plasma cells for decades [10].

The other B-2 cell subset is the marginal zone B cell [11]. In mice, MZ B cells express the phenotype of $IgM^{hi}IgD^{low}CD21^{hi}CD23^{-}CD24^{dim}$. They are located in the marginal sinus region (marginal zone) of the spleen. MZ B cells also originate from T2 B cells, although how their fate is determined (FO vs. MZ) is not fully elucidated [12]. MZ B cells are vital in the general first-line defense against blood-borne antigens. Upon recognition of T cell independent antigens they start to proliferate and differentiate into plasma cells that produce low-affinity antibodies. Thus, MZ B cells function as "innate-like lymphocytes" [13]. Human MZ B cells express $IgM^{hi}IgD^{low}CD1c^{+}CD21^{hi}CD23^{-}CD27^{+}$ phenotype [13, 14]. The ontogeny and functions of human marginal zone B cells remain controversial. B cells identical to splenic MZ B cells are also present in human peripheral blood, suggesting that human MZ B cells recirculate [15], unlike murine marginal zone B cells that are restricted to the spleen [13]. Also, a significant proportion of $IgM^{+}CD27^{+}$ MZ B cells have been shown to undergo somatic mutations in humans [16].

B-1 B cell is the third type of mature B cells with a distinct cell-surface phenotype, at least in mice [17, 18]. B-1 B cells are predominantly present in the pleural and peritoneal cavities. Additionally, there is a very small population of B-1 cells in the spleen. B-1 B cells are further divided into two subpopulations, B-1a ($CD11b^{+}CD5^{+}$) and B-1b cells ($CD11b^{+}CD5^{-}$). B-1a cells develop mainly from fetal-liver–derived haematopoietic stem cells. B-1 B cells have a unique self-renewing capacity. B-1 B cells preferentially produce polyreactive IgM and IgA, and like MZ B cells known as "natural antibodies," play an important role as "innate-like B cells." Whereas the B-1 B cell population is distinct in mice, the existence of human B-1 B cells has been controversial. However, recent studies have demonstrated $CD20^{+}CD27^{+}CD43^{+}CD70^{-}$ as a human B-1 cell phenotype [19].

7.4 B Cell Activation

B cells respond to diverse chemical and environmental cues. BCR can bind a vast variety of antigens, including native proteins, glycoproteins, and polysaccharides. Mature B cells are essentially activated by the recognition of antigen. Classically, B cell activation is classified into T-dependent activation and T-independent activation. In T-dependent response, the B cell is activated by antigen and also by T-cell interaction through CD40/CD40L [20]. T-independent antigens can induce B cell activation without T-cell help.

B cell fate is determined by BCR signaling [21]. Transmembrane Ig serves as the antigen-binding subunit of the BCR. Ig expressed on the cell surface is noncovalently associated with the CD79a (Igα) and CD79b (Igβ) heterodimer, which serves as a signal transducing subunit [22, 23]. CD79a and CD79b cytoplasmic domains contain immunoreceptor tyrosine-based activation motifs (ITAMs). When a B cell encounters antigen, ITAMs within CD79a/CD79b are phosphorylated by Src-family protein tyrosine kinases (PTKs), Lyn, Fyn, and Blk [24]. Whereas Lyn is a predominant Src-family PTK in B cells [25, 26], Blk is also a B cell-specific Src-family PTK. Blk deficiency in mice does not exhibit an overt abnormal phenotype, however, Blk polymorphisms are closely associated with several connective tissue diseases including SLE [27]. Phosphorylated ITAMs then recruit other PTKs, such as Syk and Btk, which further phosphorylate the ITAMs, as well as other signaling molecules, that mediate downstream signaling events which determine the fate of the cell during development, activation, and proliferation. Among them, BANK1 is an adaptor protein primarily expressed in B cells. BANK1 polymorphism is also associated with SLE and systemic sclerosis [28, 29].

B cell responses are further fine-tuned by other surface molecules (Fig. 7.2). These molecules are roughly categorized into positive regulators and negative regulators. Positive regulators include CD19, CD40, and CD45. CD19 is a cell-surface protein expressed in B cells and follicular dendritic cells. CD19 is associated on the B-cell membrane with CD21. CD21 is the receptor for C3d and Epstein–Barr virus [30]. CD21 generates transmembrane signals through CD19 and informs the B cell of inflammatory responses within microenvironments. Once the complement is activated, C3d-bearing antigens efficiently activate B cells as BCR and CD19 are colligated [31]. CD19 has nine conserved tyrosine residues in the cytoplasmic domain. Lyn phosphorylates these tyrosines, which then mediate PI3-kinase activation and Lyn kinase activity amplification [32, 33].

CD40 is expressed on B cells as well as a variety of cells including monocytes and dendritic cells. CD40 serves as a critical survival factor for GC B cells and is the ligand for CD154 (CD40 ligand; CD40L) expressed by activated T cells [20]. CD40 ligation by CD40L promotes B-cell activation, maturation, differentiation, and Ig production. It is critical to germinal center formation and promotes memory B cell development. CD40/CD40L interactions rescue immature B cells from negative selection and germinal center B cells from apoptosis.

Toll-like receptors (TLRs) are also expressed on B cells [34]. Some TLRs, such as TLR2 and TLR4, which are triggered by lipothecoic acid and lipopolysaccharide (LPS), respectively, are present on the plasma membrane, whereas others, such as TLR7 and TLR9, which recognize single-stranded RNA and CpG-containing DNA, respectively, are present in the endosomal compartment. TLR, alone or with other stimuli, can activate B cells. Although human naïve B cells do not express significant levels of TLRs, human memory B cells constitutively express TLR2, TLR6, TLR7, TLR9, and TLR10 [34–36].

B cell response is also regulated by cytokines, such as IL-4, IL-5, IFN-γ, and TGF-β. These cytokines differentially induce a class switch. IL-21 secreted by

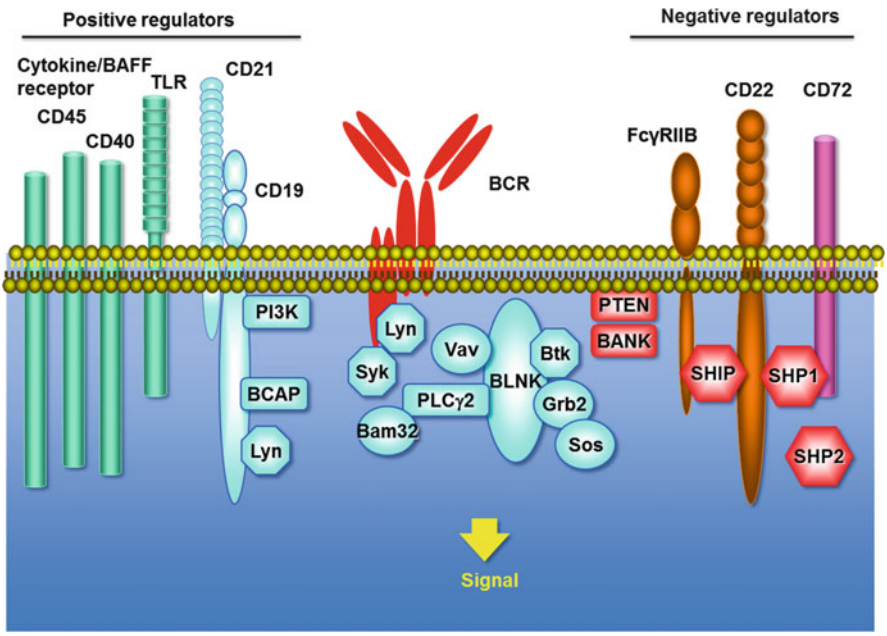

Fig. 7.2 B cell signaling. The BCR consists of membrane-bound Ig associated with the CD79a (Igα) and CD79b (Igβ) heterodimer. Upon BCR ligation, Src-family protein tyrosine kinases (Lyn etc.), Syk, and Btk are activated, and mediate downstream signaling cascades that determine the fate of the cell during the development, activation, and proliferation. These responses are further fine-tuned by B-cell surface molecules, which are roughly categorized into positive regulators and negative regulators. Positive regulators include CD19/CD21, CD40, CD45, Toll-like receptors (TLR), and cytokine receptors including BAFF-R. Negative regulators include CD22, CD72, and Fcγ receptor IIB (FcγRIIB), which are characterized by ITIMs (immunoreceptor tyrosine-based inhibitory motifs) in the cytoplasmic domain

follicular dendritic T cells is also important for germinal center formation in addition to IL-4 [37].

B-cell activating factor (BAFF), a member of the tumor necrosis factor family of cytokines, is particularly important in B-cell survival and differentiation [38]. BAFF is produced by DCs and monocyte/macrophages and binds three receptors, B cell maturation antigen (BCMA), transmembrane activator and calcium-modulator and cyclophilin ligand interactor (TACI), and BAFF-R. The BAFF signal induces immature B-cell survival and mature B-cell proliferation within peripheral lymphoid tissues when coupled with BCR ligation. Transgenic mice that overexpress BAFF develop a lupus-like disease. Belimumab, a monoclonal antibody against BAFF, has been approved for the treatment of SLE by the Food and Drug Administration (FDA).

By contrast, there are several negative regulators that dampen B cell activation signals [39]. CD22 functions as a mammalian lectin for α2,6-linked sialic acid [40]. CD22 has ITIMs (immunoreceptor tyrosine-based inhibitory motifs) in the

cytoplasmic domain that recruit SHP-1 phosphatase. CD19 and CD22 reciprocally regulate their functions, and their balance appears to determine the susceptibility of autoimmunity [41]. CD72 also functions as a negative regulator of signal transduction through the ITIM motif and as the B-cell ligand for Semaphorin 4D (CD100) [42]. Fcγ receptor IIB (FcγRIIB), which binds the Fc portion of IgG, is another important negative regulator of B cells that contains an ITIM [43].

Other important B cell-surface proteins include CD20, a mature B cell-specific molecule that functions as a membrane-embedded Ca^{2+} channel, although their specific significance remains unclear [44]. Note that CD20 is the target of ritixumab, a chimeric CD20 mAb which was approved first by the FDA for clinical use in cancer therapy. CD23 is a low-affinity receptor for IgE expressed on activated B cells. CD24 is a pan-B-cell molecule, although this unique GPI-anchored glycoprotein's function remains unknown. CD38 is a membrane-associated enzyme that may increase BCR signaling by its interaction with CD19.

7.5 Antibodies

The B cell is a central player of humoral immunity by its capacity to differentiate into cells that can secrete antibodies. Antibodies are the secreted form of the BCR. Antibodies, i.e., Igs, are tetrameric molecules composed of two pairs of polypeptide chains (Fig. 7.3). Ig has two identical light chains with a molecular weight of 25 kD and two identical heavy chains with a molecular weight of 50–75 kD. There are two types of light chains, kappa (κ) and lambda (λ). Kappa chains are more common than λ chains in humans (60 %) and mice (95 %), although no functional differences have been found between the κ and λ chains. Antibody molecules consist of three portions presenting a roughly Y shape. Two arms of the Y are identical and termed Fab fragments containing a variable region of the light and heavy chains. The light and heavy chains of Fab fragments consist of VL and CL domains and VH and CH1 domains, respectively. Antigen-binding sites are formed by the paired VL and VH domains. The variable regions are responsible for the recognition of a great diversity of antigens. The tail of the Y is termed the Fc fragment and consists of constant regions, CH2 and CH3 domains. IgM and IgE have an extra C domain. The constant regions of the heavy chains determine the Ig class or isotype. The antibody molecule exerts effector functions such as complement activation and binding to Fc receptors. These functions vary significantly with antibody class. There are five known isotypes in mice and humans: IgM, IgG, IgA, IgE, and IgD (Table 7.1).

IgM accounts for approximately 10 % of the serum Ig pool. It is the first Ig generated during the primary immune response and thus plays a pivotal role in the primary immune response. IgM forms a pentamer in serum, which helps to enhance its avidity for antigen as IgM antibody generally has low affinity.

IgG is the most abundant Ig isotype that makes up approximately 75 % of the serum Ig pool. In humans, there are four subclasses of IgG: IgG1, IgG2, IgG3, and IgG4. IgG1 is the most abundant (>50 % of total IgG) and the major component of

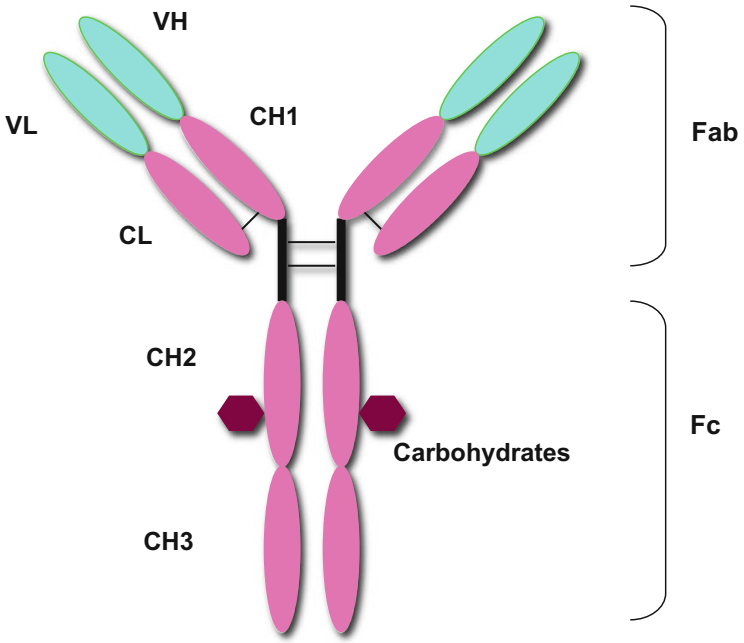

Fig. 7.3 The structure of antibody. Antibodies (Igs) are tetrameric molecules composed of two pairs of polypeptide chains, presenting roughly a Y shape. Ig has two identical light chains and two identical heavy chains. Antibody molecules consist of three portions. Two arms of the Y are termed Fab fragments containing a variable region of the light and heavy chains. The light and heavy chains of Fab fragments consist of VL and CL domains and VH and CH1 domains, respectively. The variable regions are responsible for the recognition of great diversity of antigens. The tail of the Y is termed Fc fragment consisting of constant regions, CH2 and CH3 domains

Table 7.1 Structure of immunogloburin G

Ig isotypes in humans								
	IgM	IgG1	IgG2	IgG3	IgG4	IgA	IgE	IgD
Molecular weight (kD)	970	146	146	165	146	160	188	184
Half-life (day)	10	21	20	7	21	6	2	3
Neutralization	+	++	++	++	++	++	–	–
Opsonization	+	++	+/–	++	+	+	–	–
Sensitization for killing by NK cells	–	++	–	++	–	–	–	–
Sensitization of mast cells	–	+	–	+	–	–	++	–
Activation of complement system	+++	++	+	++	–	+	–	–
Transport across epithelium	+	–	–	–	–	++	–	–
Transport across placenta	–	++	+	++	+/–	–	–	–
Diffusion into extravascular sites	+/–	++	++	++	++	+	+	–
Mean serum levels (mg/ml)	1.5	9	3	1	0.5	3	0.0003	0.03

the response to protein antigens, whereas IgG2 is produced in response to polysaccharide antigens. IgG3 is considered to be important in the response to respiratory viruses. IgG4 is the least abundant (<5 %) and undergoes "Fab-arm exchange," resulting in bispecific antibody. IgG4 is associated with Th2 response, as is IgE. Functional properties are different among the subtypes based on the Fc difference. IgG1 and IgG3 strongly activate the complement system and can bind to mononuclear cells and neutrophils. The half-life of IgG3 (7 days) is shorter than that of the other subtypes (21 days). IgG is the major Ig that causes autoimmune diseases as it can penetrate into tissues. Recently, accumulated evidence has established a concept of "IgG4-related disease" characterized by a lymphoplasmacytic infiltrate composed of IgG4+ plasma cells [45], although the molecular mechanisms of how IgG4 participates in the pathogenesis remains unclear.

IgA is the predominant isotype at mucosal surfaces [46]. It makes up approximately 15% of the serum Ig pool. IgA exists as two subclasses, IgA1 and IgA2. IgA1 is mainly a monomer in serum, and IgA2 exists as a dimer linked by a J chain and linked to a peptide known as a secretory component. IgA binds to pIgR and transits through secretory epithelium. Having this component, polymeric IgA is resistant to enzymatic degradation. IgA activates complement through the alternative pathway. IgA deposition is observed in Henoch–Schonlein purpura. IgA antibodies also have a pathogenic role in mucous membrane pemphigoid and herptiform dermatitis.

IgE is the least abundant Ig in the serum of nonatopic individuals [47]. IgE is mainly sequestered in tissues and binds in the monomeric form to the high affinity FcεR on basophils and mast cells. Antigen binding to cell-bound IgE leads to degranulation and release of mediators which causes immediate hypersensitivity reactions, such as urticarial and anaphylaxis. Increased IgE levels are required for the upregulation of the expression of the high-affinity IgE receptor (FcεRI) by mast cells homing to mucosal surfaces.

IgD is also less abundant in serum. IgD is expressed at high density on the mature B cell surface, and induces strong BCR signals. Its physiologic function as a soluble form is not well understood, however, a recent study has demonstrated that IgD binds to basophils and mast cells and activates these cells to produce antimicrobial factors to participate in a respiratory immune defense in humans. In an autoinflammatory syndrome, "hyper-IgG syndrome," patients present increased levels of serum IgD, although the mechanism remains unknown.

Antibodies recognize entering foreign antigens and trigger a biological response to eliminate the antigen utilizing three types of response (Fig. 7.4). The first, known as neutralization, antibodies prevent the adherence of pathogens and toxins to host cells. Second, antibody coats the surface of a pathogen, and promotes phagocytosis, which is called opsonization. Antibodies bound to the pathogen are recognized by Fc receptors expressed on phagocytes [43]. Third, antibodies coating a pathogen can activate the complement system by classical pathways. Complement proteins bound to the pathogen surface enhance opsonization and lyses some bacteria [48].

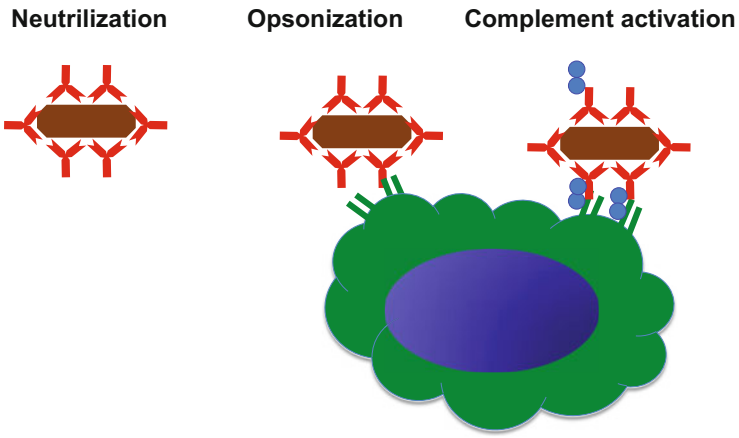

Fig. 7.4 Functions of antibody. Antibodies recognize antigens (*brown*) and trigger a biological response in three ways. (i) Neutralization, antibodies prevent the adherence of pathogens and toxins to host cells. (ii) Opsonization. Antibody coats the surface of a pathogen, and promotes phagocytosis. Antibodies bound to the pathogen are recognized by Fc receptors expressed on phagocytes (*green* cell). (iii) Complement activation. Complement proteins (*blue*) bound to the pathogen surface with antibody. Antibody enhances opsonization and lyses some bacteria

7.6 Cytokines

In addition to antibody production, antibody-independent functions of B cells have been identified. These include antigen presentation, costimulation, and lymphoid tissue neogenesis and structure regulation. B cells can produce a variety of cytokines [49]. They secrete cytokines constitutively, or in response to antigen, TLR ligands, T cells, or their combinations [50]. Cytokines produced by B cells include proinflammatory cytokines such as IL-1, IL-2, IL-4, IL-6, IL-12, IL-13, IFN-γ, TNFα, and LTα, hematopoietic growth factors such as GCSF, GMCSF, and IL-7, and immunosuppressive cytokines such as TGFβ and IL-10 [51]. It has been proposed that cytokine-producing B cells can be subdivided into B effector-1 (Be-1) cells and B effector-2 (Be-2) cells based on their cytokine profile in analogy with Th1 and Th2 cells [52]. B cells primed by T_{H1} cells and antigen differentiate into Be-1 cells capable of producing cytokines associated with T_{H1} responses, such as IFN-γ and IL-12. Be-1 cells do not secrete substantial amounts of IL-4, IL-13, or IL-2, but can secrete IL-10, TNF, and IL-6. Conversely, B cells primed by T_{H2} cells and antigen differentiate into Be-2 cells that produce cytokines associated with TH2 responses, such as IL-2, IL-4, and IL-13. Be-2 cells produce minimal amounts of IFNγ and IL-12, but can also secrete IL-10, TNF, and IL-6 [50]. Current data suggest that effector B cells are derived from conventional FO B cells [53, 54]. Finally, B cells that only produce immunosuppressive IL-10 are called regulatory B cells, or B10 cells, and are discussed in the next section.

Effector B cells can also modulate T cell mediated immune responses. In mice, Be-1 and Be-2 B cells generated in vitro can promote the in vitro activation and differentiation of naive T cells into effector T_H1 and T_H2 cells, respectively, through a cytokine-dependent mechanism [52]. Similarly, IL-12-producing human B cells and IL-4-producing human B cells are shown to promote T_H1 and T_H2 cell responses, respectively [55–57].

7.7 Regulatory B Cell

In addition to the effector B cell subsets that enhance immune responses, there are also certain B cell subsets exhibiting regulatory functions [58, 59]. The first evidence for the suppressive function of B cells was documented in the 1970s. Depletion of B cells from splenocyte preparations eliminated the ability of adoptively transferred cell preparations to inhibit DTH responses in guinea pigs [60]. Adoptive transfer of Ag-activated B cells or B-cell blasts could also induce tolerance in recipient naive mice and induce the differentiation of suppressor T cells [61–63]. In 1996, a protective function of B cells was demonstrated against experimental autoimmune encephalomyelitis (EAE), a mouse model for human multiple sclerosis, in B10.PL mice [64]. The B cell-deficient μMT mice were unable to recover from EAE. Another study elucidated that the exacerbation of EAE in μMT mice is due to a deficiency in IL-10-producing B cells [65]. The term "regulatory B cells" was first used in 2003 to designate B cells with inhibitory properties [66, 67]. Regulatory B cells mediate their diverse regulatory functions mainly via the production of IL-10. In mice, splenic $CD1d^{hi}CD5^{+}$ B cells are shown to exert potent immunosuppressive function through IL-10 production, and are thus termed "B10 cells" [68]. These "B10 cells" have a phenotype resembling MZ B cells. T2-MZ precursor cells ($CD19^{+}CD21^{high}CD23^{+}CD24^{high}CD93^{+}$) have also been proposed as regulatory B cells [59]. Therefore, it is likely that regulatory B cells are derived from the MZ B cell lineage within B2 cells in mice. Additionally B1 cells may also exert immunosuppressive effects via IL-10. In humans, $CD19^{+}CD24^{hi}CD27^{+}$ B cells and $CD19^{+}CD24^{high}CD38^{high}$ B cells are proposed as regulatory B cells [69, 70].

Regulatory B cells have been shown to play a potent suppressive function in various murine disease models. In skin inflammatory diseases, regulatory B cells have been reported to suppress inflammatory reactions in contact hypersensitivity in mice [71]. B cell infiltration is observed at the site of CHS in mice [72]. Regulatory B cells also appear to have a critical role in murine connective tissue disease models including arthritis, lupus, and scleroderma [73–75].

7.8 B Cell and Disease

B cells play a critical role in host defense against various infections. This is mainly mediated by antibody production, but nonantibody functions may be also important. Genetic defects in molecules expressed in B cells and/or other cells cause inherited immunodeficiency related to B cell dysfunction [76]. Btk deficiency results in Bruton's X-linked agammaglobulinemia, and CD40/CD40L deficiency results in hyper-IgM syndrome, which are among the best examples. Immunodeficiency caused by genetic defects in BLNK, CD19, ICOS, and AID is also reported [76].

By contrast, B cells play a pathogenic role in several diseases [77]. Type I hypersensitivity is an essential component of urticarial and anaphylaxis. Binding antigen to IgE cross-links FCεRs on mast cells, leading to the release of chemical mediators, such as histamine and leukotrienes. Type II and type III hypersensitivity responses are mediated by IgG antibodies.

Autoantibodies are the hallmark of the B-cell contribution to autoimmune diseases. Some autoantibodies are clearly pathogenic, however, the pathogenicity remains unclear in others. The former includes autoantibodies against transmembrane proteins found in autoimmune blistering diseases such as antidesmoglein, one third antibodies in pemphigus and anti-BP180 antibody in bullous pemphigoid [78, 79]. The titer of these antibodies generally correlates with disease activity. By contrast, most antinuclear antibodies detected in connective tissue diseases including systemic lupus erythematosus, scleroderma, and dermatomyositis are categorized into the latter. Nonetheless, many of these antinuclear and anticytoplasmic antibodies are disease-specific, and serve as sensitive diagnostic markers.

Nonclassical B cell functions independent of antibody production are likely to be important in the manifestation of autoimmune diseases. In mice, a series of studies has demonstrated that, in lupus-prone MRL/lpr mice, elimination of B cells results in a complete abrogation of nephritis, vasculitis, and skin disease [80]. Furthermore, MRL/lpr mice with B cells that cannot secrete antibodies still develop nephritis and vasculitis. The pathological relevance of antibody-independent functions is currently less obvious in humans, although the efficacy of B cell-depletion therapy has been observed clinically even in a variety of diseases that are not considered to be mediated by antibodies, such as type I diabetes and multiple sclerosis.

7.9 Therapeutic Intervention of B Cells

There are an increasing number of molecular targeted therapies related to B cells [81, 82]. The approaches that are currently available or in development include: (i) antibodies that are directed against cell-surface proteins expressed preferentially by B cells (e.g., CD20, CD22), (ii) inhibitors of cytokine signals that are crucial for

B-cell activation and survival (e.g., BAFF), and (iii) low-molecular–weight compounds that inhibit intracellular signaling molecules (e.g., Btk, Syk). Antibodies to B-cell surface proteins can either deplete B cells by direct killing, or modulate B cell functions by interrupting activation/survival signals or inducing negative signaling. Syk and Btk are intracellular PTKs that mediate BCR signaling [24]. Low-molecular–weight compounds that inhibit kinase activity of these PTKs can attenuate activation signaling. Regarding Ig as a target, an antibody therapy against IgE is also prevalent. Finally, there are also many drugs that indirectly affect B cell functions, including IL-6, CD40L, and CTLA4 as targets.

7.9.1 Anti-CD20

Rituximab is a chimeric anti-CD20 monoclonal IgG1 antibody that has widely been used for the treatment of non-Hodgkin follicular lymphoma since it was approved by FDA in 1997 [82]. More recently, rituximab has also been approved for the treatment of RA refractory to anti-TNF therapy, Wegener's granulomatosis, and microscopic polyangiitis. Rituximab efficiently depletes B cells mainly by Fc receptor-mediated Ab-dependent cell-mediated cytotoxicity [83]. Newer human or humanized anti-CD20 monoclonal antibodies are also under development (e.g., ofatumumab, ocrelizumab).

The efficacy of Rituximab in various autoimmune disorders has been reported, although two multicenter large clinical trials for the treatment of SLE failed to prove significant efficacy [84, 85]. In the dermatology field, anti-CD20 therapy has mainly been reported for the treatment of autoimmune bullous diseases, mostly pemphigus vulgaris and pemphigus folliaceous [86, 87].

7.9.2 Anti-CD22

Epratuzumab is a humanized, monoclonal IgG_1 anti-CD22 antibody that binds to the third cytoplasmic Ig domain [82]. In contrast to the anti-CD20 antibody, Epratuzumab treatment leads to a modest reduction of peripheral B cells [89]. Therefore, Epratuzumab is considered to modulate B cell function by inducing negative signals through CD22 and/or altering CD22 expression. Currently, clinical trials are being conducted in several diseases including SLE and primary Sjogren's syndrome.

7.9.3 Anti-BAFF

Belimumab is a fully human IgG_1 anti-BAFF monoclonal antibody that targets soluble BAFF [82]. Belimumab was FDA approved for the treatment of SLE in 2011, based on two definitive Phase III clinical trials demonstrating superiority to the standard of care but which did not include patients with active nephritis or CNS disease. Other approaches using BAFF receptor-Ig fusion protein (BR3-Ig) or a decoy receptor, TACI-Ig, are currently under development.

7.9.4 Anti-IgE

Omalizumab is a humanized monoclonal IgG1 antibody against the cε3 domain of IgE near the binding site for FcεRI and FcεRII. Omalizumab is approved for the treatment of allergic asthma, and the efficacy for urticaria has also been reported [88].

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Chapter 8
Mast Cells and Basophils

Atsushi Otsuka

Abstract Mast cells and basophils are potent effector cells of the innate immune system and are functionally similar to cell types that are generally associated with T helper 2 (Th2) immune responses. Although their in vitro functions are well studied, these functions remain poorly understood. Recently, newly genetically modified mouse strains that specifically target mast cells or basophils have been developed. These advances have expanded our knowledge of cutaneous immune responses over the past few years. For example, the role of mast cells in contact hypersensitivity has become apparent through studies of mice engineered to deplete mast cells conditionally. In addition, studies of newly developed basophil-deficient mice have revealed that basophils cause IgG1-mediated systemic anaphylaxis, that they contribute to protective immunity against *Trichuris muris*, and that they enhance humoral memory responses in the spleen. In this review, we discuss the recent advances related to mast cells and basophils in cutaneous immune responses and discuss the development and future direction of this updated mechanism.

Keywords Mast cell • Basophil • Th2 • Contact hypersensitivity • Atopic dermatitis • Basophil-depend delayed type hypersensitivity

8.1 Introduction

Mast cells and basophils are similar cell types that function alike and are generally associated with T helper 2 (Th2) immune responses, which are mainly characterized by the presence of Th2 cells and high levels of immunoglobulin E (IgE). Th2 immunity develops in response to allergens and parasites. Mast cells and basophils express several effector molecules in common, including mast-cell–associated proteases, vasodilating substances such as histamine, various cytokines, proinflammatory chemokines, and lipid mediators. Many of these effector molecules are rapidly released in response to the activation of the high-affinity receptor

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	Mast cells	Basophils
Development	Bone marrow	Bone marrow
Distribution	Peripheral tissue	Blood
Survival time	Long (weeks)	Short (2〜5 days)
Proliferation	Yes	No
Surface marker	FcεR I α^+, c-kit$^+$	FcεR I α^+, c-kit$^-$, DX5$^+$

Fig. 8.1 The differences between mast cells and basophils. Mast cells and basophils have some differences, for instance, in their development, distribution, and proliferation

for IgE (FcεRI) or other surface receptors that are expressed on mast cells and basophils [1, 2].

In addition, previous studies have reported the involvement of mast cells in other processes such as protection of the host against a range of parasitic and bacterial infections, degradation of toxins, induction of tolerance to skin transplants, and tumor rejection. Basophils can contribute to protection against helminths and ticks [3], but they can also play a pivotal role during IgE-mediated chronic allergic inflammation of the skin and are implicated in the late-phase response of allergic asthma [4].

In addition to their effector functions, mast cells and basophils can rapidly respond to environmental signals and might function as modulators of immune responses by enhancing, suppressing, or polarizing adaptive immunity [2, 5–7]. Mast cells and basophils also have some differences, for instance, in their development, distribution, and proliferation (Fig. 8.1).

Although a number of functions of mast cells or basophils have been reported as mentioned above, the true in vivo functions of both cell types are still largely unknown due to the fact that these cells are rare and are difficult to isolate as pure populations. Furthermore, there have been no ideal tools to evaluate their in vivo role, such as conditional depletion systems. Recently, new types of genetically modified mouse strains have been developed, and previously proposed functions of mast cells and basophils have been revisited extensively. In this review, we summarize these recent studies of mast cells and basophils and focus on their role in cutaneous immune response.

8.2 Characteristics of Mast Cells

Mast cells are derived from hematopoietic stem cells, but they do not ordinarily circulate in their mature form [8]. Instead, the precursor cells differentiate and mature locally after their migration into the vascularized tissues in which mast cells will ultimately reside [8]. Mast cells are widely distributed throughout tissues, especially near surfaces exposed to the environment, such as the skin, airways, and gastrointestinal and genitourinary tracts, where pathogens, allergens, and other environmental agents are encountered [9]. This distribution permits these cells, along with dendritic cells (DCs) and tissue macrophages, to be among the first cells in the immune response system to interact with environmental antigens and allergens [10]. Mast cells can survive for a long time, and some tissue mast cells proliferate in situ in response to certain forms of stimulation [8] (Fig. 8.1). Recent study has shown that mast cell maturation is driven via a group III phospholipase A2-prostaglandin D2-DP1 receptor paracrine axis [11].

Mast cells can participate in many cycles of activation for the release of mediators and can be activated to release distinct patterns of mediators or cytokines, depending on the type and strength of the activating stimuli [9]. Tumor necrosis factor (TNF)-α is a major cytokine stored and released by mast cells. It upregulates endothelial and epithelial adhesion molecules, increases bronchial responsiveness, and has antitumor effects. Other cytokines produced by mast cells include interleukin (IL)-3, granulocyte macrophage colony-stimulating factor (GM-CSF), and IL-5, all of which are critical for eosinophil development and survival, and IL-6, IL-10, and IL-13 [8, 9].

8.3 Characteristics of Basophils

Basophils are the least abundant leucocytes and are primarily found in the circulation system. They comprise only a small percentage (<0.5%) of circulating blood cells under steady-state conditions but rapidly expand in the bone marrow in response to inflammatory signals, whereupon they are mobilized to the blood, spleen, lungs, and liver. They are generated from the granulocyte–monocyte progenitors in the bone marrow and populate the periphery as fully mature cells [12]. The lifespan of basophils is short [13] (Fig. 8.1). For many years, basophils have been a somewhat enigmatic immune cell type and questions regarding their role in protective immunity as well as the specific pathogens or insults that elicit basophil responses are not fully answered. Studies of basophils have been hindered by the lack of information about how they can be distinguished both phenotypically and functionally from developmentally related mast cells.

Basophils can be activated by an array of signals, including those mediated by antibodies (IgE, IgG, and IgD), cytokines (IL-3, IL-18, and IL-33), proteases, Toll-like receptor (TLR) ligands, and complement factors [12, 14]. Activated basophils

are capable of producing a variety of secreted factors including the cytokines IL-4, IL-13, IL-6, TNF-α, and thymic stromal lymphopoietin (TSLP), preformed mediators such as histamine and leukotriene C4 (LTC4), antimicrobial peptides, and chemotactic factors known to recruit multiple immune cell types [15, 16].

8.4 Mast Cell-Specific Depletion Model

The selective deletion of mast cells or basophils in vivo is a useful approach through which to address the role of these cell types during immune responses. Classic models used to study mast cell functions are based on Kit-mutant mouse strains. In addition to their mast cell defect, Kit^{W/W^v} mice have multiple hematopoietic abnormalities that include compromised fitness of the hematopoietic stem and progenitor cells [17], severe macrocytic anemia [18], impaired T development in the thymus [18], and a shift in intraepithelial T cells in the gut in favor of T cell receptor (TCR) $\alpha\beta^+$ cells and against TCR $\gamma\delta^+$ cells [19]. It is important to note that this *Kit* mutant is neutropenic, which may be a major factor affecting immune responses in this strain [20]. New mouse models have recently been developed to avoid these problems. Several groups have reported the generation of mice expressing Cre recombinase under the control of mast cell protease genes [21–23], and, in 2011, four laboratories used their mice or additionally generated lines to obtain Kit-independent mast-cell–deficient mouse strains [6, 24–26]. Some groups have used the diphtheria toxin (DT) system for depletion of the mast cell lineage [6, 24] and other groups have ablated mast cells constitutively by exploiting the genotoxicity of Cre-recombinase [25]. Furthermore, another group depleted mast cells by the Cre-mediated deletion of an apoptosis suppressor gene [26]. Strain-specific characteristics are summarized in Table 8.1.

Table 8.1 Mast cell and basophil depleted model

Mouse strain	Description	Model of depletion	Deleted Population	Remarks	Refs
Mast cells					
Mcpt5-Cre	BAC transgene (129 kb, Cre inserted after the Mcpt5 start codon)	Cross to Cre-iDTR mice or R-DTA mice	CTMCs (>90 %)	MMCs and basophils are not deleted	[23, 24]
Cpa3-Cre	Promoter transgene	Mcl-$1^{fl/fl}$ mice causes impaired survival	CTMCs and MMCs (>90 %), basophils (60–80 %)	Mice develop splenic neutrophilia and macrocytic anemia	[26]

(continued)

Table 8.1 (continued)

Mouse strain	Description	Model of depletion	Deleted Population	Remarks	Refs
Chm:Cre	Promoter transgene (600 bp baboon α-chymase promoter)	NR	NA	Marks mast cells in the lung and the colon	[22]
$Cpa3^{Cre}$	Knock-in of Cre before the first exon of Cpa3	Constitutive depletion	CTMCs and MMCs (100 %), basophils (60 % in the spleen)	NA	[25]
Mas-TRECK	DTR transgene (under control of 5′ enhancer, promoter, and intronic enhancer of IL4)	DT injection	CTMCs, MMCs, and basophils (90–100 %)	Basophils are restored 2 weeks after DT treatment	[6, 27]
Basophils					
Basoph8	Knock-in of IRES-YFP-Cre cassette before the Mcpt8 start codon	Cross to R-DTA mice	Basophils (>90 %)	NA	[28]
Mcpt8-Cre	BAC transgene (228 kb, Cre inserted after the Mcpt8 start codon)	Constitutive depletion	Basophils (>90 %)	NA	[29]
$Mcpt8^{DTR}$	Knock-in of IRES-DTR-EGFP cassette in 3′ UTR of Mcpt8	DT injection	Basophils (>90 %)	NA	[30]
P1-Runx1	Knockout	P1-Runx1 seems to be important for the basophil lineage	Basophils (>90 %)	NA	[31]
Bas-TRECK	DTR transgene (under control of 5′ enhancer, promoter, and intronic enhancer of IL4)	DT injection	Basophils (>90 %)	NA	[27]

BAC bacterial artificial chromosome, *BMMCs* bone marrow-derived mast cells, *CTMCs* connective tissue mast cells, *DTR* diphtheria toxin receptor, *EGFP* enhanced green fluorescent protein, *iDTR* inducible DTR, *IL4* interleukin-4, *IRES* internal ribosome entry site, *P1-RUNX1* distal promoter-derived runt-related transcription factor 1, *Mcpt* mast cell protease, *MMCs* mucosal mast cells, *NA* not applicable, *NR* not reported, *R-DTA* ROSA-diphtheria toxin-α, *UTR* untranslated region, *YFP* yellow fluorescent protein

8.5 Basophil-Specific Depletion Model

Due to the lack of any natural mouse mutants with basophil deficiencies, antibodies that deplete this population of cells have often been used to study the contribution of basophils in different experimental settings. These antibodies recognize either FcεRI (clone MAR-1) or the orphan activating receptor CD200 receptor 3 (CD200R3; clone Ba103), which are both mainly expressed by basophils and mast cells. Although both antibody clones can efficiently deplete basophils, they can also activate mast cells [29, 32]. Furthermore, the depletion of basophils by Ba103 is FcR-dependent and might therefore activate myeloid cells and natural killer (NK) cells [33]. MAR-1 also depletes a subset of FcεRI-expressing DCs [34]. Several in vivo functions have been attributed to basophils on the basis of studies using these depleting antibodies and this has led to several conclusions: first, that basophils cause IgG1-mediated systemic anaphylaxis [35], second, that they contribute to protective immunity against *Trichuris muris* [36], and third, that they enhance humoral memory responses in the spleen [37]. Several new mouse strains with a constitutive or inducible depletion of basophils have recently been generated, and studies using these mice could confirm some of the previously proposed effector functions of basophils.

8.6 Mast Cell and/or Basophil Involvement in Several Skin Diseases

Various models of severe inflammatory autoimmune diseases reveal that neutrophil infiltration into sites of local inflammation and tissue destruction critically depend on mast cells. Involvement of mast cells has been suggested in several animal disease models, such as psoriasis, rheumatoid arthritis (RA) [38–40], and bacterial infection [41, 42]. These observations seem to be highly relevant in terms of our understanding of human diseases, as large numbers of activated mast cells infiltrate the tissues in the corresponding human diseases, such as allergic contact dermatitis, psoriasis, and RA [43–45]. In addition to these diseases, mast cells are involved in multiple inflammatory and malignant diseases (Table 8.2). On the other hand, it is still largely unclear in which skin diseases basophils are involved. Recent studies have shown infiltration of basophils in several skin diseases, such as atopic dermatitis (AD), prurigo, and urticaria [46]. It is notable that skin lesions of bullous pemphigoid, classical eosinophilic pustular folliculitis (Ofuji's disease), and Henoch–Schönlein purpura also frequently show tissue basophilia [46–49] (Table 8.3).

Table 8.2 Dermatological diseases with evidence for mast cell involvement

Anaphylaxis
Hay fever
Urticaria
Localized mastocytomas
Disseminated mastocytosis
Mast cell leukemia
Contact dermatitis
Psoriasis and psoriasis arthritis
Atopic dermatitis
Bullous autoimmune diseases (bullous pemphigoid)
Autoimmune vasculitis
Systemic lupus erythematodes
Systemic sclerosis and morphea
Chronic graft-versus-host disease
Morbus Morbihan and rosacea
Skin infections
Bacteria, fungi
Parasites (Leishmania major)
Skin tumors (basal cell carcinoma, spinocellular carcinoma, angiosarcoma)

Table 8.3 Dermatological diseases with evidence for basophil infiltration

Atopic dermatitis
Prurigo
Urticaria
Pemphigus vulgaris
Bullous vulgaris
Drug eruption
Henoch–Schönlein purpura
Insect bite (tick bite)
Scabies
Dermatomyositis
Eosinophilic pustular folliculitis
Leprosy (LL type)

8.7 The Role of Mast Cells During Contact Hypersensitivity

Contact hypersensitivity (CHS) has been widely used as a model to study cutaneous immune responses, inasmuch as it is a prototype of delayed-type hypersensitivity mediated by antigen-specific T cells [50, 51]. CHS is classified as the sensitization phase and the elicitation phase. An essential step in the sensitization phase for CHS is the migration of hapten-bearing cutaneous DCs, such as epidermal Langerhans

cells (LCs) and dermal DCs, into the skin-draining lymph nodes (LNs). After completing their maturation, mature DCs present antigens to naive T cells in the LNs, thus establishing the sensitization phase. In the subsequent challenge phase, re-exposure to the cognate hapten results in the recruitment of antigen-specific T cells and other non-antigen–specific leukocytes.

Mast cells are a candidate DC modulator because they express and release a wide variety of intermediaries, such as histamine, TNF-α, and lipid mediators. It has been reported that activated human cord-blood–derived mast cells induce DC maturation in vitro [52], that IgE-stimulated mast-cells–derived histamine induces murine LC migration in vivo [53], and that MC-derived TNF-α promotes cutaneous murine DC migration in vivo in an IgE-independent manner [54]. On the other hand, prostaglandin (PG) D_2 is abundantly produced by mast cells in response to allergens [55] and inhibits LC migration [56]. Therefore, MCs might have bidirectional effects on DC activity in a context-dependent manner and the question of the mechanisms by which DCs are modulated by mast cells is an important issue to pursue.

Although basophils operate irrespectively of the development of CHS [7], the role of mast cells in CHS remains controversial. In some studies, mast-cell–deficient mice have exhibited reduced inflammation in TNCB-induced CHS [57, 58]. Other studies reported undiminished CHS induced with TNCB or DNFB [59, 60]. Furthermore, a recent publication reported that mast cells have regulatory roles through their production of IL-10, as mast-cell–deficient mice exhibited enhanced urushiol and DNFB-induced CHS [61]. In these studies, however, mice carrying mutations in the stem cell factor or its receptor c-Kit were used as mast-cell–deficient mice (C57BL/6-$Kit^{W-sh/W-sh}$ or WBB6F1-$Kit^{w/w-v}$). Although these mice lack mast cells, they also have various other immunological alterations, making it difficult to form a conclusion regarding the role of mast cells in CHS based solely on studies using these mice.

In new mast cell depletion models [7, 24], it has been reported that mice depleted of mast cells exhibited reduced CHS induced with FITC, Oxazolone, or DNFB [7, 24]. In addition, mast-cell–specific deletion of IL-10 did not result in exacerbated CHS. Without mast cells, skin DC migration and/or maturation and T-cell priming in the sensitization phase were impaired. Mast cells stimulated DCs via ICAM-1 or lymphocyte function-associated antigen one interaction and by membrane-bound tumor necrosis factor α on mast cells (Fig. 8.2). Interestingly, activated DCs in turn increased Ca^{2+} influx in mast cells, suggesting that mast cells and DCs interact to activate each other. In the elicitation phase, mast cell deficiency resulted in an impaired CHS response, probably as a result of reduced vascular permeability caused by a loss of histamine release from mast cells [24].

To date, it remains unknown why there is such a discrepancy between the reports using stem cell factor-deficient or c-Kit deficient models and those using conditional mast cell ablation models. One of the differences between these two models is the existence of melanocytes and hematopoietic stem cells. Recently, melanocytes were shown to express TLRs to modulate immune responses and to produce IL-1α and IL-1β [62, 63]. In addition, because of the congenital absence of mast cells in $Kit^{W/Wv}$ and $Kit^{W-sh/W-sh}$ mice, a compensatory mechanism may exist such

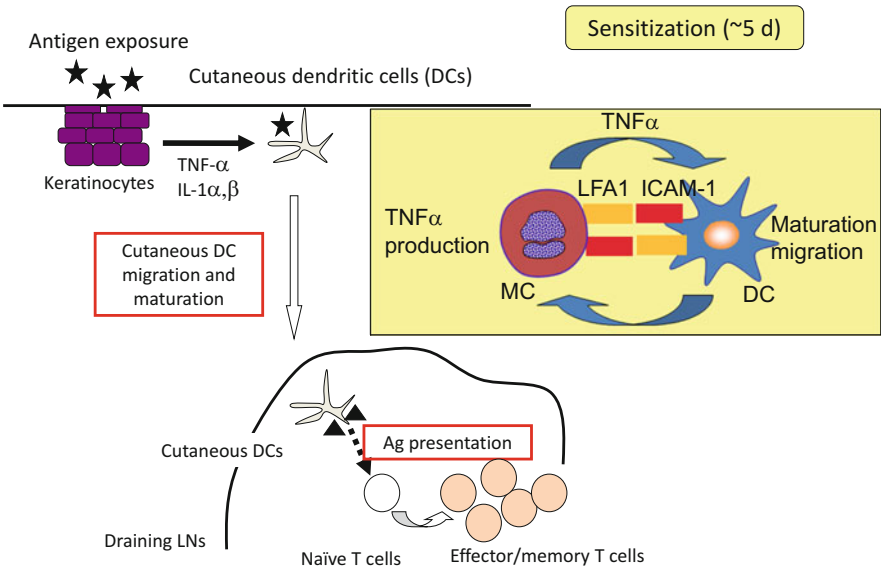

Fig. 8.2 Schema of contact dermatitis. During sensitization phases, mast cells bind to DCs in the dermis and stimulate DCs via ICAM-1 or lymphocyte-function–associated antigen one interaction as well as membrane-bound tumor necrosis factor α on mast cells

as the repopulation of the skin with basophils [64]. Therefore, $Kit^{W/Wv}$ and $Kit^{W-sh/W-sh}$ mice may not necessarily be appropriate to evaluate the exclusive roles of mast cells.

8.8 The Role of Mast Cells During AD Pathogenesis

AD is characterized by skin inflammation, impaired skin barrier function, and IgE-mediated sensitization to food and environmental allergens. The etiology of this disease is not yet understood completely, but it is multifactorial; the disease, moreover, is characterized by complex interactions between genetic and environmental factors. Recently, two major hypotheses have come to the fore as possible explanations for the pathogenesis of this heterogeneous disease: (I) one assumes that the primary defect is an immune dysregulation that causes Th2-predominant inflammation and IgE-mediated sensitization [65]. In the other hypothesis (II), an intrinsic defect in the skin barrier function such as a filaggrin mutation is underscored as a primary cause of the disease [66, 67].

As most studies have shown increased numbers of mast cells in skin lesions in the AD models, it is generally assumed that mast cells contribute to skin inflammation. However, few studies have directly addressed whether, to what extent, or by what mechanism, mast cells play a role in the development of AD-like skin

lesions. A cutaneous ovalbumin (OVA) patch model showed that skin inflammation is comparable in wild-type and $Kit^{W/W-v}$ mice [68]. On the other hand, skin inflammation induced by cutaneous sensitization with cedar pollen antigens was abolished in $Kit^{W/W-v}$ and $Kit^{Sl/Sl-d}$ mice [69]. However, mast cell reconstitution experiments have not been performed in either study. Interestingly, a recent study showed that FcεRI and FcRγ are involved in a cutaneous OVA patch model [70]. Analysis using new cell-specific depletion models may answer this question.

8.9 New Role of Basophils During Th2 Skewing

The induction of Th2 immune responses was previously considered to depend mainly on DCs [71]. However, this dogma has recently been challenged because basophils might also play a pivotal role in this process [36, 72, 73]. It has been reported that $CD49b^+$ $Fc\varepsilon RI^+$ $c\text{-}Kit^-$ basophils migrate into draining LNs from the site of helminth infection or papain injection and thus act as APCs by taking up and processing antigens [36, 72, 73]. In addition, basophils express MHC class II and costimulatory molecules and secrete IL-4 and thymic stromal lymphopoietin (TSLP), which are critical for Th2 development. Therefore, basophils alone are considered to induce Th2 polarization from naïve T cells without requiring DCs under certain conditions. In contrast, another group has found that IL-4-producing basophils were recruited to the mediastinal LNs upon primary exposure to house dust mites. In this case, they contributed to the strength of the Th2 response in the lungs but, in this model, basophils could not present antigens or express the chaperones involved in antigen presentation [34]. Therefore, the authors claimed that DCs were necessary and sufficient for inducing Th2 immunity to house dust mites in the lungs without the requirement of basophils. It has consistently been reported that Th2 responses are severely impaired either after *Schistosoma mansoni* egg injection or during active *Schistosoma. mansoni* infection by the depletion of $CD11c^+$ cells but not by the depletion of basophils using anti-FcεRIα antibody [74]. Therefore, the role of basophils in the development of the Th2 response has been controversial.

We have demonstrated that basophils are responsible for Th2 skewing to haptens and peptide antigens but not protein antigens using, Bas TRECK Tg mice, a new basophil-deficient model [7] (Fig. 8.3). Interestingly, basophils expressed MHC class II, CD40, CD80, CD86, and IL-4 in the hapten-induced Th2 model, but could not take up or process OVA protein sufficiently using the DQ-OVA system. Although the experimental models differ in several ways (e.g., in the routes of antigen administrations), we assume that the discrepancy stems from the different types of antigens used such as proteins, peptides, and haptens. Hapten antigens may bind to MHC class II on the surface of basophils directly, and peptides can be acquired and presented by basophils, whereas protein antigens are not presented efficiently by basophils because the protein is hardly digested by basophils. In fact, previous reports have demonstrated that basophils promote Th2 induction using the

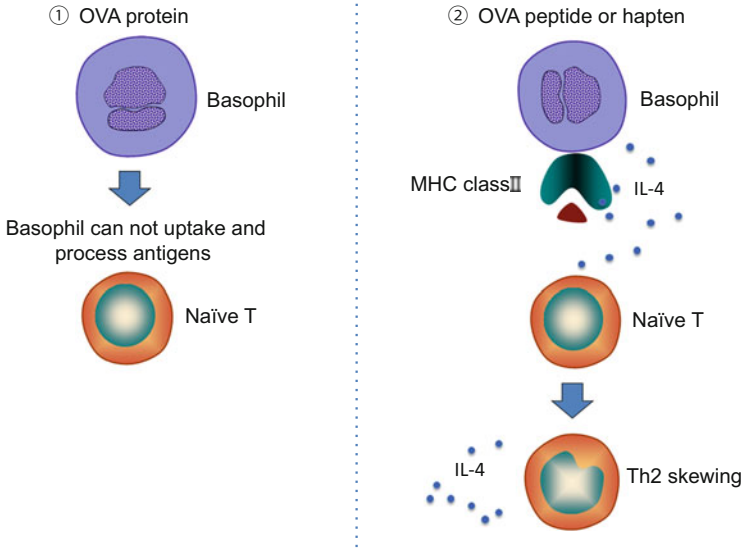

Fig. 8.3 Basophils promote Th2 skewing with haptens and peptide antigens but not protein antigens. Basophils expressed MHC class II, CD40, CD80, CD86, and IL-4 in the hapten-induced Th2 model but they could not take up or process OVA protein, which is one reason why basophils cannot promote Th2 skewing with protein antigens

OVA peptide but not the OVA protein in vitro [72, 73]. The protease allergen papain reaches the LNs after cutaneous immunization and induces MHC class II expression on basophils in accord with the preparation of OVA peptide antigens from OVA protein in vivo [72]. Another group has reported that basophils pulsed with anti-2, 4-dinitrophenyl (DNP IgE) exhibit, enhanced Th2 skewing upon exposure to DNP-conjugated OVA by taking up DNP-OVA–IgE anti-DNP immune complexes [73]. Although house dust mites also contain cysteine protease activity, they are not sufficient for Th2 induction because they do not upregulate MHC class II on basophils in this model even though cysteine protease may work to prepare peptide antigens from protein antigens in vivo [34].

Additionally, our group reported that basophils increase the frequency of IL-4-positive $CD4^+$ T cells by the aid of DCs [7]. Inasmuch as basophils cannot take up or process protein antigens efficiently, DCs may prepare peptides to be presented by basophils or may promote basophils to produce IL-4 to skew Th2. In line with this, our study has demonstrated that Langerhans cells, an epidermal DC subset, mediate epicutaneous sensitization with OVA protein antigens to induce Th2-type immune responses [75]. In addition, Th2 reaction was reduced upon sensitization with protein antigens or schistosome infections in a CD11c-depletion model [34, 74]; therefore, DCs seem to be necessary for Th2 induction both in vivo and in vitro

upon protein antigen exposure. Given that basophils were found in the vicinity of T-cells in the T-cell zone of the draining LNs, it is possible that basophils, T-cells, and DCs promote Th2 induction in a coordinated way. It would be intriguing to further evaluate whether DCs present peptides to basophils directly or by trogocytosis.

8.10 Basophil-Dependent Delayed Type Hypersensitivity

A cutaneous delayed-type hypersensitivity reaction containing large basophil infiltrates was extensively studied in the 1970s [76]. It was termed Jones–Mote hypersensitivity (JMH) in humans or cutaneous basophil hypersensitivity (CBH) in guinea pigs [77]. CBH is distinct from the classical delayed-type hypersensitivity in several aspects [77]. In general, CBH is elicited by the immunization of proteins in incomplete Freund's adjuvant (without mycobacterial components), whereas immunization using complete Freund's adjuvant (with mycobacterial components) is usually needed to elicit the classic hypersensitivity. CBH is characterized by erythema and a slight thickening; it peaks at 18–24 h after the antigen challenge and fades by 48 h. Çlassic delayed-type hypersensitivity, on the other hand, is characterized by erythema and induration; it reaches its maximal intensity within 24–30 h, and remains indurated as long as 48–72 h [78].

It was reported that basophils contributed to a novel type of chronic IgE-mediated allergic inflammation in mice in 2005 [79]. Although basophils are not essential for the immediate- and late-phase responses that occur after multivalent antigens are administered via a single subcutaneous injection into the ear, they are required for the IgE-mediated chronic allergic inflammation that follows. IgE-mediated chronic allergic inflammation (IgE-CAI) was found to be independent of mast cells and T cells but was dependent on an FcεRIα^{+}, CD49b^{+} cell population identified as basophils [79]. Interestingly, although basophils accounted for only 1–2 % of the cellular infiltrate at the site of the skin lesion, their depletion led to a dramatic reduction in inflammation associated with a decrease in the number of eosinophils and neutrophils and a marked reduction in ear thickness [79]. Recent studies have shown that inflammatory monocytes recruited to IgE-CAI lesions acquire an anti-inflammatory M2 phenotype via basophil-derived IL-4 [80]. Collectively, these results illustrate the potent inflammatory effects of small numbers of basophils and suggest a novel, nonredundant role for basophils in the initiation and maintenance of chronic IgE-mediated inflammatory responses in mice.

8.11 Conclusion

Although studies with newly developed mast-cell–deficient or basophil-deficient mice, our understanding of the mechanisms of cutaneous immune reaction has advanced significantly beyond our understanding a decade ago. At the same time, however, some key questions remain unanswered, such as what role basophils play in pathogenic processes, where they are detected in the lesional skin, and how DCs present peptides to basophils during Th2 skewing. In addition, there still remains a compelling need to determine whether these findings in mouse models are relevant to humans. The newly developed mast-cell–deficient or basophil-deficient models can provide us with valuable information on the mechanisms of cutaneous diseases. Future studies focusing on this topic will enable us to develop novel therapeutic approaches to controlling cutaneous inflammatory diseases.

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Chapter 9
Neutrophils

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

Abstract Neutrophils are essential components of the innate immune system. They participate in a variety of tissue reactions, including antimicrobial responses and damage repair. Neutrophils are exquisitely sensitive to migratory stimuli, which enables them to rapidly home into injured tissues, including the skin, where they exert their effector functions. The latter include the release of preformed mediators from granules, production of reactive oxygen species, and release of DNA traps into the extracellular space. Based on these activities, neutrophils play a crucial role in cutaneous immune responses, and patients with neutrophil defects are prone to bacterial and fungal skin infections. Nevertheless, neutrophils may also directly cause tissue damage, and are the driving force behind the pathology of a number of inflammatory skin conditions. This chapter describes the molecular mechanisms underlying neutrophil function in the skin, and reviews our current understanding of the role of neutrophils in cutaneous biology and disease.

Keywords Skin • Innate immunity • Neutrophil • Infection • *Staphylococcus aureus* • Neutrophilic dermatoses and neutrophil deficiencies

9.1 Introduction

The skin, with its sentinel immune cells, forms the first line of defence against invading pathogens and noxious insults. The presence of specialized immune cell subsets resident in the epidermis, for example, Langerhans cells, coupled with a vast array of antimicrobial peptides secreted by epithelial cells, deters the growth of pathogenic microbes [43, 53, 58]. Although the epidermis and its constituents act as

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an efficient barrier against microbes and environmental factors, occasionally these mechanisms fail due to either pathogen-mediated neutralisation of host defence or due to physical barrier breach. The detection of these microbes (commensal or pathogenic) and/or tissue damage results in the activation of a pro-inflammatory cascade leading to the rapid recruitment of leukocytes from the bloodstream. Neutrophils are the prime responders to such stimuli due to their high numbers in the circulation and their exquisite sensitivity for chemoattractants. These cells are replete with an array of cytotoxic granules containing preformed mediators that can be rapidly unleashed within tissues. As such, neutrophils are essential for the clearance of bacterial and parasitic pathogens as well as tissue debris. Nevertheless, in cases where the influx of neutrophils persists over prolonged periods of time or where negative regulatory mechanisms fail neutrophils may also contribute to tissue damage. In this chapter we outline the role of neutrophils in cutaneous immune responses and discuss conditions associated with impaired neutrophil functions and their consequences on skin physiology and pathology.

9.2 Neutrophil Residence in Bone Marrow and Release into Circulation

Neutrophils are short-lived bone marrow-derived cells whose development and release are intimately linked to signals originating at inflammatory sites. Neutrophils are produced in the haematopoietic cords in the bone marrow and their production is tightly regulated by cytokines and a large number of transcription factors [12]. Normal adult human and murine bone marrow produces 10^{11} and 10^{7} neutrophils per day, respectively [13, 31], with only 1–2% of these cells being present in the circulation. Their production is tightly regulated by the IL-23/IL-17A/G-CSF cytokine axis [70, 108, 130]. IL-23 produced by macrophages in the periphery stimulates γδ T cells and a subset of NKT cells to produce IL-17A, which in turn stimulates production of G-CSF from bone marrow stromal cells leading to granulocytopoiesis [70, 119]. Neutrophil release from the bone marrow is mediated by differential signalling via the CXCR4 and CXCR2 chemokine receptors. CXCL12 expression in osteoblasts results in CXCR4-mediated signalling that promotes retention of neutrophils in the bone marrow, whereas CXCL1/2 expression in endothelial cells facilitates CXCR2-mediated neutrophil egress [35, 76, 106, 109, 120, 122, 133]. During inflammation, G-CSF produced by immune cells in the periphery skews the chemotactic response towards CXCR2 by increasing the expression of CXCL2 on endothelial cells in the bone marrow while reducing both CXCL12 producing osteoblasts [26, 35, 133] resulting in rapid mobilisation of neutrophils into systemic circulation. While in the blood stream, neutrophils have a short half-life (<12 h in adult mice). In addition to their central antimicrobial role (discussed below), during their short circulatory life neutrophils can also perform housekeeping functions such as intraluminal removal of damaged endothelial cells in coordination with Nr4a1-dependent patrolling monocytes

[18]. Apoptotic and senescent neutrophils are cleared from the circulation by resident macrophages in the liver, spleen, and bone marrow; a study using radiolabelled neutrophils revealed that all three organs contribute equally in clearing neutrophils [41, 102]. Although terminal trafficking of neutrophils into the intestinal lumen has also been reported [66], its physiological relevance in neutrophil clearance is unknown.

9.3 Neutrophil Entry into the Skin and Interstitial Dermal Migration

Following an insult in peripheral organs, such as the skin, neutrophils are rapidly recruited into the damaged interstitium. The inflammatory cascade underlying this recruitment has traditionally been viewed as being initiated by skin-resident cells. These cells include dendritic cells, macrophages, and mast cells, as well as epithelial cells and stromal cells, all of which are capable of sensing the presence of tissue damage or pathogens via germline encoded pattern recognition receptors (PRRs) that bind to damage-associated molecular patterns (DAMPs) or pathogen-associated molecular patterns (PAMPs). In addition, low numbers of neutrophils themselves have been found to scan the noninflamed dermis actively, where they likely serve as first-line responders to tissue damage [71, 90]. Signalling via PRRs in skin-resident cells leads to the release of pro-inflammatory mediators, including the cytokines TNFα, IL-1β, IL-17 family members, histamine, and/or lipid mediators (Fig. 9.1a). This is followed by the activation of blood vessel endothelium and recruitment of neutrophils into the interstitium via the 'leukocyte adhesion cascade'.

Neutrophil recruitment into the dermis, as in other organs, occurs through postcapillary venules in a process that closely follows the steps of the classical 'leukocyte adhesion cascade' [64, 69, 96]. Under inflammatory conditions endothelial cells increase their expression of E- and P-selectins. This allows circulating neutrophils, which express P-selectin glycoprotein ligand-1, to interact with endothelial selectins, and results in neutrophil tethering and rolling along the blood vessel wall (Fig. 9.1b). Neutrophils are then able to interact with chemokines that are presented on the surface of endothelial cells. This triggers conformational changes in integrins, such as LFA-1 and Mac-1, on neutrophils resulting in high affinity binding of integrins to ICAM1/2 on endothelial cells and, consequently, firm arrest (Fig. 9.1b). Additionally, under some experimental conditions, VLA-4 is also induced on neutrophils and this can interact with VCAM-1 expressed on the endothelium [60]. Thereafter, neutrophils crawl along the luminal surface of the venules in an integrin-dependent manner and search for a preferred point of transmigration. Focal deposits of chemokines along the blood vessel wall are thought to play a role in directed movement, although the precise mechanisms underlying this process are incompletely understood. Firmly adherent neutrophils

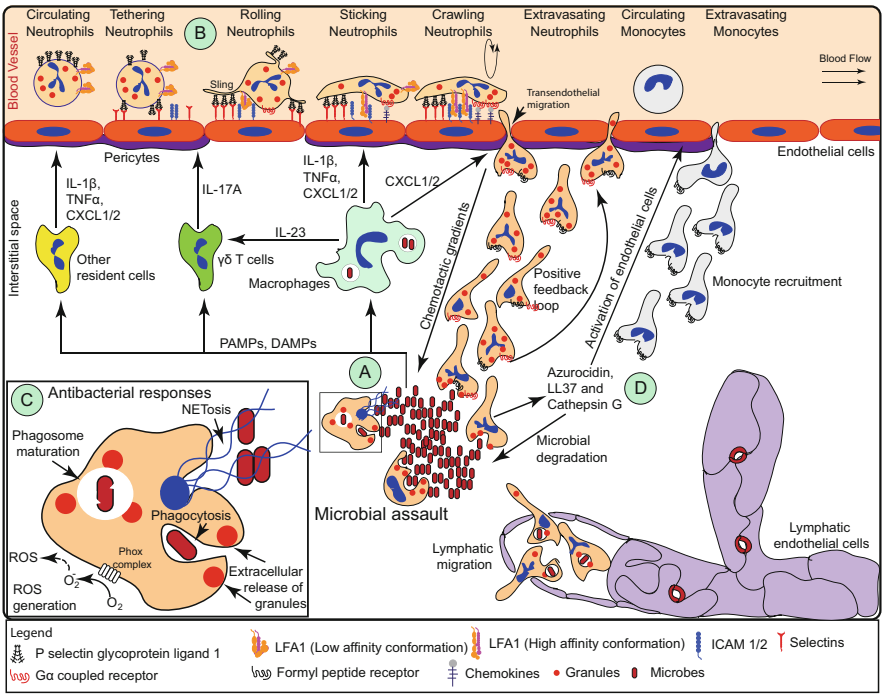

Fig. 9.1 Neutrophils in the skin. Schematic representation of molecular and cellular processes initiated during neutrophil antibacterial response in the dermis. The antibacterial response is initiated by sensing of DAMPs and PAMPs by resident cells in the interstitium (**a**) resulting in rapid recruitment of neutrophils from circulation via leukocyte adhesion cascade (**b**). These recruited neutrophils initiate multiple antibacterial responses (**c**) and activate endothelial cells for recruitment of monocytes and other immune cells (**d**). In addition to these functions, neutrophils can also transport microbial pathogens to draining lymph nodes via afferent lymphatics

then traverse the blood vessel wall and enter the dermal interstitial space. This involves negotiating multiple barriers. Initially, the penetration of the endothelial barrier requires neutrophils to either pass through the body of endothelial cells (transcellular pathway), or through the tight junctions between them (paracellular pathway). Upon reaching the subendothelial space, neutrophils migrate along pericyte processes in an integrin-dependent manner before exiting through gaps between pericytes [100]. These gaps become enlarged in response to cytokine stimulation via remodelling of basement membrane proteins [128, 131] and are associated with increased expression of integrin ligands and neutrophil-attracting chemokines on the pericyte [100].

Upon entering the dermis, neutrophils show strong directional migration towards gradients of chemotactic molecules emanating from the site of injury [79, 118]. Capillary and arteriolar pericytes have also been implicated in guiding neutrophils to the site of injury by upregulating ICAM-1 on the pericyte cell surface and releasing macrophage migration-inhibitory factor (MIF) [118]. Depending on the type of

noxious insult, a vast array of chemotactic molecules may be present in the interstitium; although activation of endothelial cells and macrophages induces production of various chemokines, a large number of DAMPs and PAMPs emanate from damaged cells or pathogens at the site of injury. It is important to note that recent studies have shown the existence of positive feedback loops which augment the attraction of neutrophils towards the site of injury. For example, neutrophil sensing of nicotinamide dinucleotide (NAD^+), which is released from dying cells and acts as a DAMP, has been shown to amplify the accumulation of neutrophils following sterile injury in the dermis [90]. Furthermore, neutrophil-derived leukotriene-B4 (LTB4) acts in a feedforward manner to increase the radius of attraction of the neutrophil population [67, 92]. The multiple chemoattracting cues provided to neutrophils in the interstitial space are integrated, and their migratory behavior is determined by a hierarchical arrangement of signalling patterns [79, 96]. For instance, signalling via formyl peptide receptor-1 supersedes signalling induced via chemokine receptor CXCR2 [79, 96]. Such a hierarchical organisation of signalling patterns assists in the guidance of neutrophils to the precise region of insult.

9.4 Neutrophil Functions at the Site of Tissue Injury

Neutrophils arriving from circulation need to neutralize invading microorganisms rapidly and clear tissue debris. This is achieved via particle uptake (phagocytosis), release of prestored granule contents, generation of reactive oxygen species (ROS), and the deployment of neutrophil extracellular traps (NETs; Fig. 9.1c). Circulating neutrophils are transcriptionally inactive but their interaction with pathogens reactivates transcription and triggers the secretion of chemotactic factors that are crucial for recruitment of other immune cells [107]. The amount of chemokines or inflammatory mediators secreted per neutrophil is relatively small compared to other resident immune cells, but their significantly higher numbers at the site of injury compensate for this low expression [88].

9.4.1 Phagocytosis

During inflammation, neutrophils take up particulates such as microorganisms into membrane-bound vesicles (phagosomes) in a process termed phagocytosis, and particles are then destroyed inside these organelles. The initial process of uptake is strongly enhanced by soluble factors such as antibody or activated complement that mark (‘opsonise’) particulates for phagocytosis. Neutrophils express a range of Fc receptors, in particular FcγIIA and FcγIIIB [42], and aggregation of these receptors by antibodies that are presented in multimeric arrays, such as on microbial cell surfaces, causes neutrophil activation [84, 103]. Additionally, binding of

complement-opsonized particles to CR3 (αMβ2-integrin/CD11b/Mac-1) leads to integrin 'outside-in' signalling and induction of phagocytosis [72]. Furthermore, phagocytosis can also be initiated nonopsonically through receptors that bind directly to microbial components, the details of which depend on the specific microorganism and the surface products that it expresses. In particular, recognition of microbial carbohydrate structures by neutrophil membrane receptors that contain lectin domains, such as dectin-1, Clec-2, or CR3 can mediate phagocytosis of a range of microorganisms [47, 63, 104]. Once internalised, the antimicrobial arsenal of the neutrophil is focused on the particulates by fusion of the phagosome with neutrophil granules.

9.4.2 Granule Composition and Release

The direct antimicrobial activity of neutrophils is largely due to the coordinated release of their granule cargo either into the extracellular milieu or the nascent phagosome. In both cases, high concentrations of antimicrobial proteins, as well as reactive oxygen species, are unleashed [2]. The formation of granules and synthesis of their contents occurs in the bone marrow and is tightly regulated during the development and maturation of neutrophils. A list of granules and their antimicrobial contents as well as the mechanism of action are detailed in Table 9.1. The release of these granules is a tightly regulated process that ensures safe delivery of their content only in close vicinity of pathogens, thereby limiting collateral damage to host cells. The mechanism regulating differential mobilization is incompletely understood though the role of intracellular calcium levels has been implicated in the process [110]. Another type of vesicle is secretory vesicles, which are not strictly granules, but are formed by endocytosis of plasma membrane during neutrophil maturation and contain proteins crucial for homing and sensing pathogens [2].

Although neutrophils possess a striking range of granule proteins, their biological activities can be summarised largely based on their mechanism of antimicrobial action [2]. The first group includes cationic proteins that bind directly to bacterial membranes and mediate lysis; these include α-defensins [44], LL37 [125], and bactericidal/permeability-increasing protein (BPI) [68]. In contrast, a number of proteins with enzymatic activity, such as lysozyme [44], neutrophil elastase (NE) [8, 124], and cathepsin G [124] exert antimicrobial activity through enzymatic degradation of microbial products. The products targeted by these enzymes may be essential components of the cell itself (e.g. lysozyme M activity against the *Micrococcus luteus* peptidoglycan cell wall [45]; neutrophil elastase-mediated degradation of the *Escherichia coli* outer membrane protein OmpA [8]), or may represent extracellular virulence factors (e.g. neutrophil elastase-mediated degradation of *S. flexerni* secreted proteins/toxins [132]). Lastly, antimicrobial activity may also be mediated by sequestration of essential nutrients; a key illustration of this is the multiple approaches used by neutrophils to limit iron availability. Lactoferrin, a component of secondary granules, has direct iron-binding activity,

Table 9.1 Neutrophil granules and their antimicrobial content (Adapted from [2])

Granules	Antimicrobial agents	Mechanism of actions
Primary/ azurophilic	Lysozyme	Degrades bacterial cell walls
	Myeloperoxidase	Generation of reactive oxygen species
	Defensin	Disruption of membrane bilayers. Inhibition of DNA, RNA, protein and cell wall synthesis
	Neutrophil elastase and cathepsin G	Cleaves virulence factors as well as membrane proteins
	Bactericidal/permeability-increasing protein (BPI)	Increases bacterial permeability and hydrolysis bacterial phospholipids
	Other proteins are azurocidin, silaidase, β- glucuronidase	
Secondary/ specific	Lysozyme	Degrades bacterial cell walls
	Lactoferrin	Binds to iron rendering it unavailable for bacterial absorption and utilization. Increases membrane permeability by binding to LPS
	Other proteins are Gp91phox/p22phox, hCAP 18, CD11b, collagenase, NGAL, B12BP, SLPI, haptoglobin, pentraxin 3, oroscomucoid, heparanase, β2 microglobulin, CRISP3	
Tertiary/ gelatinase	Gelatinase	Metalloprotease and can degrade extracellular matrix to increase neutrophil migration
	Other proteins are Gp91phox/p22phox, CD11b, MMP25, arginase-1, β2 microglobulin, CRISP3	
Secretory vesicles	Complement receptor 1	Phagocytosis of complement opsonized microbes
	FcγRIII (CD16)	Phagocytosis of immunoglobulin opsonized microbes
	Other proteins are Gp91phox/p22phox, CD11b, MMP25, C1q-R, FPR, alkaline phosphatase, CD10, CD13, CD14, plasma proteins	

which limits free iron available to growing microorganisms [78, 91]. Alternatively, lipocalin (NGAL) is able to bind to a range of bacterial siderophores and inhibits bacterial iron uptake [39]. These multiple proteins act in concert with reactive oxygen species, which are also generated during degranulation, and exert potent microbicidal activity [89].

The second major antimicrobial component of the degranulation response is the production of reactive oxygen species (reviewed in [7, 32, 134]). The assembly of functional NADPH oxidase (phagocytic oxidase/phox) enzyme from individual components is the initiating step in ROS formation. Under resting conditions, $Gp91^{phox}$ and $p22^{phox}$ reside within secondary granules and the other key components of the enzyme, including $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$ and the small GTPase

rac2, are present within the cytoplasm. Upon neutrophil activation, the cytoplasmic components are translocated to the phagosome that has formed by the fusion of the plasma membrane and granules (or to the plasma membrane), thereby bringing all components of the enzyme together [7, 134]. On becoming functional, NADPH oxidase catalyzes the key initial step in the generation of ROS, which is the conversion of molecular oxygen (O_2) to superoxide ($2O_2^-$). Once formed, superoxide rapidly dismutates to hydrogen peroxide (H_2O_2) and this is acted upon by myeloperoxidase (MPO), which is also released into the phagosome during degranulation, to form hypochlorous acid ($HOCl^-$). In addition, a range of oxidant species including chloramine, hydroxyl radicals ($OH^\bullet$), singlet oxygen ($^1O_2$), and ozone (O_3) is formed [127]. Although all of the different ROS produced have varying degrees of antimicrobial activity, hypochlorous acid is particularly potent at causing oxidative damage to most biological molecules. It is generally believed that this powerful oxidation is one of the key mediators of neutrophil antimicrobial activity [32].

9.4.3 Neutrophil Extracellular Traps (NETs)

Neutrophils can also 'ensnare' pathogens by releasing DNA-based molecular traps (referred to as neutrophil extracellular traps or NETs). These released DNA NETs are associated with histones, myeloperoxidase, neutrophil elastase, lactoferrin, pentraxin-related protein 3 (PTX3), matrix-metalloproteases (MMPs), and other granule proteins all of which can orchestrate antimicrobial functions [15, 16, 137]. DNA NETs can act as molecular scaffolds that entrap pathogens and enhance their interaction with microbicidal proteins. Although neutrophils mostly expel genomic DNA for NET formation [15], mitochondrial DNA can also be used for this process [139]. The process of NET formation is generally considered to be 'suicidal' and is also referred to as 'NETosis', similar to other cell death mechanisms such as apoptosis, necrosis, and pyroptosis [16, 137].

The molecular processes required for NET formation are incompletely understood. It has been shown that activation of NADPH oxidase and subsequent generation of ROS is crucial for NET formation [15, 40, 80]. Upon ROS generation, the granule enzymes NE and MPO translocate to the nucleus and degrade histones [93]. Uncondensed nuclear material is then released by perforation of the cell membrane leading to cell lysis [15, 16, 137]. Furthermore, there is increasing evidence for a second type of NETosis that does not require cell membrane perforation and lysis but instead uses vesicular exportation of DNA for release, referred to as 'vital NETosis' [137]. The released 'anuclear' neutrophil cytoplast retains pathogen-sensing, chemotactic migration, and phagocytosing capabilities [138]. These differences between 'suicidal' and 'vital' forms of NETosis could be due to differences in the activating signal or in the 'maturity' of neutrophils. Nonetheless, both forms of NETosis are important for mediating neutrophil antibacterial responses [137].

9.5 Neutrophil Recirculation and Transport of Antigen

Increasing evidence suggests that neutrophils may enter peripheral tissues, including the skin, even under noninflammatory conditions, and that they may traffic into lymphatic vessels to draining lymph nodes both during steady state and inflammation. During their classic lymphatic cannulation studies in sheep, Morris and colleagues observed neutrophils in lymph fluid, in both the lymphatics draining the hind limb [52] and in the efferent lymph of the popliteal lymph node [50]. It was, however, not until the advent of multiphoton microscopy that such 'scouting' neutrophils were directly observed within the skin during steady state [90]. Labeled neutrophils intravenously injected into sheep could be recovered from the lymphatics within 3–4 h of intravenous injection, indicating that neutrophil recirculation from the blood into the lymph occurs rapidly, in contrast to the delayed kinetics (6–20 h) of lymphocytes [90]. Although it remains uncertain what proportion of neutrophils recirculates under physiological conditions, a clear consequence of this phenomenon is that, in addition to their role as rapid responders, neutrophils may also serve as a primary sensor population for damage and infection [90].

In addition to this physiological recirculation, numerous studies have reported the trafficking of pathogen-bearing neutrophils from the site of infection to draining lymph nodes, including from the skin to cutaneous lymph nodes [1, 11, 73, 121, 136], and that such migration facilitates antigen delivery for ongoing adaptive immune responses [1, 6, 121]. Only a relatively small proportion of recruited neutrophils appears to traffic into draining lymphatics [121], and it remains unclear what differentiates this population from the majority that remain and die within the infected tissue. Similarly, the cellular and molecular pathways that regulate neutrophil entry into the lymphatics remain largely unknown. Although it has been reported that neutrophil migration to the lymph node is CCR7-dependent [5], the potential role for other chemokine receptors has yet to be investigated. CXCR2 and CXCR4 are of particular interest, given their well-documented role in coordinating neutrophil trafficking from the bone marrow [35, 76]. Moreover, CXCR2 has already been implicated in neutrophil trafficking directly into the lymph node from the blood during inflammation [14], whereas CXCR4 is upregulated on the surface of neutrophils following their extravasation into inflammatory tissue [135].

9.6 Neutrophil Apoptosis and Resolution of Inflammation

Neutrophils are generally regarded as pro-inflammatory cells but a large body of evidence indicates that they also have a crucial role in resolving inflammation. During the resolution phase, due to their phagocytic activity, neutrophils remove cellular debris from the site of injury, paving the way for tissue repair [64]. MMP-9 released by neutrophils can degrade various intracellular cytoskeletal components

released from damaged cells, thereby clearing DAMPs [64], and can also promote vascularisation of tissue by activating vascular endothelial growth factor (VEGF) [9, 25]. Neutrophils also initiate recruitment of monocytes via activating endothelial cells to produce CCL2 or by releasing azurocidin, LL37 and cathepsin G that can activate monocyte formyl peptide receptors (FPRs) (Fig. 9.1d) [114, 116, 117]. These monocytes then phagocytose apoptotic neutrophils during tissue resolution.

Although neutrophils are short-lived cells, factors released at the site of injury can enhance their viability. For example, the presence of LPS or GM-CSF increases the expression of antiapoptotic proteins Mcl-1 and A1, thereby leading to an extended neutrophil lifespan (detailed in [2, 62]). In contrast, the neutralisation of noxious insults and microbes leads to decreased signalling from these pathways and results in increased neutrophil apoptosis. At the initial stages of apoptosis neutrophils secrete 'find me' signals to guide monocytes and macrophages to the site of apoptosis and later display 'eat me' signals for rapid phagocytic uptake [115]. Phagocytosis of apoptotic neutrophils by tissue-resident macrophages results in decreased production and release of IL-23 leading to decreased IL-17A levels. The reduced IL-17A results in less secretion of G-CSF by bone marrow stromal cells, decreased granulocytopoiesis, and increased retention of mature neutrophils in the bone marrow [119].

Soluble lipid mediators are important both in neutrophil recruitment and in termination of the acute inflammatory response. At early stages of inflammation, prostaglandins (prostaglandin I2) and leukotrienes (LTB4) are produced, resulting in rapid recruitment of neutrophils, but as inflammation progresses, a molecular switch in lipid metabolism results in production of lipoxins, resolvins, and protectins [2, 64, 111]. These mediators assist in monocyte influx and suppress neutrophil recruitment and antimicrobial functions. In addition, lipid mediators result in nonphlogistic uptake of apoptotic cells by monocytes and macrophages resulting in IL-10 and TGF-β production. Together, these processes promote the resolution of inflammation and tissue restitution.

9.7 Neutrophil Deficiencies

Deficiencies in either the absolute number of circulating neutrophils or their correct functioning typically lead to enhanced susceptibility to infection, particularly within the skin. This is highlighted in acquired neutropenia, such as in patients undergoing chemotherapy, where the severity of neutrophil deficiency is an important determinant of susceptibility to infection with many bacterial and fungal pathogens [10, 74]. A number of molecular deficits that affect neutrophil development or release from the bone marrow can lead to primary neutropenia or functional defects (reviewed in [34]). Such genetic deficiencies include: chronic granulomatous disease (CGD) or MPO deficiency in which ROS generation is affected [54, 127], compromised granule formation/function (e.g. Chédiak–Higashi

syndrome) [4], or leukocyte adhesion deficiency syndromes, which result in decreased neutrophil recruitment [51]. Furthermore, defects in cytokine pathways that contribute to the recruitment of neutrophils also manifest as increased susceptibility to cutaneous infection. For example, mutations that lead to either diminished IL-17 production or IL-17 signalling, including in *IL17RA*, *TYK2*, *STAT3*, or *STAT1*, predispose to chronic mucocutaneous candidiasis [83, 101, 126]. Taken together, the increased susceptibility to infection, particularly to a subset of microorganisms that includes *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Candida albicans*, and *Aspergillus* spp, highlights the critical importance of neutrophils in protection from these pathogens.

9.8 Neutrophilic Dermatoses

Although neutrophils are central to the acute inflammatory response that is initiated following tissue damage or pathogen entry, in some individuals inappropriate recruitment and activation of these cells may occur. This can then contribute to pathology in the absence of an identifiable cause. In some diseases, for example, psoriasis, neutrophils are found in large numbers, but it is unclear whether this represents an epiphenomenon or a true pathogenic event [95, 123]. Alternatively, autoimmune conditions such as lupus erythematosus, cutaneous vasculitides, and neutrophilic dermatoses are prominent examples of conditions where neutrophils appear to contribute to pathology. In vasculitides, the skin can either be the primary organ of involvement, for example, in small vessel leukocytoclastic vasculitis, or part of systemic syndromes, such as in Wegener's granulomatosis where autoantibodies are directed against neutrophils [19, 59]. These conditions have diverse aetiology and their detailed discussion is beyond the scope of this chapter.

Neutrophilic dermatoses are a set of clinical entities that share the common feature of neutrophil influx within the skin in the absence of infection. Several conditions are recognised, but those most commonly described are Sweet's syndrome (SS), pyoderma gangrenosum (PG), and subcorneal pustular dermatosis (SCD; reviewed in [28]). In SS and PG, neutrophil influx occurs predominantly within the dermis, although in PG it may extend to subcutaneous tissues. Variable influx of other inflammatory cell populations, including eosinophils, lymphocytes, and giant cells may also occur depending on the subclassification. In contrast, in SCD neutrophils are confined primarily to the subcorneum [27, 28, 105].

The underlying aetiology of neutrophilic dermatoses is unclear; however, there are a number of striking disease correlations. These include, depending on the classification: prior infections, drug treatment, inflammatory bowel disease, autoimmune conditions, and malignancies [27, 28]. A number of lines of evidence implicate Th1 and/or Th17 inflammatory cytokines in mediating disease, at least in SS and PG. Observations supporting Th1 involvement include increased serum IL-2 and IFNγ [46], whereas elevated levels of IL-1, IL-3, IL-6, and IL-8 have also been noted [27, 77]. A clear pro-pathogenic role for TNF, in particular, has been

reported, and a number of studies have shown the benefit of anti-TNF therapy [17, 28]. More recently the expression of IL-17 and/or IL-23 in SS and PG has been reported, suggesting potential involvement of this cytokine axis in pathology [38, 49, 77]. Consistent with this, case studies that report the successful use of anti-IL-12/23 therapy for the treatment of PG have begun to emerge [37, 49]. Whether this cytokine–disease link holds true in larger studies remains to be seen.

9.9 Neutrophils During Cutaneous Infection: *S. aureus* as a Model Pathogen

The distinct subset of pathogens that cause disease in individuals with neutrophil deficiencies suggests that there are shared protective pathways involved in the responses to these species. In the following section we use the major human pathogen *S. aureus* as an example to illustrate the role of neutrophils in cutaneous immunity.

Data from clinical, rodent, and in vitro studies show remarkable agreement in support of the essential role of neutrophils in protection from *S. aureus*. As described in Sect. 9.7 above, a range of defects in either neutrophil number or function is associated with increased susceptibility to cutaneous *S. aureus* infection. Note that this is mirrored in rodents, as mice deficient in NADPH-oxidase components develop spontaneous cutaneous lesions, or fail to control experimental infection [36, 57, 99]. Moreover, depletion of circulating neutrophils prevents control of cutaneous *S. aureus* infection [85]. In vitro, phagocytosis of *S. aureus* by neutrophils is enhanced by serum opsonins [3, 61, 94], and their subsequent killing is mediated by the NADPH-oxidase dependent production of ROS [36, 75]. A number of granule proteins have direct anti-*Staphylococcal* activity in vitro. These include the α-defensins HNP1-3, as well as LL-37, PG-1, S100A8/9, and lactoferrin [21, 29, 125], whereas others, including azurocidin and lysozyme are ineffective [21]. Lastly, extracellular DNA NETs are released by neutrophils in response to *S. aureus*. The NETs are capable of trapping bacteria, and the NET-associated histones and granule proteins then mediate killing [15, 40, 98].

Rodent models have been particularly informative in identifying the pathways that lead to neutrophil recruitment. Initial recognition of *S. aureus* infection by resident immune sentinel cells occurs via TLR2, NOD2, and the NLRP3 inflammasome [55, 82, 112], resulting in the induction of pro-inflammatory cytokines. Dominant amongst the initial cytokines produced are IL-1β [81, 82] and IL-17A/F [23], both of which have been shown to drive neutrophil accumulation and activation in a range of disease models [65, 113]. Both humans [97, 129] and mice [22, 24, 81] with deficits in the TLR/IL-1β signalling pathway control *S. aureus* infection poorly. The initial source of IL-1β in the skin is unclear: whereas inflammasome-dependent IL-1β release in response to *S. aureus* in vitro is typically

associated with monocyte/macrophages [30, 86, 112], in vivo much of the IL-1β produced appears to be derived from recruited neutrophils, at least during later stages [22]. As with IL-1β, deficiencies in IL-17 signalling (mutations in *STAT3* and *TYK2*) also predispose to *S. aureus* infection [23, 33, 48, 56, 83, 87]. This IL-17A and IL-17F is produced by skin resident γδ-T cells in an IL-23–dependent manner, and during *S. aureus* infection γδ-T-cell–deficient mice show decreased neutrophil recruitment [23]. Ultimately, the combination of IL-1β and IL-17A/F stimulates local production of CXCL1 and CXCL2 from resident cells. These chemokines specifically recruit neutrophils to the infection site, and under normal circumstances this results in bacterial clearance [22, 23, 81, 82].

9.10 Concluding Remarks

The essential role of neutrophils as a key player in the innate immune response to injury/infection within the skin is well recognised, however, it is becoming clear that this is only one aspect in their short life span. Neutrophils under homeostasis perform myriad functions ranging from removing damaged endothelial cells [18] to regulating the stem cell pool in the bone marrow [20]. In particular, the identification of 'scouting' neutrophils that are capable of sensing the presence of tissue damage [90], and the finding that they can recirculate [50, 52, 90] and influence the development of immune responses is furthering our understanding of skin immunity. Although these findings suggest that neutrophils may have previously unrecognised functions in the initiation of both innate and adaptive immune responses, the fate of these neutrophils in the lymph nodes is not completely understood. Moreover the presence of these 'scouting' neutrophils in uninflamed dermis challenges our current understanding about neutrophil recruitment in the skin. Taken together, the increasingly apparent complexity of neutrophil biology argues that continued investigation into this often underestimated cell is likely to lead to improved strategies for treatment of a broad range of cutaneous diseases.

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Chapter 10
Macrophages

Sho Hanakawa and Akihiko Kitoh

Abstract Macrophages strategically adopt a variety of functional phenotypes in response to local microenvironmental factors and perform important immune surveillance activities, including phagocytosis, antigen presentation, and immune suppression. In contrast to circulating macrophages that are homogeneous, tissue-resident macrophages are heterogeneous for the purpose of tissue-specific and microanatomical niche-specific functions. Macrophages have a variety of fundamental roles in biology, from homeostasis and tissue repair to regulation of immune systems. During the early phase of inflammation, macrophages exert proinflammatory functions such as antigen presentation, phagocytosis, and the production of inflammatory cytokines, and they produce growth factors that facilitate the resolution of inflammation in the later phase. Persistence of macrophage activation and polarization is often observed in chronic inflammatory skin diseases such as atopic dermatitis and may be involved in the disease pathogenesis. This chapter summarizes current knowledge of functional macrophage subsets in wound healing and atopic dermatitis, thereby providing more insights into macrophage function and possible interventions in this process. Finally, we briefly discuss the recently reported function and characterization of macrophage heterogeneity in humans.

Keywords Atopic dermatitis • Extracellular matrix • Fibroblast • Inflammation • Macrophage • Phenotype • Wound healing

10.1 Introduction

In addition to providing a structural barrier, the skin contains several types of immune cells that can be activated by invading pathogens or skin damage. One of the most important immune cells involved in inflammation and wound healing is the macrophage, which exhibits different immunological functions in the skin, including phagocytosis and antigen presentation. Macrophages also produce many cytokines and chemokines that stimulate new capillary growth, collagen

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synthesis, and fibrosis [27]. These immune cells are thought to orchestrate the resolution of inflammation and the wound healing process throughout the different phases, such as homeostasis, inflammation, proliferation, angiogenesis, and re-epithelialization as well as remodeling [11, 26].

Macrophages were first identified by Elie Metchnikoff at the end of the nineteenth century as immune cells that engulf and destroy harmful matters such as bacteria [28]. Their precursors are released into the blood as monocytes, and they seed tissues throughout the body [14]. When monocytes migrate from the circulation into the skin, they differentiate into dermal macrophages or dendritic cells (DCs) [20]. Infiltrated monocytes replenish the pool of skin-resident macrophages and DCs in steady state and in response to inflammation. In addition, recent studies have revealed that epidermal Langerhans cells also belong to the monocyte–macrophage lineage. Langerhans cells are established before birth from hematopoietic stem cells or yolk sac-derived macrophages [18]. In contrast to circulating monocyte-derived macrophages, skin-resident macrophages regulate homeostasis by acting as sentinels or professional phagocytic cells along with DCs, neutrophils, and mast cells, and responding to pathological and physiological changes [8]. Macrophages exhibit various functional characters in inflammatory states (Sect. 10.2). These include the classically activated macrophage (CAM) and alternatively activated macrophage (AAM) categories, which are also referred to as M1 and M2 macrophages, respectively [15, 42]. These two states are defined by responses to the cytokine interferon (IFN)-γ and activation of Toll-like receptors (TLRs), and to interleukin (IL)-4 and IL-13, respectively.

M1/M2 macrophage phenotypes influence the wound healing process in different ways, depending on the microenvironment in which they exert their function (Sect. 10.3). Therefore, it is likely that a balance between the two phenotypes is important in the different phases of wound healing. Initially, under the pro-inflammatory phase of wound healing, M1 macrophages scavenge debris and kill any possible invading pathogens. On the other hand, in the later phase in which new tissue formation is pronounced, M2 macrophages exhibit anti-inflammatory activity and produce growth factors that stimulate epithelial cells and fibroblasts to regenerate damaged tissue.

Macrophages play key roles in inflammation [48]. During the onset of the inflammatory process, these phagocytic cells become activated and have destructive effects. Macrophage activation, which involves the induction of more than 400 genes, results in an increased capacity to eliminate bacteria and to regulate many other cells through the release of cytokines and chemokines. However, excessive activation may have destructive effects, such as septic shock, which can lead to multiple organ dysfunction syndrome and death. On the other hand, attenuated pro-inflammatory activity is observed in chronic type 2 inflammations such as atopic dermatitis (AD) [32].

There are several reviews on macrophage responses to pathogens and/or macrophage phenotypes [15, 30]. Therefore, in this chapter, we summarize the functional subsets of macrophages briefly and provide an overview on how

macrophages contribute to physiological and pathophysiological adaptations in mammals by examining wound healing (Sect. 10.3) and AD (Sect. 10.4).

10.2 Functional Diversity of Macrophage Subsets

Macrophages are highly plastic cells that adopt a variety of activation states in response to stimuli in the local environment. During pathogen invasion, tissue injury, or exposure to environmental irritants, local tissue macrophages often adopt an activated or inflammatory phenotype [30]. These cells are commonly called M1 macrophages because they were the first activated macrophage population to be formally defined. These macrophages are activated by IFN-γ and/or TLR engagement, leading to the activation of the NF-κB and STAT1 signaling pathways [29]. This in turn increases the production of reactive oxygen and nitrogen species, and pro-inflammatory cytokines, such as TNF-α, IL-1, and IL-6, which enhance antimicrobial and antitumor immunity, but may also contribute to the development of insulin resistance and diet-induced obesity [42; Table 10.1]. In contrast, some epithelium-derived alarmins, such as IL-25, IL-33, and thymic stromal lymphopoietin (TSLP), and the type 2 cytokines IL-4 and IL-13 induce the M2 macrophage, which is associated with wound healing, fibrosis, insulin sensitivity, and immunoregulatory functions. M2 macrophages promote wound healing and pro-fibrotic response by transforming growth factor β1 (TGFβ1), platelet-derived growth factor (PDGF), and various matrix metalloproteinases that regulate myofibroblast activation and the deposition of extracellular matrix (ECM) components [11]. M2 macrophages also express a variety of immunoregulatory proteins, such as arginase 1 (Arg1), resistin-like molecule α (RELMα; also known as RETNLα or FIZZ1), programmed death 1 ligand 2 (PD-L2), and IL-10 that regulate the magnitude and duration of immune responses (Table 10.1). These cells also scavenge collagen and ECM components, and thus the ECM is remodeled [30]. Therefore, in contrast to M1 macrophages that activate immune defenses, M2 macrophages are typically involved in the suppression of immunity and the re-establishment of homeostasis. Although type 2 cytokines are important inducers of suppressive or immunoregulatory macrophages, it is now clear that several additional mechanisms can also contribute to the activation of macrophages with immunoregulatory activity. Indeed, IL-10-producing regulatory T cells, Fcγ receptor engagement, engulfment of apoptotic cells, and prostaglandins have also been shown to increase the numbers preferentially of regulatory macrophages that suppress inflammation and inhibit antimicrobial and antitumor defenses [29]. Thus, when stimulated, macrophages adopt context-dependent phenotypes that either promote or inhibit host antimicrobial defense, antitumor immunity, and inflammatory responses.

It is generally believed that macrophages represent a spectrum of activated phenotypes rather than discrete stable subpopulations. Indeed, many studies have reported flexibility in their programming, with macrophages switching from one

Table 10.1 Functional diversity of macrophage subsets

Marker	Function	Comments
Classically activated macrophage (M1 macrophage)		
IL-6	Promotes inflammatory response	Induced by IFN-γ
IL-12	Induces Th1 development	Induced by IFN-γ
iNOS	Produces NO to kill microorganisms	Induced by IFN-γ
TNF-α	Promotes inflammatory response	Induced by IFN-γ
Alternatively activated and regulatory macrophage (M2 macrophage)		
Arg1	Counteracts iNOS	Induced by STAT3/6 pathways
IL-10	Anti-inflammatory cytokine	Induced by TLRs-signaling
PD-L2	Inhibits T cell activation	Induced by STAT6 pathway
RELMα	Promotes deposition of ECM	Induced by IL-4 and IL-13
TGFβ	Anti-inflammatory cytokine	Induced by IL-13
YM1	Binds to ECM	Induced by IL-4

Arg1, RELMα and YM1 are not expressed in human macrophage
Arg1 Arginase-1, *ECM* extracellular matrix, *IFN-γ* interferon-γ, *IL* interleukin, *iNOS* inducible nitric oxide synthase, *NO* nitric oxide, *PD-L2* programmed death 1 ligand 2, *RELMα* resistin-like molecule-α, *STAT* signal transducer and activator of transcription, *TGFβ* transforming growth factor β, *Th* T helper, *TLR* Toll-like receptor, *TNF-α* tumor necrosis factor α

functional phenotype to another [16, 23, 44]. In addition, although the M1/M2 classification is a useful heuristic that may reflect extreme states, this classification is unable to represent diverse forms of macrophage activation in vivo, which is induced by the variable microenviromental signals of the local milieu. Indeed, transcriptional profiling of resident macrophages by the Immunological Genome Project (http://www.immgen.org/) represents that these populations have high transcriptional diversity with minimal overlap, suggesting that there are several unique classes of macrophages [13]. Thus, macrophages are a diverse set of cells that constantly shift their functional state to new metastable states in response to changes in tissue physiology or environmental challenges.

10.3 Macrophages in Wound Healing

An emerging dogma in tissue repair is that M1 macrophages are the predominant population present during the first few days after injury, corresponding to the inflammatory and early proliferative phases, whereas M2 macrophages are the primary effectors of later stages of repair or the later proliferative and remodeling phases [11, 30; Fig. 10.1]. The pattern of pro-inflammatory cytokine production by tissue-repair macrophages is perhaps the strongest evidence for the early-M1/late-M2 paradigm or at least for switching from a pro- to an anti-inflammatory phenotype.

The M1 macrophage activator IFN-γ is upregulated rapidly after injury of the skin and is required for proper healing of this tissue [40, 46]. The M1 macrophage-

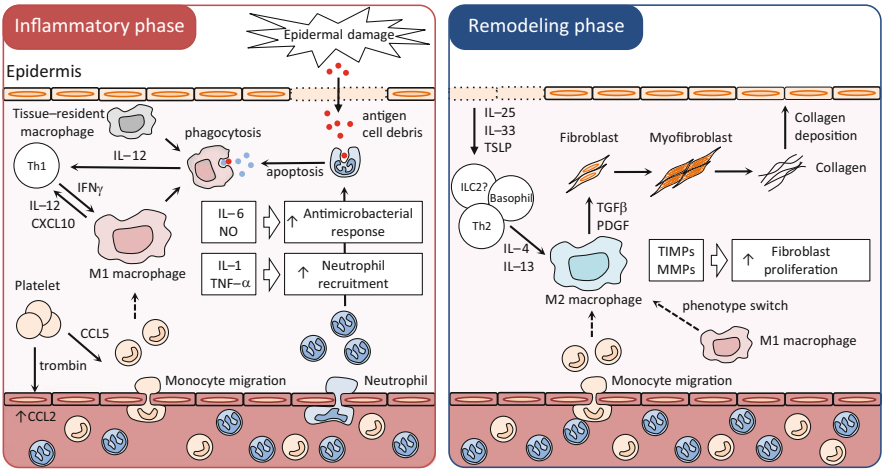

Fig.10.1 Macrophages in wound healing, different phenotypes in different phases. Schema of molecular and cellular processes with a focus on macrophages in wound healing. Tissue-resident cells initiate the immune response against exogenous antigens and recruit neutrophils from the circulation to the dermis. These recruited neutrophils trigger macrophage differentiation to M1 macrophages via antigen processing in the acute phase (inflammatory phase). If the tissue-damaging irritant persists, activated M1 macrophages can further exacerbate the inflammatory response by recruiting neutrophils. The damaged epithelial cells release alarmins, including IL-25, IL-33, and TSLP, which induce IL-4 and IL-13 production by various innate and adaptive immune cells. When the antigens are eliminated, M1 macrophage activation diminishes, and Th2-type cytokines, IL-4 and IL13, convert the inflammatory phase to the wound healing phase (remodeling phase). Th2-type cytokines differentiate M1 macrophages and recruited monocytes to M2 macrophages. M2 macrophages promote wound healing and fibrosis through the production of matrix metalloproteinases (MMPs), tissue inhibitors of metalloproteinases (TIMPs), and various growth factors. M2 macrophages also promote the resolution of wound healing by antagonizing the inflammatory M1 response. *CCL* CC-chemokine ligand, *CXCL* CXC-chemokine ligand, *ILC* innate lymphoid cell, *PDGF* platelet-derived growth factor

associated cytokines IL-1β, TNF-α, IL-6, and IL-12 are expressed by macrophages during the first few hours to days after acute injury of the skin [10, 43]. Within a few days of wound debridement by phagocytic cells, the injured dermis starts to gain volume by the formation of granulation tissue. As recently reported, the dominating macrophage population in the wound at day 5 is the M2 macrophage [26] and M1 macrophages can convert into anti-inflammatory macrophages with an M2 wound-healing phenotype [5]. In contrast to pro-inflammatory and antimicrobial M1 macrophage responses, M2 macrophages exhibit potent anti-inflammatory activity and have important roles in wound healing [51].

M2 macrophages produce growth factors that stimulate epithelial cells and fibroblasts, such as TGFβ1 and PDGF [3]. TGFβ1 contributes to tissue regeneration and wound repair by promoting fibroblast differentiation into myofibroblasts, by enhancing expression of tissue inhibitors of metalloproteinases (TIMPs) that block the degradation of ECM, and by directly stimulating the synthesis of interstitial fibrillar collagens in myofibroblasts [37]. PDGF also stimulates the proliferation of

activated ECM-producing myofibroblasts [41]. M2 macrophages can also regulate wound healing independently of their interactions with myofibroblasts. For example, M2 macrophages produce matrix metalloproteinases (MMPs) that control ECM turnover [49]. M2 macrophages not only recruit Th2 cells and regulatory T cells by secreting CCL17 and CCL22 [9, 19] but also serve as antigen-presenting cells that propagate antigen-specific Th2 and regulatory T-cell responses, which promote wound healing while limiting the development of fibrosis [39]. In addition, they express immunoregulatory proteins, such as IL-10, RELMα, and chitinase-like proteins, which have been shown to decrease the magnitude and duration of inflammatory responses and promote wound healing [33, 36].

Rodent M2 macrophages also express Arg1, which has been suggested to be important for tissue repair [29]. Arginine metabolism through the Arg1 pathway produces polyamines, which are important for cell proliferation, and proline, which is a major component of collagen. However, proline produced via the Arg1 pathway may not be a limiting factor for collagen synthesis [6], and the role of Arg1 in collagen production and fibrosis in vivo is complex and likely context-dependent [50]. Although Arg1 is expressed in macrophages during injury and tissue repair, Arg1 can be produced by cells other than macrophages [21]. In addition, human macrophages do not produce Arg1 [34] and therefore Arg1 appears to be derived entirely from nonmacrophage cell types in humans [6].

10.4 Macrophages in Atopic Dermatitis

Atopic dermatitis (AD) is one of the most frequent chronic inflammatory skin disorders with an increasing prevalence affecting 15–30 % of children and 2–10 % of adults in industrial countries [2]. The hallmarks of AD are a chronic relapsing form of skin inflammation accompanied by a Th2 cytokine-predominant milieu, a disturbance of epidermal-barrier function and hyperproduction of IgE to environmental allergens [4, 22].

Although emphasis has been placed on the regulatory role of T cells in AD research, recent studies indicate that macrophages are alternatively activated in the lesional skin, which may be associated with the pathogenesis of AD. In acutely and chronically inflamed AD skin, CD36 expression by macrophages is elevated [24]. In addition, macrophages expressing mannose receptors [24] and CD163 [45] are significantly increased in lesional skin of AD, compared to normal skin. Collectively, these findings indicate that alternatively activated (M2) macrophages are increased in AD skin lesions. However, it remains unclear whether alternative activation of macrophages actively contributes to the development and maintenance of chronic skin inflammation in AD.

More than 90 % of patients with AD are colonized with *Staphylococcus aureus* in the lesional skin [7], whereas most healthy individuals do not harbor the pathogen [38]. In addition, staphylococcal colonization density in AD patients positively correlates with the disease activity of AD skin lesion [25]. Recent

study has shown that *Staphylococcus* δ-toxin induces skin inflammation by activating mast cells [31], indicating that increased susceptibility to skin colonization with *S. aureus* is a risk factor for AD. Increased susceptibility to skin colonization with *S. aureus* can be caused by several factors including not only skin barrier dysfunction and reduced skin lipid content, but also dysregulated innate and adaptive immune responses [22]. The TLR2 expression and TLR2-mediated production of pro-inflammatory cytokines by macrophages, which play a major role in combating *S. aureus* [Takeuchi et al. 1998, 46], are impaired in patients with AD [32]. In addition, the single nucleotide polymorphism R753Q in the *TLR2* gene has been shown to be associated with the severity of AD [1]. Taken together, dysfunction of macrophages may exacerbate skin inflammation in AD via promoting skin colonization with *S. aureus*.

Although a functional disturbance of macrophages is observed in AD, no conclusive data are available on the pathogenic role of these cells in AD. Therefore, further studies are needed to clarify the precise role for macrophages in the development and persistence of AD skin lesions.

10.5 Perspectives

Macrophages are critical orchestrators of repair and regeneration in numerous tissues and may also contribute to chronic skin disorders including fibrosis and AD. Variable stimuli can induce a broad spectrum of functional macrophage phenotypes. Because this plasticity likely contributes to almost all skin diseases, especially chronic inflammatory skin diseases, to manipulate macrophage functions represents an attractive therapeutic approach. For manipulating macrophage functions properly, however, it is necessary to further understand the functional and phenotypic diversity of macrophages, by determining their proteomes and transcriptomes, as was recently performed for resident macrophages [13]. The advances in proteome and transcriptome analyses at the single-cell level may provide better understanding of a wide spectrum of macrophage activation states.

Functional regulation of macrophages by a variety of cytokine milieux complicates the precise identification of macrophage phenotypes in vivo, compared to macrophages activated in stable and polarized cytokine conditions in vitro. For example, in vivo differentiated tissue-repair macrophages do not necessarily conform to in vitro defined phenotypic categories. Although macrophages exhibit a pro-inflammatory profile in the early stage of tissue repair compared to the late stage, they may lack iNOS expression or express CD206, Ym1, and IL-10 in addition to pro-inflammatory cytokines, indicating that those macrophages do not exhibit an entire M1 phenotype. Similarly, macrophages in the later stage show an increased expression of anti-inflammatory cytokines and some M2-associated markers such as CD206, but other M2 markers, such as Ym1, may be downregulated, indicating that macrophages during the later stages of tissue repair are not entirely the M2 phenotype. Thus, tissue-repair macrophages exhibit

complex and heterogeneous phenotypes that change throughout the repair process and do not correspond to in vitro defined M1/M2 categories.

Significant research effort has been concentrated on the identification of genetic factors and dysregulated immunologic pathways that could lead to the manifestation of AD. Although the pathogenic roles of various immune cells such as T cells, eosinophils, and mast cells in AD have been intensely investigated, much less is known about how macrophages contribute to the chronic skin inflammation in AD. Because of their possible versatile roles in the pathophysiology of AD, their multifaceted character, and their capacities to both promote and prevent the manifestation of allergic skin inflammation, macrophages represent promising therapeutic targets for AD in the future as well as for cancer, fibrosis, and multiple sclerosis [17].

Finally, rodent and human macrophages do not exhibit entirely similar responses to in vitro activation [29, 35]. It also remains to be determined whether there are species-dependent differences in macrophage function in vivo inasmuch as macrophage biology in humans is poorly developed because of the technical limitations of obtaining fresh material for flow cytometric analysis. Notable differences also exist between human and mouse macrophages, for example, the inability of human macrophages to increase Arg1 expression, which is an important marker of IL-4–regulated macrophages in mice. Human macrophage diversity has begun to be defined [12]. Several sequencing efforts are in progress and these will begin to address the essential need to translate mouse biology into the human context. As macrophages are known to play important roles in normal and impaired wound healing, an improved understanding of the reciprocal regulation of the macrophage phenotype and the various disease environments will provide insight into novel therapies based on manipulation of macrophage functions.

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Chapter 11
Myeloid Derived Suppressor Cells

Taku Fujimura and Alexander H. Enk

Abstract Myeloid derived suppressor cells (MDSCs) comprise a phenotypically heterogeneous population of cells, which can be found in tumor bearing mice and in patients with cancer. MDSCs play a central role in the induction of peripheral tolerance. Together with regulatory T cells (Tregs) they promote an immunosuppressive environment in tumor-bearing hosts. In addition, MDSCs convert into M2 like tumor-associated macrophages (TAMs) in the tumor to establish an immunosuppressive, tumor microenvironment. In peripheral blood organs, MDSCs contribute to systemic tolerance by producing immunomodulatory cytokines (e.g. IL-10, TGFβ). The phenotype of MDSCs differs in humans and mice, and the exact mechanisms of their suppressive function are still controversially discussed. In summary, MDSCs are a group of phenotypically heterogeneous cells of myeloid origin that have common biological activities. In this section, we discuss the definition of MDSCs, the proposed mechanisms of expansion and the recruitment and activation of MDSCs, as well as their biological activities in tumor bearing hosts to assess the potential therapeutic applications.

Keywords Myeloid derived suppressor cells • Regulatory T cells • Tumor microenvironment • Peripheral tolerance • Tumor associated macrophages • Immunosuppressive macrophages

11.1 Introduction

Myeloid-derived suppressor cells (MDSCs) comprise a phenotypically heterogeneous population of cells, which can be found in tumor-bearing mice and in patients with cancer [1–4]. MDSCs play a central role in the induction of peripheral tolerance, together with other immunosuppressive cells, such as regulatory T cells (Tregs), tumor associated macrophages (TAMs), or M2 macrophages (Fig. 11.1).

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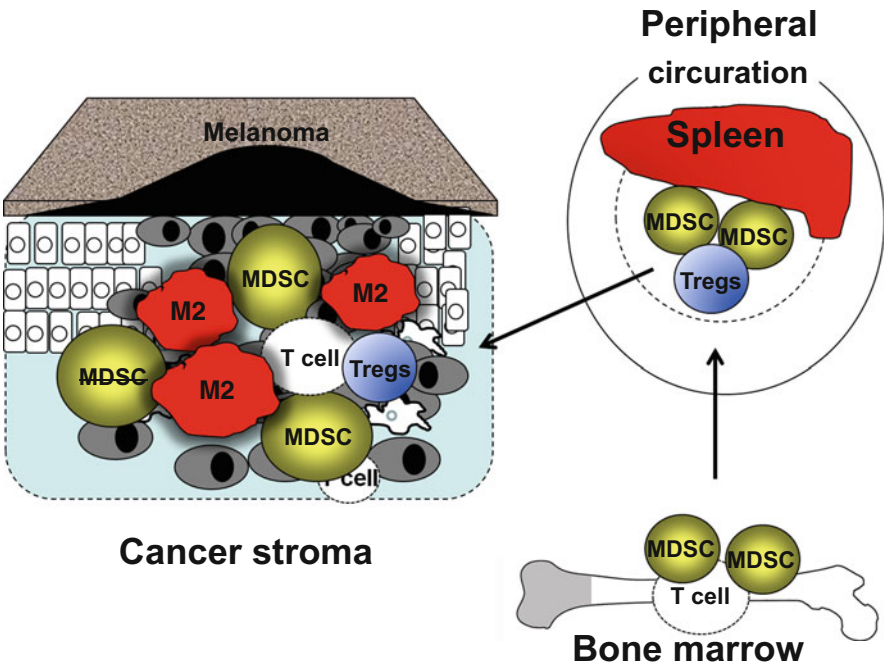

Fig. 11.1 MDSCs are a heterogeneous population of immature myeloid cells in a tumor-bearing host that suppress acquired and innate antitumor immunity through several different mechanisms. MDSCs are differentiated into M2 macrophages and, together with Tregs, maintain the immunosuppressive microenvironment in the tumor-bearing host

Recent reports suggested that MDSCs convert into M2-like TAMs in the tumor to establish an immunosuppressive tumor microenvironment [5, 6]. In peripheral blood organs, MDSCs contribute to systemic tolerance by producing immunomodulatory cytokines (e.g., IL-10, TGFβ) [1–4]. The phenotypes of MDSCs differ in humans and mice, and the exact mechanisms of their suppressive function are still controversial. In summary, MDSCs are a group of phenotypically heterogeneous cells of myeloid origin that have common biological activities. In this section, we discuss the definition of MDSCs, proposed mechanisms of their expansion, and the recruitment and activation of MDSCs, as well as their biological activities in tumor-bearing hosts to assess potential therapeutic applications.

11.2 Definition of MDSC

MDSCs are defined by function rather than as a lineage of myeloid cells, and encompass immature $CD11b^{+}$ $Ly6C^{hi}$ $Ly6G^{neg}$ monocytic (MO-MDSC) and $CD11b^{+}$ $Ly6C^{int}$ $Ly6G^{hi}$ granulocytic (PMN-MDSC) cells with a common

immunosuppressive capacity, albeit through different mechanisms [1–4]. Originally, in a mouse model, MDSCs were described as a population of $CD11b^+$ $Gr1^+$ cells that accumulate in the blood and lymphoid organs during tumor growth [1]. However, recent studies also suggested that positivity for CD11b and Gr-1 staining are not unique to MDSCs and not all $CD11b^+$ $Gr1^+$ cells are immunosuppressive [7]. For example, this $CD11b^+Gr\text{-}1^+$ cell population also includes mature neutrophils, $Ly6C^{hi}$ inflammatory monocytes, $Ly6C^{int}$ $MHCII^{neg}$ $CCR3^{hi}$ eosinophils, and TNF/iNOS-producing (Tip) DC [7]. Therefore, functional suppressive markers for MDSCs were indispensable for further definitions of MDSCs. Several markers, such as CD115 (M-CSF receptor), CD124 (IL-4Rα), B7-H1 (PD-L1), and retinoic acid early inducible-1 gene (RAE-1) are also described as being functional markers for subsets of MDSCs (Fig. 11.2) [1–3, 8]. Thus, these markers suggest the phenotypic heterogeneity of MDSCs and underscore the importance of assessing functional activities for separating MDSCs from other $CD11b^+$ $Gr1^+$ cell populations.

In humans, MDSCs are even less defined and no specific markers are yet known. For instance, human cells do not express a marker homologous to mouse Gr1. Instead of $Gr1^+$, the phenotype of MDSCs in humans is defined as $CD11b^+CD14^-CD33^+$, $CD11b^+CD14^-CD15^+$, or Lin^- $HLA\text{-}DR^-$ $CD33^+$ [9, 10]. In addition, MDSCs could morphologically be divided into two different

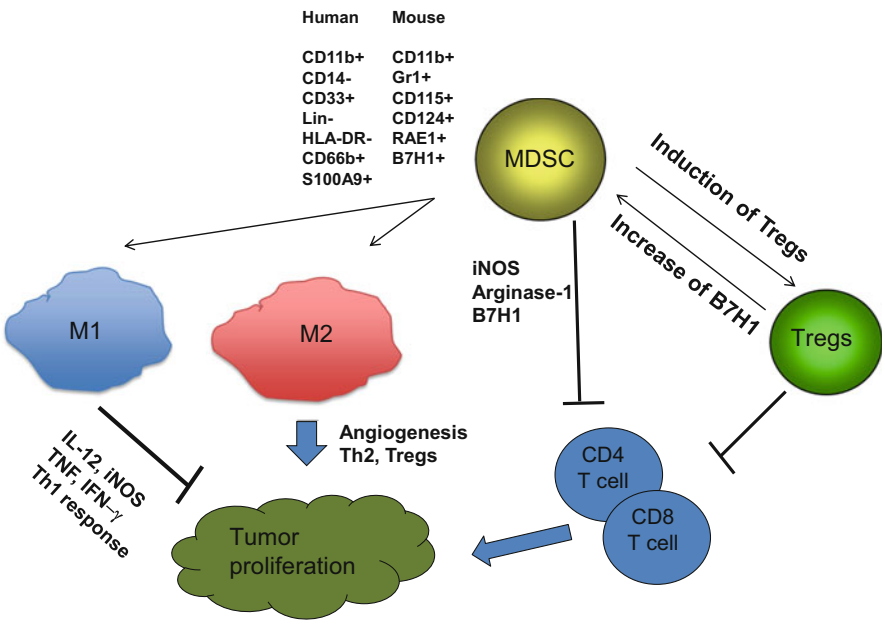

Fig. 11.2 MDSCs are a heterogeneous population of immature myeloid cells in both human and mouse that suppress acquired and innate antitumor immunity through several different mechanisms. MDSCs are differentiated into M2 macrophages and, together with Tregs, maintain the immunosuppressive microenvironment in the tumor-bearing host

phenotypes: polymorphonuclear (P-)MDSCs and monocytic (M-)MDSCs [4]. P-MDSCs possess CD66b in addition to the above MDSC phenotypes, whereas M-MDSCs were defined as $CD33^{+}CD14^{+}HLA\text{-}DR^{low/-}$ phenotypes of MDSCs. CD66b, a member of the carcinoembryonic antigen (CEA)-like glycoprotein family present on granulocytes, is reported to serve as a marker for a subpopulation of $CD11b^{+}CD14^{-}CD33^{+}CD66b^{+}$ MDSCs, which possess clearly enhanced suppressive capacity, in patients with renal cell carcinoma, bladder carcinoma, and urothelial cancer [11, 12]. More recently, $CD14^{+}S100A9^{+}$ inflammatory monocytes in patients with non-small–cell lung carcinoma were reported to represent a distinct subset of MDSCs that suppress T cells by arginase1 (Arg1), inducible nitric oxide synthase (iNOS), and the IL-13/IL-4Rα axis [13]. As MDSCs are a group of phenotypically heterogeneous cells that have only immunosuppressive activities in common, additional markers, such as S100A9 and CD66b, are noted for more clearly specifying the phenotype (Fig. 11.2). Because there are no common markers for human MDSCs in different types of tumors, further investigations are required to assess the functions of MDSC-subtypes during tumor growth and to correlate them with the prognosis of cancer patients.

11.3 Expansion, Recruitment, and Differentiation of MDSCs

To assess the immunosuppressive function of MDSCs in tumor models, it is important to investigate their systemic expansion, recruitment to the tumor site and enhancement of the suppressive function. In this section, we discuss the expansion, recruitment, and differentiation of MDSCs that are related to tumor-associated inflammation, angiogenic factors, and other chemoattractants.

11.3.1 Tumor-Associated Inflammation

Previously, pro-inflammatory cytokines, such as IL-1β, IL-6, and bioactive lipid PGE2, were reported to induce the accumulation of MDSCs, which supports the notion that tumor-associated inflammation is of importance in the expansion of MDSCs in tumor-bearing hosts. Tumor-derived IL-1β expands $Gr1^{+}CD11b^{+}$ immature myeloid cells in the spleen, which facilitates tumor growth and survival [14]. Other results indicate that this tumor-derived IL-1β does not directly activate MDSCs. Instead, IL-1β is believed to trigger an inflammatory cascade, which as a consequence induces the MDSCs by the release of other cytokines in tumor-bearing hosts [15]. As a possible candidate IL-6 is discussed. IL-6 is a cytokine located downstream of IL-1β in inflammatory responses, and Bunt et al. demonstrated that the induction of MDSCs is partially restored by IL-6 in IL-1β–deficient mice

[16]. This report clearly suggests that inflammation is able to induce MDSCs by activation via IL-1β/IL-6 pathways in tumor-bearing hosts and to facilitate tumor growth.

Elevated concentrations of TNF- α, another pro-inflammatory cytokine, are detected in pathologies characterized by chronic inflammation, including tumor-associated inflammation. A recent report suggested that TNF-α augmented the suppressive function of MDSCs during chronic inflammation via the S100A8 and S100A9 inflammatory proteins and their corresponding receptor (RAGE), which leads to the dysfunction of T and NK cells by downregulating the T-cell receptor ζ chain [17]. The authors concluded that TNF has a suppressive function on MDSCs through several pathways, including iNOS, Arg1, and radical oxygen species (ROS), and therefore has a fundamental role in promoting the immunosuppressive environment generated during chronic inflammation. In another report, S100A8/A9 proteins are reported to be able to induce MDSCs, leading to the accumulation of MDSCs at the tumor site [18, 19]. Cheng et al. demonstrated that S100A9 blocks the differentiation of myeloid precursors into functional dendritic cells or macrophages through a STAT3-dependent pathway [18]. They suggested that mice lacking this protein exhibit reduced MDSC numbers and rejected transplanted EL-4 lymphoma spontaneously. Sinha et al. also reported that S100A8/A9 complexes contribute to the recruitment of MDSCs to tumor sites through NF-κB–dependent pathways [20]. Moreover, the role of S100A8/A9 proteins is of particular interest because MDSCs produce S100A8/A9 proteins by themselves, suggesting that S100A8/A9 proteins provide an autocrine feedback loop for accumulating MDSCs at tumor sites.

In addition to cytokines, bioactive lipids, such as prostagrandin E2 (PGE2) and cyclooxygenase 2 (COX2), are produced by many tumors and are known as major contributors to the inflammatory tumor milieu. The effects of these substances on MDSC are also apparent, as Rodriguez et al. previously demonstrated that PGE2 and COX-2 amplify the Arg1 levels in MDSCs. Moreover, genetic or pharmacologic inhibition of COX-2 blocked the expression of Arg1 and induced an antitumor immune response [21]. Sinha et al. also reported that tumor-derived PGE2 and/or COX-2 significantly induced MDSCs from bone marrow precursor cells through the EP2/4 receptor. Along these lines it has also been demonstrated that treatment of tumor-bearing mice with COX-2 inhibitors reduced the frequency of MDSCs in tumors and blood and slowed down tumor growth [22]. In aggregate, these reports suggest a relationship between the expansion of MDSCs and inflammation that is mediated by the arachidonic acid cascade.

11.3.2 Angiogenetic Factor

Previous reports indicated that the vascular endothelial growth factor (VEGF) released by tumors is one of the main factors responsible for the expansion of $CD11b^{+}Gr1^{+}$ immature myeloid cells by the inhibition of DC maturation

[23, 24]. This effect seems partly to be mediated by matrix metalloproteinase-9 (MMP-9), as this protease remodels the extracellular matrix and consequently promotes the growth of new blood vessels by stimulating the production of VEGF. Indeed, Yang et al. reported the requirement of MMP-9 for the expansion and maintenance of MDSCs [25]. Furthermore, in murine mammary carcinoma models, tumor resident $CD11b^{+}Gr1^{+}$ MDSCs facilitate tumor cell invasion and metastasis through MMP activity by inducing TGFβ1 in the tumors [26]. More recently, pharmacological inhibition of MMP-9 by amino-biphosphonate, which decreases pro-MMP-9 and VEGF in the serum, was reported to abrogate the induction of MDSCs [27]. Thus these reports clearly indicate the contribution of $CD11b^{+}Gr1^{+}$ MDSC-derived MMPs to tumor invasion and metastasis.

11.3.3 Chemoattracttant Factors

Chemokines are important factors involved in shaping the tumor microenvironment. Several reports suggested that CCL2 (MCP-1)/CCR2 is necessary to attract MDSCs into the tumor microenvironment [28]. Sawanobori et al. further demonstrated that CCR2 attracted mainly macrophage-like MDSCs ($CD11b^{+}Gr\text{-}1^{int/dul}Ly\text{-}6C^{hi}$), which directly interfere with tumor growth [28]. More recently, Gehad et al. reported the expression of CCR2 on MDSCs from patients with cutaneous squamous cell carcinoma (SCC) [29]. They concluded that SCC itself expressed human β-defensin, which contributed to the migration of CCR2-expressing MDSCs at the tumor site. Thus CCR2 may shape the tumor-infiltrating MDSCs towards a macrophage-like, tumor-promoting phenotype. In another report, Pan et al. demonstrated that tumor-derived stem cell factor (SCF) leads to myelopoiesis and the expansion of MDSCs by inhibiting the differentiation of myeloid precursor to functional dendritic cells [30]. Thus, these data may suggest that, in the absence of other DC-differentiating factors, the default pathway of some myeloid precursors may be the development of MDSC subpopulations. SDF-1/CXCR4 and CXCL5/CXCR2 contribute to the recruitment of MDSCs to the tumor site [26]. In humans, increased levels of PGE2 are associated with the accumulation of MDSC via SDF1/CXCR4 pathways [31]. However, in contrast to SCF, these chemokines specifically recruit already fully developed MDSCs and do not interfere with their differentiation from precursors to mature MDSCs.

11.4 Mechanisms of Suppressive Function of MDSC

To exert their suppressive activity, MDSCs must be activated. Indeed, several factors have been reported to stimulate the suppressive function of MDSCs. IL-4, IL-13, IFN- γ, IL-1β, and TGF−β are known to activate several different pathways in MDSCs that involve STAT6, STAT1, and nuclear factor-κB (NF-κB). STAT1,

STAT3, and STAT6 have been described as having distinct roles in macrophage polarization [2]. STAT3 regulates the expansion of MDSCs through the S100A9/N-glycan pathway [18] and the accumulation of reactive oxygen species (ROS) [32], however, STAT6 and STAT1 regulate the activation of MDSCs to express Arg1 and iNOS [1, 33, 34]. IL-4 and IL-13 were reported to activate STAT6 signaling through the IL-4Rα pathway [34], whereas IL-1β and IFN-γ activate the STAT1 signaling cascade [35]. In addition to inducing Arg1 and iNOS, the IL-4Rα/STAT6 pathway also elicits the production of TGF-β by MDSCs [35].

Part of the suppressive function of MDSCs is mediated by the metabolism of L-arginine. It has been suggested that MDSCs express high levels of Arg1, which inhibits T-cell proliferation [1–4, 36, 37]. Arg1 enhances L-arginine catabolism, which causes a shortage of L-arginine in the tumor microenvironment and inhibits T-cell proliferation by decreasing their expression of the CD3-ζ chains [38] and by the induction of cell cycle arrest in proliferating T cells in the G_0–G_1 phase [38]. More recently, Mace et al. reported that the suppressive function of Arg1 in both circulating and infiltrating MDSCs is a downstream target of activated STAT3 [39]. In another study, they also reported that cancer stromal cells, such as pancreatic-cancer–associated fibroblasts, drive the differentiation of MDSCs through STAT3-dependent pathways in pancreatic cancer [39], which suggested that MDSCs could augment their suppressive function by soluble factors produced by stromal cells in the tumor microenvironment.

Immunomodulatory costimulatory molecules, such as B7-homologue families, are other pathways for the suppressive mechanisms of myeloid cells. Recently, several reports suggested that the expression of immunomodulatory costimulatory molecules, B7H1 (also reported as PD-L1, CD274), on MDSCs are essential for antigen-specific tolerance induction [3, 8, 40]. The uptake of specific antigens induces the production of IL-10 from myeloid cells, which leads to upregulation of the expression of B7H1 on myeloid cells [40]. In addition, the expression of B7H1 not only directly suppresses T-cell proliferation via B7H1/PD-1 pathways, but also induces tolerance by maintaining regulatory T cells [8, 40]. Furthermore, another B7-family member, B7H4, was also reported to affect the immunosuppressive activity of MDSC [41]. Leung et al. reported that $CD11b^+$ splenic MDSCs from B7H4 KO tumor-bearing mice had much more potent suppressive activities against T-cell proliferation compared with their WT counterparts [41]. The production of ROS also contributes to the activity of suppressive macrophages, as increased ROS levels in macrophages induce the upregulation of several subunits of NADPH oxidase. Further effects of macrophage-derived ROS include (i) induction of DNA damage in immune cells resident in the tumor microenvironment, (ii) inhibition of the differentiation of macrophages into functional DCs, and (iii) recruitment of macrophages to the tumor site [1–4]. Moreover, extracellular ROS catalyze the nitration of TCR, which consequently inhibits the T cell–peptide–MHC interaction, resulting in T-cell suppression [42].

11.5 Immunosupportive Therapy by Targeting MDSCs

After expansion in the periphery, MDSCs migrate to tumor sites and become activated to express Arg1 and their suppressive factors, such as IL-10 and TGF-β, to induce an immunosuppressive tumor microenvironment [2, 3, 5, 43]. Indeed, a previous report suggested that the levels of Arg1 in tumor sites are much higher as compared to the periphery [2]. Another report also suggested that MDSCs differentiate into matured M2 macrophages in tumor sites [5, 6]. These reports indicated that a chemoattractant factor produced in tumor sites was indispensable for the migration of these suppressive macrophages and could be a target for the immunotherapy for cancer patients. From the above reports, MDSC-targeted antitumor therapies are based on: (i) depletion of MDSCs, (ii) differentiation of MDSCs into immunogenic macrophages, and (iii) inhibition of the recruitment of MDSCs to tumor sites.

11.5.1 Depletion of MDSCs

Depletion of immunosuppressive macrophages has been reported as one of the possible supportive therapies for tumor-bearing hosts [1–4, 44–46]. Among them, Rogers et al. reviewed the therapeutic effect of bisphosphonate on breast cancer [46]. They concluded that bisphosphonate, such as zomedronic acid, can target tumor cells to increase apoptotic cell death and decrease proliferation, migration, and invasion as well as target immunosuppressive macrophages to inhibit the production of pro-angiogenic factor MMP9 and trigger the reversal of the TAM phenotype from M2 to M1 [46]. Previous reports also suggested the additional effects of cytotoxic chemotherapeutic drugs on MDSCs [1–3, 47, 48]. For example, a noncytotoxic dose of paclitaxel decreased MDSCs and even blocked the immunosuppressive potential of MDSCs in a mouse melanoma model [47]. In another report, Suzuki et al. described that gemcitabine was able to reduce specifically the number of $CD11b^{+}Gr1^{+}$ MDSCs in the spleen [49]. In contrast, another chemotherapeutic drug, cyclophosphamide, increased monocytic MDSCs and had no beneficial antitumor effects on mouse spontaneous melanoma, and even a decrease of Tregs was observed [50]. Bruchard et al. reported that the administration of 5-Fluorouracil (5-FU) decreased MDSCs at an early time point in the therapy, but later increased the production of IL-1β from MDSCs, and promoted Th17-mediated tumor growth in vivo [51]. More recently, Görgün et al. reported that lenalidomide decreased the number of MDSCs in patients with multiple myeloma without modifying the suppressive function of MDSCs [52]. Furthermore, an additional immunological effect of a molecular targeting drug, Vemurafenib, was that it decreased both CD14-CD66b+Arg1+ granulocytic MDSCs and CD14+HLADR-/low monocytic MDSCs in patients with advanced melanoma [53]. These reports

strongly suggest the importance of assessing the effects of chemotherapeutic drugs on MDSCs when clinicians develop novel protocols for cancer patients.

11.5.2 Differentiation of MDSCs into Immunogenic Macrophages

Because MDSCs represent a group of cells of myeloid lineage at different stages of differentiation, the induction of differentiation into less harmful phenotypes of macrophages or functional dendritic cells (DC) is another therapeutic approach to avoid the immune suppression of antitumor responses. Previous reports suggested that the polarization of macrophages largely depends on the local cytokine profiles [1, 2, 10, 45]. It is known that attenuated NF-kB activation in TAMs mediates their immunosuppressive M2 property and that NF-kB reactivation can redirect TAMs to a tumoricidal M1-like phenotype [54]. In another report, Kodumudi et al. suggested that docetaxel treatment polarized MDSCs towards an M1-like phenotype, resulting in upregulation of macrophage differentiation markers such as MHC class II, CD11c, and CD86 in an in vivo mouse mammary tumor model [5]. Bisphosphonate, such as zomedronic acid, can also trigger the reversal of the TAM phenotype from M2 to M1, as we described above [47]. All-*trans* retinoic acid (ATRA) was reported to induce the differentiation of MDSCs into functional DC or macrophages [55]. The mechanism of all-*trans* retinoic acid-mediated anti-immunosuppressive effects may be twofold: at first, the administration of ATRA results in the differentiation of immunosuppressive macrophages into functional DC [56], which can then contribute to the augmentation of the anticancer immune responses by stimulating effector T cells [57]; secondly, ATRA reduces the ROS levels in MDSCs, which significantly reduces the recruitment of MDSCs to tumor sites [55].

11.5.3 Inhibition of Recruitment of Macrophages to the Tumor Site

After expansion in the periphery, MDSCs migrate to tumor sites and become activated. Several chemokines and cytokines are reported to co-relate with the recruitment of macrophages to tumor sites. Among them, CCL2 (MCP-1)/CCR2 was reported as one of the main chemotactic factors in the migration of macrophages to the tumor environment, which even correlated with a poor prognosis in cancer patients [58, 59]. Roca et al. reported the role of CCL2 and IL-6 in the survival of myeloid monocytes recruited to the tumor microenvironment and their differentiation towards tumor-promoting M2-type macrophages via inhibition of caspase-8 and enhanced autophagy [58]. In a mouse model, Sawanobori et al. demonstrated that CCR2-attracted MDSCs are mainly macrophage-like

MDSCs ($CD11b^{+}Gr\text{-}1^{int/dul}Ly\text{-}6C^{hi}$), which directly interfere with tumor growth [28]. As a clinical application, recently, a CCL2-targeting drug, trabectedin, has been applied to ovarian cancer and myxoid liposarcoma to suppress the recruitment of monocytes to tumor sites and inhibit their differentiation [26, 60]. CXCL12/CXCR4 and CXCL5/CXCR2 contribute to the recruitment of MDSCs to the tumor site [31]. But these chemokines specifically recruit already fully developed MDSCs and do not interfere with their differentiation from precursors to mature MDSCs. VEGF and CCL5 are other chemoattractants for macrophages, which can be potential targets for antitumor treatment [45]. Indeed, Dinnen et al. reported that selectively inhibiting VEGFR-2 reduced macrophages and prevented angiogenesis in orthotropic pancreatic tumors [61]. In aggregate, the inhibition of various types of chemoattractants for macrophages could be novel targets for the induction of antitumor immune responses in the tumor microenvironment.

11.6 Concluding Remarks

MDSCs are a group of heterogeneous immature myeloid cells that have common biological activities, i.e., the suppression of immune responses. Although several studies suggested that the presence of high numbers of MDSCs in tumor-bearing individuals is associated with poor prognosis, further investigations will be required to quantify the impact of MDSCs on survival in different cancers. Clinical studies for targeting MDSCs are limited and the expanding phenotypes of MDSCs (e.g., granulocytic cells, monocytic cells, polymorphonuclear cells) are difficult to discriminate in tumors of different origin. In this aspect, MDSCs must be more clearly characterized to make them suitable as a clinical target. Because the abrogation of the MDSC function in tumor-bearing mice has been shown to amplify antitumor immune responses, targeting of MDSCs may be an optimal supportive therapy for cancer immunotherapies in humans.

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Chapter 12
Lymphatic Vessels

Satoshi Hirakawa and Michael Detmar

Abstract Lymphatic vessels play important roles in maintaining skin homeostasis and immune surveillance. Cutaneous lymphatic vessels promote the recruitment and transport of immune cells to regional lymph nodes. Pathological impairment of lymphatic vessel function increases the risk of infection and of the development of certain types of tumors. New lymphatic vessel growth called lymphangiogenesis occurs during embryonic development and in pathologic settings. A major part of cutaneous lymphatic vessels develops from veins. Prox1 and vascular endothelial cell growth factor (VEGF)-C play an essential role in the initial formation of lymphatic vessels. A subpopulation of progenitors for lymphatic endothelial cells contributes to the development of cutaneous lymphatic vessels in mice. Pathologic lymphangiogenesis occurs during tumor development and in chronic inflammation. Tumor-associated lymphangiogenesis promotes tumor metastasis in draining lymph nodes and is associated with reduced overall patient survival. Note that new lymphatic vessel growth occurs in the lymph nodes prior to tumor metastasis, facilitating the progression of tumor development. Chronic skin inflammation in mice induces hyperplasia of lymphatic vessels with functional impairment, leading to the reduced transport of lymphatic fluid and prolonged inflammation. Recovery of lymphatic vessel function by VEGF-C treatment results in marked reduction of cutaneous inflammation, indicating that lymphatic vessels play a key role in the regulation of the inflammatory response in the skin. This chapter introduces recent discoveries of the molecular mechanisms in lymphatic vessel biology and of the pathological roles of lymphatic vessels, leading to a better understanding of skin immunology.

Keywords Lymphangiogenesis • Prox1 • Vascular endothelial growth factor-C • Tumor progression • Chronic inflammation

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12.1 Introduction: An Essential Role of Lymphatic Vessels in the Skin

Many organs including skin contain lymphatic vessels that absorb interstitial tissue fluid and macromolecules, and transport them to venous circulation [1]. The skin is a barrier that protects the body from the environment outside. Therefore, the immune system is required for the skin to avoid bacterial or viral infections. Furthermore, numerous pathogens including insects contact our body surface and induce inflammatory tissue responses in the skin. Therefore, different types of immune cells are recruited and play a key role in promoting immune surveillance. Cutaneous lymphatic vessels arise in the dermis and connect the skin to regional lymph nodes. In addition to fluid transport, these lymphatic vessels take up immune cells such as Langerhans cells, which migrate to the lymph nodes and induce an inflammatory tissue response.

12.2 Development of Cutaneous Lymphatic Vessels in Mice

Emerging evidence reveals that lymphatic vessels arise from veins during embryonic development in mammals. Florence Sabin, an anatomist in the early twentieth century, proposed this concept based on her findings in pig skin [2, 3]. The fundamental finding was confirmed by molecular biology techniques utilizing knockout mice in the last decade of the twentieth century [4]. Lymphatic vessel development requires several key molecules that induce progenitors of lymphatic endothelial cells, leading to the development of primitive lymph sacs. Prox1, a homeobox transcription factor, promotes the reprogramming of venous endothelial cells to lymphatic endothelial cells. Genetic inactivation of *prox1* leads to the loss of lymphatic vessels and embryonic lethality in null mice. Lack of *prox1* impairs the sprouting of lymphatic endothelial cell progenitors, leading to the formation of lymphedema in null mice. During mouse embryogenesis at E9.5, Prox1 is induced in a subpopulation of endothelial cells in the cardinal vein [4]. Thereafter, Prox1-positive lymphatic progenitors sprout from cardinal veins and further form primitive lymph sacs. Thus, the crucial findings in *prox1* knockout mice confirmed Sabin's theory. Another key molecule that induces the development of lymphatic vessels is vascular endothelial growth factor (VEGF)-C. An essential role of VEGF-C in lymphatic vessel development was shown by genetic inactivation in mice [5]. VEGF-C is a ligand for VEGF receptor-2 as well as VEGFR-3 which specifically promotes new lymphatic vessel development during mouse embryogenesis. Genetic inactivation of *vegf-c* causes the lack of primitive lymph sacs during embryonic development, leading to the loss of lymphatic vessels in null mice. Activation of VEGFR-3/Flt4, a tyrosine kinase with *trans*-membrane domain, induces lymphangiogenesis in physiological conditions [6]. A missense mutation in the tyrosine kinase domains in *Flt4* impairs lymphatic vessel development in

humans, leading to the formation of primary lymphedema called Milroy's disease. Recent studies identified that several types of missense mutation are found in the pedigrees that develop Milroy's disease [6].

FOXc2, a member of the forkhead box family of transcription factors, plays a key role in promoting valve formation during lymphatic vessel development [7]. Genetic inactivation of *Foxc2* results in the loss of valves in collecting lymphatic vessels and the abnormal accumulation of pericytes surrounding initial lymphatic vessels, leading to the formation of lymphedema in null mice [7]. Mutations in human *Foxc2* are associated with primary lymphedema in the lymphedema–distichiasis syndrome which also includes accessory eyelashes [6]. Patients with primary lymphedema suffer from common infections such as warts and tinea, indicating that impaired development of cutaneous lymphatic vessels allows the increased incidence of viral and fungal infection in the skin. Indeed, cutaneous lymphatic vessels are essential for host defense and maintenance of immune surveillance.

Recent studies have challenged the concept that the lymphatic vessels arise from veins. The initial concept proposed by Sabin was independently confirmed by several groups, and has been widely accepted [4, 8, 9]. However, the exact origin of lymphatic vessels in mice remains controversial and may be heterogeneous in several organs including the skin [10, 11]. Dermal lymphatic vessels in cervical and thoracic areas predominantly have a venous origin, whereas a subpopulation of nonvenous-derived progenitors contributes to the formation of cutaneous lymphatic vessels in the lumbar areas [11]. Mesenterium and dorsal subcutaneous tissue of transgenic mice that express enhanced green fluorescent protein (EGFP) under control of the Prox1 promoter show a well-developed lymphatic vessel network by stereoscopic fluorescence microscopy (SMZ25, Nikon, Tokyo). Neonatal mice 7 days after birth demonstrate a prominent network of subcutaneous lymphatic vessels in the lateral sides of the lumbar region (Fig. 12.1a). These lymphatic vessels migrate to the midline, whereas no connections are found between the left and right side. A high-power field image shows the active migration of lymphatic vessels in the midline as compared with the lateral side (Fig. 12.1b). In contrast, adult mice 1 year after birth show a consistent network of subcutaneous lymphatic vessels that are connected in the midline (Fig. 12.1c). A high-power field image shows the thin structure of lymphatic vessels in the subcutaneous tissue (Fig. 12.1d). Accordingly, lymphatic vessels are present in the intestine and prominent in the mesenterium in adult transgenic mice (Fig. 12.1e). Within the lymphatic vessels of Prox1-EGFP mice, valves strongly express EGFP (Fig. 12.1f), indicating that Prox1 is required for the maintenance of valves in the lymphatic vessels during the postnatal period.

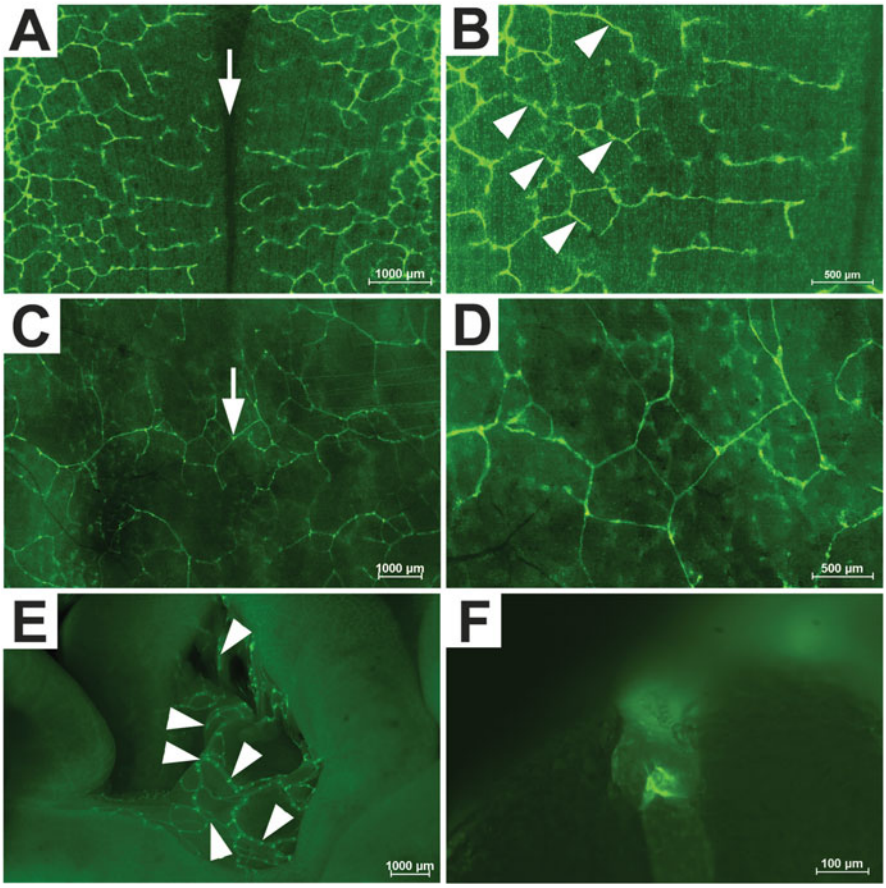

Fig. 12.1 Lymphatic vessels and valves shown by enhanced green fluorescent protein driven by Prox1 promoter in transgenic mice. (**a**) Cutaneous lymphatic vessels are found in dorsal skin 1 week after birth. Lymphatic vessels are absent in the center median site (*arrow*) (**b**) High-power view. Lymphatic vessels show enlargement in the lateral side (*arrowheads*) and penetration to the median site. (**c**) Cutaneous lymphatic vessels in the dorsal skin 1 year after birth. Lymphatic vessels are connected at the median site (*arrow*) (**d**) High-power image demonstrates the junction of the lymphatic vessels. (**e**) Lymphatic vessels in mesentery (*arrowheads*) in transgenic mice. (**f**) Lymphatic valves are prominently found in mesenteric lymphatic vessels. Scale bars: 1,000 μm (**a**–**e**); 500 μm (**b**–**d**); 100 μm (**f**)

12.3 Role of Tumor Lymphangiogenesis in Cancer Metastasis

Lymphangiogenesis is induced in pathological conditions such as tumor progression [12]. Recent studies have identified that several types of cancer including cutaneous malignant melanoma induce new lymphatic vessel growth in the primary site [13, 14]. Note that tumor lymphangiogenesis actively promotes sentinel lymph

node metastasis in melanoma, leading to reduced patient survival. VEGF-C, a key growth factor in physiological lymphatic vessel development, was found to induce tumor lymphangiogenesis and enhanced lymph node metastasis in experimental animal models [15, 16]. Tumor lymphangiogensis is also induced in several types of human cancer, and numerous studies found that increased expression levels of VEGF-C are found in the primary sites, and are associated with increased incidence of sentinel lymph node metastasis. The tumor microenvironment recruits several types of inflammatory cells including macrophages, which also express VEGF-C [17, 18].

Tumor-induced lymphangiogenesis was originally identified in experimental animal models. These studies used VEGF-C or VEGF-D as lymphangiogenic growth factor in overexpression models [15, 16, 19]. Moreover, targeted overexpression of VEGF-A in mouse skin also promotes tumor lymphangiogenesis as well as angiogenesis [20]. VEGF-A promotes distant organ metastasis by induction of new blood vessel formation in several types of cancer. Thus, VEGF-A and its receptors are common molecular targets for clinical therapies. Bevacizumab, a neutralizing antibody against human VEGF-A, and several tyrosine kinase inhibitors targeting VEGF receptors are currently utilized for patients undergoing metastatic malignancy such as colon cancer. Thus, lymph node metastasis may be another target for the drugs that functionally impair VEGF receptors and their signal pathways.

12.4 Role of Lymph Node Lymphangiogenesis in Tumor Progression

Lymph node metastasis is associated with reduced overall patient survival in several types of cancer. Primary tumors may induce new lymphatic vessel growth in draining lymph nodes prior to metastasis. Targeted overexpression of either VEGF-A or VEGF-C in mouse skin showed active promotion of lymph node lymphangiogenesis in a standard multistep chemically induced skin carcinogenesis model [20, 21]. It is important to note that lymph node lymphangiogenesis also promoted enhanced metastasis to distant sites, indicative of an advanced tumor progression.

Lymph node lymphangiogenesis represents a premetastatic niche that promotes the initial steps of cancer metastasis. Furthermore, recent studies showed that the premetastatic niche plays a key role in maintaining the survival of cancer stem cells as well as the promotion of metastasis. Lymphatic endothelial cells have been found to suppress immune response in lymph nodes [22]. Therefore, further studies are needed to investigate the significance of lymphatic vessel development in lymph nodes by focusing on the interaction between tumor cells and lymphatic endothelial cells.

12.5 Role of Lymphatic Vessels in Inflammatory Diseases

In contrast to the well-defined role of the blood vascular system in mediating skin inflammation [23, 24], the role of the lymphatic system has been poorly researched and has remained largely unknown. Similar to blood vessels [23], lymphatic vessels have been found to be enlarged in psoriatic skin lesions [25]. However, enlargement of lymphatic vessels might reflect impaired drainage function as well as enhanced fluid uptake, and several diverging functions of lymphatic vessels in inflammation have been proposed. Lymphatic vessels might contribute to inflammatory reactions based on the transfer of antigen-presenting cells to draining lymph nodes, and based on the local release of pro-inflammatory mediators [24]. On the other hand, lymphatic vessels might contribute to the resolution of inflammation by draining inflammatory mediators and cells away from the site of inflammation. Recent studies indicate that lymphatic vessels might indeed mediate anti-inflammatory effects.

The first evidence for this came from a model of chronic skin inflammation, where sensitization of keratin 14 (K14)/VEGF-A transgenic mice with oxazolone, followed by challenge of the ear skin with oxazolone 5 days later, led to a chronic, psoriasis-like skin inflammation [25]. In this model, the lymphatic drainage function was greatly impaired in the chronically inflamed skin [26]. Treatment with an antibody against VEGFR-3 resulted in enhanced inflammatory skin swelling [26]. Conversely, when K14/VEGF-A transgenic mice were crossed with K14/VEGF-C mice, which express the potent lymphangiogenesis factor VEGF-C in the skin and have an increased number of cutaneous lymphatic vessels, the oxazolone-induced inflammatory reaction was reduced and there was no development of the chronic, psoriasis-like skin inflammation [26]. Note that this was associated with improved lymphatic clearance function [26], indicating that enhanced drainage of inflammatory mediators and cells from the inflamed skin contributed to the anti-inflammatory effect. A similar effect was seen when the K14/VEGF-A transgenic mice were crossed with K14/VEGF-D transgenic mice that express the related lymphangiogenic factor VEGF-D in the skin. Intralesional injection of recombinant VEGF-C protein over 7 days also resulted in significantly decreased skin inflammation [26]. These results were supported by the finding that the draining function of lymphatic vessels is reduced in chronic UV damage of mouse skin which includes inflammatory changes [27]. It remains to be investigated whether the observed improvement of diabetic wound healing in mice after VEGF-C treatment [28] might also be related to the anti-inflammatory effects of VEGF-C–induced lymphatic vessel activation in the skin.

Beyond chronic inflammation, activation of lymphatic vessels was also found to reduce acute skin inflammation in mice. Induction of contact hypersensitivity reactions in K14/VEGF-C and K14/VEGF-D transgenic mice, which have an increased number of dermal lymphatic vessels, was associated with reduced tissue swelling [29]. Similarly, the inflammatory response to acute UVB irradiation was also reduced in both genetic mouse models [29]. Intradermal injection of a mutated

VEGF-C protein that specifically recognizes VEGFR-3 significantly inhibited the inflammatory cutaneous swelling after a single UV irradiation, associated with reduced numbers of CD11b-positive inflammatory cells [27].

The identification of an important role of lymphatic vessels in limiting inflammation also has importance beyond dermatological diseases. Indeed, it was found that the lymphatic drainage function is impaired in rheumatoid arthritis, and that adeno-associated virus (AAV)-mediated delivery of VEGF-C attenuates joint damage in chronic experimental inflammatory arthritis [30]. Lymphatic vessels are also enlarged in inflammatory bowel disease, and inhibition of lymphatic function with an anti-VEGFR-3 antibody resulted in increased inflammation in an experimental inflammatory bowel disease model [31]. Conversely, very recent results indicate that VEGF-C reduces inflammation in experimental inflammatory bowel disease [32].

In summary, there is increasing evidence for an anti-inflammatory activity of cutaneous lymphatic vessels that show reduced activity in chronic skin inflammation. Therefore, activation of lymphatic function via VEGF-C or other mediators might represent a new therapeutic strategy to treat inflammatory skin diseases [33].

12.6 Perspective

Pathological lymphangiogenesis occurs in tumor progression and chronic inflammation. Several growth factors such as VEGF-A promote new lymphatic vessel growth and alter the physiological function in experimental models. Note that VEGF-C potently improves the lymphatic vessel function in pathological settings. Thus far, the molecular regulation of lymphatic vessel development and the functional regulation by key mediators has been studied in detail. Future investigations are needed to investigate the importance of lymphatic vessel interaction with immune cells during tumor development and inflammation, potentially leading to new therapeutic approaches for the treatment of skin diseases.

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Chapter 13
Hair Follicles

Manabu Ohyama and Keisuke Nagao

Abstract The hair follicle (HF) is a skin appendage providing physical barrier to protect mammals from external stimuli. HF has been attracting great interest as an object of developmental and stem cell research, however, little attention has been paid to HF in the light of immunology. Previous observations supported that HF might enjoy immune privilege by suppressing immune-related antigen expression and producing immunosuppressive molecules, indicating HF possesses intrinsic machinery to escape from unwanted immune response. The recent discovery that HF segments differentially express chemokines, both chemoattractive and possibly suppressive, suggested that HF is not merely an innocent bystander but may play previously unrecognized roles in the regulation of immune reactions in skin. In this chapter, basic knowledge of hair follicle morphology and physiology is summarized. The similarities and differences between mouse pelage and human scalp hair follicles is presented, which is crucial for translational research. Finally, immunological features of hair follicle segments is discussed.

Keywords Hair follicle • Immune privilege • Bulge • Chemokine • Alopecia areata

13.1 Introduction

The hair follicle (HF) is a mammalian skin appendage that produces the hair shaft and provides a physical barrier against external insults [1]. One of the most characteristic features of HF is that it cyclically self-renews throughout the life of mammals [2, 3]. After the seminal discovery that HF harbors stem cell populations in the bulge area [4] that maintain HF homeostasis and possesses multipotency to repopulate sebaceous glands and the epidermis in wound repair [5–7], investigation related to HF has centered on stem cell biology or regenerative medicine.

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In most mammals, HF is indispensable for their survival, and thus it is reasonable to speculate that HF is equipped with some machinery to escape from unwanted immune responses. Indeed, HF has been considered to be an immune-privileged (IP) structure [8]. Previous studies demonstrated downregulation of immune-related molecules and upregulation of immune inhibitors in anatomically distinct portions of HF, putatively granting IP to HF [9]. With IP, HF has been thought to be an innocent bystander of immunological events. However, the recent discovery that anatomically distinct HF segments differentially produce chemokines implies active roles of HF as a key immune regulator in skin [10].

In this section, the fundamental knowledge on HF morphology and physiology is introduced. Subsequently, immunological aspects of respective HF segments will be briefly summarized.

13.2 Fundamental Knowledge of the Hair Follicle

13.2.1 Morphology and Microanatomy

The main body of HF consists of multiple cylindrical layers of keratinocytes (KCs) surrounding the hair shaft [3, 11] (Fig. 13.1). The layers are largely divided into two distinctly differentiated hollow cylinder structures, the inner and outer root sheath, which are separated by the companion layer and individually subdivided into several layers [3, 11, 12] (Fig. 13.1). The dermal papilla, a highly specialized dermal cell aggregate regulating hair shaft production and hair cycle, locates at the proximal end of the main body [13] (Fig. 13.1). The whole structure is wrapped by a connective tissue sheath [13]. HF is accompanied by the sebaceous gland and the sebaceous duct opens into the space between the hair shaft and the outer root sheath above the level where the inner root sheath keratinocytes are terminally keratinized and lost [3, 11, 12]. A part of the outer root sheath providing an insertion point for the arrector pili muscle is called the bulge, an area that harbors HF stem cell populations [4] (Fig. 13.1).

A full-grown anagen HF is split into five anatomical substructures: infundibulum (the epidermal surface, the sebaceous duct), isthmus (the sebaceous duct, the bulge), bulge, suprabulb (between the bulge and the bulb), and bulb [3, 11, 12] (Fig. 13.1). It is important to note that those HF segments differentially express a panel of cell surface markers which enable targeted cell isolation of individual segments in mice [10], however, such markers have not been established in human HF.

13.2.2 The Bulge Area Provides the Stem Cell Niche

Using a label-retaining cell technique, quiescent follicular keratinocyte stem cells were identified in the bulge area both in mouse and human HF [4, 14, 15].

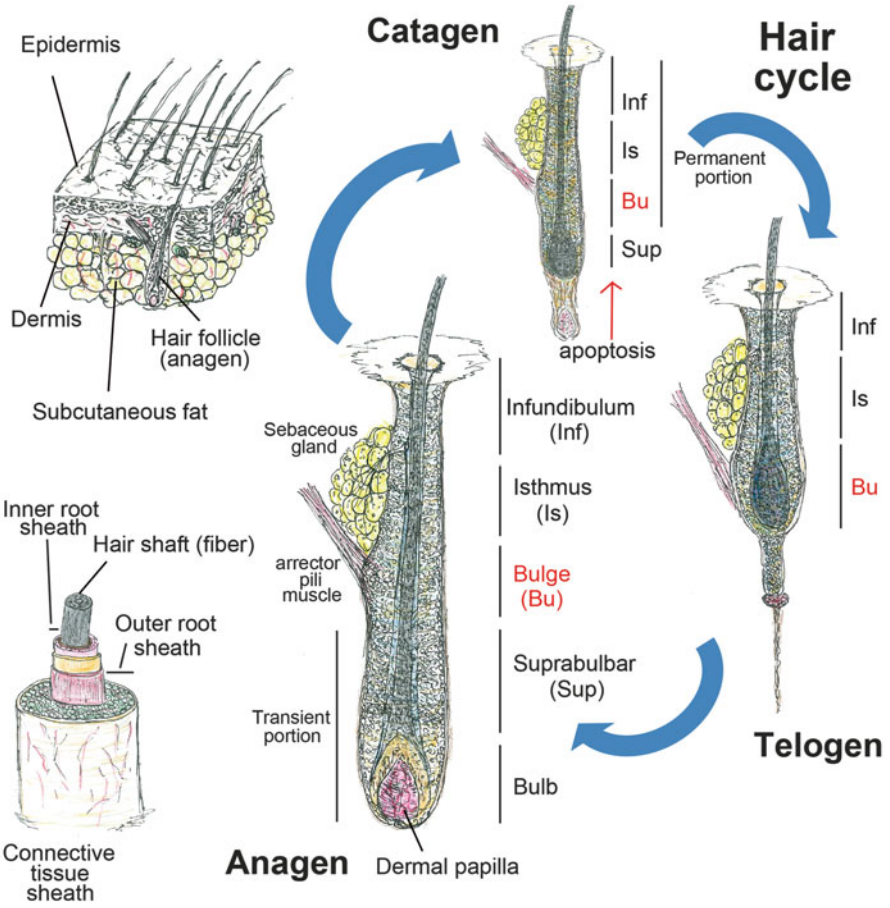

Fig. 13.1 The structure of the hair follicle and the hair cycle. Fully grown anagen hair follicle extends deeper into subcutaneous fat. Anagen hair follicle is divided into five anatomical segments: infundibulum, isthmus, bulge, suprabulbar portion, and bulb. The suprabulbar (Sup) and the bulb regress by apoptosis in catagen phase. The bulge harbors stem cells and marks the bottom of telogen hair follicle

Colony formation assay demonstrated that they possess high proliferative capacity [6, 15–17]. Lineage tracking experiments elucidated that bugle stem cells are indispensable for the maintenance of HF homeostasis and multipotent to regenerate the sebaceous gland and the epidermis, when the stem/progenitor cells maintaining those structures are damaged [7]. Keratin 15 (mouse and human) [14, 17], 19 (human) [18], alpha6-integrin (mouse) [6, 18], CD34 (mouse) [19], and CD200 (human) [15] have been used as preferential markers for bulge stem cells. Later studies elucidated that several cell populations marked by MTS24 [20], Lrig1 [21], Lgr5 [22] and 6 [23], and Gli1 [24] expression also possess stem cell capacities, represented by the potency to repopulate the pilosebaceous unit and

the epidermis. A recent study uncovered that melanocyte stem cells also reside in the bulge area, just below the KC stem cell niche [25].

13.2.3 Hair Cycle and the Change in HF Structure

Cyclic self-renewal is intrinsic to HF [3, 11]. The hair cycle is composed of three major stages: anagen (growing phase), catagen (regression phase), and telogen (resting phase) [3, 11] (Fig. 13.1). In the anagen phase, the size of HF is at the maximum. All five HF segments can be observed and the matrix cells located over the dermal papilla actively proliferate to produce the hair shaft [3, 11]. When HF shifts into catagen, proximal portions below the bulge (transient portion) start to regress by apoptosis to leave the permanent portion of HF [3, 26] (Fig. 13.1). During this process, the dermal papilla is pulled up by regressing structure to be at the bottom of telogen HF [3, 26]. The duration of anagen or telogen is greatly different among species and anatomical locations, but that of catagen is short in common [3].

13.2.4 Dissimilarities Between Mouse and Human Hair Follicles

Mice pelage HFs have been predominantly used for the studies to dissect basic HF biology, whereas human scalp HFs have been preferentially utilized as materials for clinical/translational research. Thus, it is beneficial to recognize biological distinctiveness clearly between these major resources for HF investigation.

Except for their difference in size, the fundamental morphology and physiology of HFs are conserved between mice and humans [27]. Thus, mice HFs provide useful materials to dissect human HF biology to some extent. In mice, pelage HFs concertedly cycle in waves starting from head to tail, whereas human scalp HFs randomly cycle. Most mice pelage HFs stop growing when they reach their destined length and stay in telogen, in the same way as human pelage HFs do; in contrast, human scalp HFs continue to grow over several years [11, 26, 27]. In mice pelage HF, shedding of the hair shaft is often delayed and that produced in one hair cycle before remains over the next telogen, but this phenomenon is rarely seen in human scalp HFs [27]. The bulge can easily be recognized as a perturbation of the outer root sheath in anagen HFs and epithelial sac-like structure in telogen HFs, respectively, whereas it can hardly be distinguished in human anagen HFs morphologically [4, 11, 15, 28]. Some biochemical differences have been noted between mouse and human bulge. For instance, CD34, a commonly used cell surface marker for mouse bulge stem cells, is not upregulated in human bulge [15].

Understanding of those similarities and dissimilarities is indispensable for appropriate interpretation and application of mice data to human subjects.

13.3 Immunological Features of Respective Hair Follicle Compartments

13.3.1 The Infundibulum~the Isthmus

Various leukocyte subsets, including Langerhans cells (LCs) and $CD4^+$ and $CD8^+$ T cells, tend to accumulate around the infundibulum~isthmus, an anatomical front-line against external insults, including microorganisms [29–32].

When mouse infundibular and isthmus KCs of pelage telogen HFs were isolated by differential expression of cell surface markers Sca1 and EpCAM (infundibular KCs, Sca-1^+EpCAM^{int}; isthmus KCs, Sca-1^- EpCAM^{hi}), they were shown to express higher levels of CCL20, a CCR6 ligand and CCL2, a CCR2 ligand, respectively, compared to other HFKC subsets [10]. Similarly, gene expression analysis of microdissected human HF detected upregulation of CCL2 and CCL20 in the infundibulum [10]. CC-chemokine receptor 2 (CCR2) and CCR6 have been shown to be necessary to recruit LC and its precursors to the epidermis [10].

These findings support that HFs may chemoattract leukocytes to the infudibulum~isthmus area for immune surveillance/protection.

13.3.2 The Bulge

As bulge stem cells are indispensable for the maintenance of HF homeostasis, it is reasonable to speculate that the bulge area may be the most representative anatomical location protected by immune privilege (IP) within HF.

In line with this, the MHC class I molecule was found to be downregulated in the bulge, implying that CD8+ T-cell–mediated cytotoxic immune response less likely targets this area [9, 33] (Fig. 13.2). The immunosuppressive cytokines, transforming growth factor-β (TGFβ), and macrophage migration inhibitory factor (MIF) and other immunosuppressive factors, including α-melanocortin stimulating hormone (α-MSH) and indoleamine-pyrrole 2,3-dioxygenase (IDO) were reported to be upregulated in the bulge, supporting that the bulge is an IP site [33–35] (Fig. 13.2). However, further functional assessment of those molecules adopting bulge-specific ablation or forced expression is required to confirm their contribution to IP maintenance in the bulge area.

CD200, a glycoprotein suppressing immune activity of macrophages via CD200R was shown to be overrepresented in the bulge of human scalp HF and mice pelage HF at the protein and messenger RNA levels, respectively [15, 36, 37] (Fig. 13.2). Interestingly, when *Cd200*-/- CD57BL/6 mice skin was transplanted

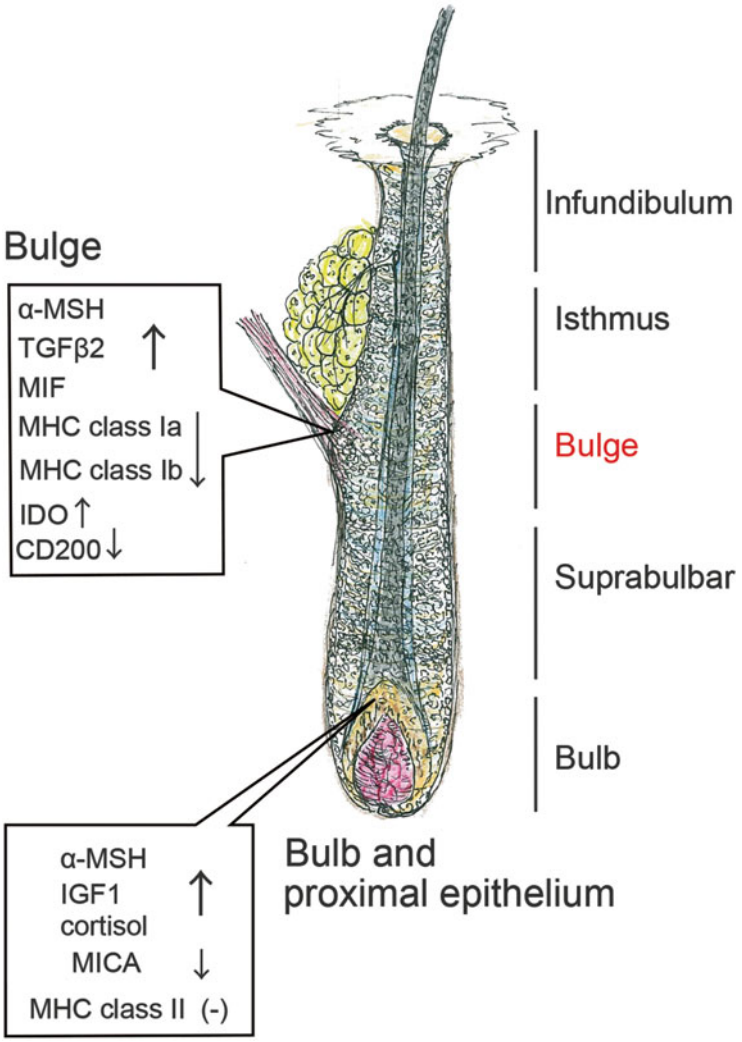

Fig. 13.2 Proposed mechanisms providing immune privilege to the bulge and the lower portion of the hair follicle. Downregulation of MHC molecules and upregulation of immunosuppressive factors may contribute to the maintenance of immune privilege in the bulge and the lower portion of the hair follicle

onto wild-type CD57BL/6 mice, lymphocytic cell infiltration was elicited in the grafted skin with resultant hair loss resembling scarring alopecia [38]. This observation implied that CD200 might contribute to the maintenance of IP in the bulge by preventing unnecessary lymphocytic inflammation. However, CD200R expression by T cells has not been convincingly demonstrated and the underlying mechanism of this observation needs to be elucidated.

In mice HF, suprabasilar bulge KCs marked by an anti-S1P1 antibody were shown to produce its ligand CCL8 in response to mechanical stress by tape-stripping, suggesting active protection of the stem cell niche by secreting immune-repulsive cytokines [10].

13.3.3 The Suprabulbar Portion~the Bulb

Similar to the bulge area, MHC class I antigen was shown to be minimally expressed in the suprabulbar~bulbar portion of HF, suggesting that this area is also immune privileged [9, 32] (Fig. 13.2). Expression of MHC class I polypeptide-related sequence A (MICA), the ligand NK-cell–activating receptor NKG2D, is suppressed as well, possibly enabling this area to avoid NK cell recognition [39]. In contrast, immunosuppressive factors α-MSH, insulin-like growth factor 1 (IGF1), and cortisol are produced in this portion of HF, which may contribute to the maintenance of IP [9, 34, 35] (Fig. 13.2). As described above, functional studies are necessary to conclude if differential expression of those molecules indeed lowers the risk of unwanted immune reaction to this area.

CCL1 and CCL8 expression was detected in the suprabulb and the infundibulum areas of human anagen HF [10]. In addition, CXCL4, CXCL9, and CXCL11, the chemokines that bind to CXCR3, were expressed with similar pattern. This finding is interesting as a possible role of CXCR3+ T cells in the development of alopecia areata (AA), a condition characterized by peribulbar dense T-cell infiltration, has been suggested in recent studies [40, 41].

13.4 Conclusion

Mainly based on clinicopathological findings of alopecia areata, HF has long been implicated as a target of immunity or an innocent bystander hiding itself from immune surveillance. Recent discovery of HF site-specific leukocyte accumulation and chemokine production uncovered previously unrecognized pivotal roles of HF in the maintenance of homeostasis of the skin immune system. We are still at the dawn of HF immunology, however, in-depth investigation should accelerate our understanding of immune regulation in the skin and may facilitate development of novel therapeutic approaches for currently intractable inflammatory skin diseases via modulating the immunoregulatory function of HF.

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Chapter 14
Platelets

Risa Tamagawa-Mineoka and Norito Katoh

Abstract Platelets contribute greatly to immune and/or inflammatory responses, in addition to having prominent roles in hemostasis and thrombosis. Platelets have numerous immune-associated molecules in their granules, rapidly express receptors such as P-selectin, CD40 ligand, and integrins on their surface upon activation, and secrete soluble mediators such as chemokines, growth factors, and lipid mediators. In this way, platelets affect the function of endothelial cells and leukocytes such as neutrophils, T cells, natural killer cells, B cells, monocytes, and dendritic cells via direct cell–cell contact and/or soluble mediators. In cutaneous inflammatory disorders including atopic dermatitis, psoriasis, and infectious diseases, platelets circulate in an activated state and are involved in the pathogenesis of these diseases. On activation, platelets bind leukocytes via P-selectin on the surface of platelets in circulating blood. The platelet–leukocyte complexes then roll along the endothelium and transmigrate into subendothelial tissue. After reaching inflamed skin tissue, platelets release soluble mediators such as chemokines at the sites of inflammation, leading to aggravation of inflammatory responses. Platelets can also recognize bacteria pathogens through interactions via Toll-like receptors, with subsequent elimination of the bacteria directly by release of microbicidal proteins or by aggregation of platelets around the bacteria. Thus, platelets play important roles in immunity and inflammation in skin through interactions with cells such as leukocytes, other platelets, and the endothelium.

Keywords Adhesion molecule • Chemokine • Immunity • Inflammation • Platelet • P-selectin

14.1 Properties of Platelets

Platelets are small subcellular fragments that are formed from the cytoplasm of megakaryocytes in human bone marrow. Platelets are released from megakaryocytes and then circulate in the blood for 7–10 days. The normal count of platelets in

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blood is widely quoted as 150–400 × 10^9/L [63]. Platelets are the smallest of the many types of cells in circulating blood, averaging only 2.0–5.0 μm in diameter and 0.5 μm in thickness, and having a mean cell volume of 6–10 fl [63].

14.2 Immune-Associated Molecules in Platelets

14.2.1 Molecules in Platelet Granules

Platelets contain three major types of intracellular granules: α-granules, dense granules, and lysosomes. Many molecules are contained in these granules. α-granules are the most abundant, with 40–80 per platelet, and mainly include adhesion molecules, chemokines, growth factors, lipid mediators, and immunoglobulins (Table 14.1). Dense granules contain nucleotides, ions, and serotonin (Table 14.2), and lysosomes contain β-glucuronidase and cathepsin. Platelets not only contain bioactive substances such as serotonin and most chemokines, but also synthesize molecules such as thromboxane A2 and platelet-activating factor [50]. Several studies suggest that platelets have a substantial amount of messenger RNA and can synthesize molecules despite the absence of a nucleus [11, 39, 64].

14.2.2 Surface Molecules Expressed on Platelets

14.2.2.1 P-Selectin

P-selectin (CD62P) is an integral membrane glycoprotein that is located in membranes of platelet α-granules [54]. Upon platelet activation, P-selectin is rapidly expressed on the cell surface and secreted into plasma [28, 45]. There are approximately 1,000 P-selectin molecules in an unstimulated platelet and 10,000 in an activated platelet [46]. By binding its major ligand, P-selectin glycoprotein-1 (PSGL-1), which is expressed on almost all leukocytes, platelet P-selectin supports adhesion of polymorphonuclear cells [36], monocytes/macrophages [17], and lymphocytes [38].

14.2.2.2 CD40 Ligand

CD40 ligand (CD154) is a member of the tumor necrosis factor (TNF) superfamily and is present in platelet α-granules. Platelets can interact with a large number of CD40-bearing immune cells such as B cells, macrophages, and dendritic cells, and nonimmune cells such as endothelial cells [49]. Platelet CD40 ligand interacts with and stimulates endothelial cells through the CD40 receptor, and this results in increased expression of adhesion molecules and chemokines (Fig. 14.1) [23].

Table 14.1 α-granule contents

Chemokines
CXCL1 (growth-related oncogene-α)
CXCL4 (PF-4)
CXCL5 (epithelial neutrophil-activating peptide-78)
CXCL7 (β-TG)
CXCL8 (interleukin-8)
CXCL12 (stromal cell-derived factor-1)
CCL1 (I-309)
CCL2 (macrophage chemotactic protein-1)
CCL3 (macrophage inflammatory protein-1α)
CCL5 (RANTES)
CCL7 (macrophage chemotactic protein-3)
CCL17 (thymus and activation-regulated chemokine)
Growth Factors
Platelet-derived growth factor
TGF-β
Vascular endothelial growth factor
Insulin-like growth factor
Cytokines
Interleukin-1β
Lipid Mediators
Platelet-activating factor
Prostaglandin E2
Microparticles
Antibacterial proteins
Thrombocidins 1 and 2
Adhesion Molecules
P-selectin
CD40 ligand
Integrins (α2β1, α5β1, α6β1, αLβ2, αIIbβ3, αvβ3)
Thrombospondin
Fibrinogen
Fibronectin
Others
Albumin
IgG
IgM
IgA

In addition, ligation of CD40 by platelet-derived CD40 ligand induces dendritic cell maturation and B-cell isotype switching, and increases $CD8^+$ T-cell responses [13].

Table 14.2 Dense granule contents

Nucleotides
Adenosine disphosphate
Adenosine triphosphate
Ions
Ca
Mg
P
Pyrophosphate
Serotonin
Epinephrine
Histamine

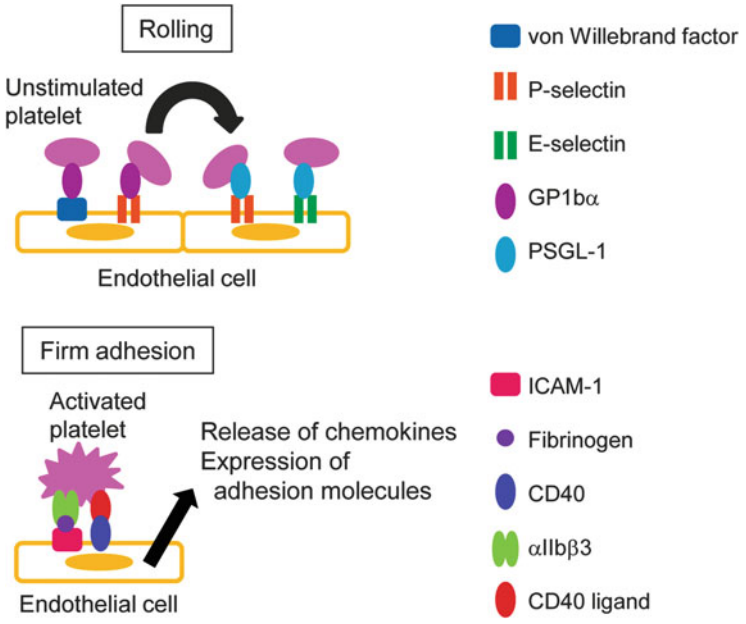

Fig. 14.1 Interaction of platelets with the endothelium via adhesion molecules

14.2.2.3 Integrins

Integrins are a family of adhesion molecules present on most cell types that mediate cell–cell and cell–matrix interactions. Platelets have six different integrins (α2β1, α5β1, α6β1, αLβ2, αIIbβ3, and αvβ3) in α-granules [9]. On platelets, αIIbβ3 (glycoprotein (GP) IIb/IIIa) is the major integral plasma membrane protein. αIIbβ3 is the only integrin expressed uniquely on platelets and mediates firm adhesion of platelets to the intercellular adhesion molecule (ICAM)-1 on the endothelium (Fig. 14.1) [8].

14.2.2.4 Chemokine Receptors

Platelets express many kinds of chemokine receptors, CCR (CCR1, CCR2, CCR3, CCR4, CCR5, CCD6, CCR7, CCR8, CCR9, and CCR10) and CXCR (CXCR1, CXCR 2, CXCR 3, CXCR4, and CXCR5) on their surface. Platelet receptors are all members of the G protein-coupled receptor family, similarly to those on other cell types. Several chemokines exert major effects on platelet functions of aggregation and adhesion [18], and platelet chemokines themselves can directly activate platelet function.

14.2.2.5 Toll-Like Receptors (TLRs)

TLRs are a family of pattern recognition receptors that are expressed by immune cells such as macrophages, dendritic cells, granulocytes, natural killer cells, and T cells, and by nonimmune cells such as fibroblasts and epithelial cells [27, 33]. Expression of TLR1-9 on platelets has been detected [51] and platelets can recognize pathogens via TLRs. Platelet-expressed TLRs can modulate sepsis-induced thrombocytopenia and TNF production in vivo [3].

14.2.2.6 High-Affinity Receptor for IgE (FcεRI)

FcεRI is expressed on the surface of mast cells, basophils, dendritic cells, and monocytes, and plays a key role in immediate hypersensitivity and IgE-mediated delayed-type hypersensitivity reactions. Platelets also express FcεRI on their surface, in addition to FcεRII and FcεRIII [21, 29]. Engagement of FcεRI on platelets induces release of serotonin (5-hydroxytryptamine, 5-HT) and regulated on activation, normal T cell expressed and secreted (RANTES, CCL5).

14.2.3 Soluble Mediators in Platelets

14.2.3.1 Chemokines

Chemokines are small chemoattractant proteins that stimulate migration and activation of cells, especially phagocytic cells and lymphocytes, and have a central role in inflammatory responses. The first platelet chemokine, platelet factor 4 (PF-4, CXCL4), was described in the 1960s [14]. A number of chemokines found in platelet α-granules are rapidly secreted upon platelet activation [18]. CXCL4 and β-thromboglobulins (β-TG, CXCL7) are particularly abundant in platelets.

14.2.3.2 Platelet-Derived Microparticles (PDMPs)

During platelet activation, microparticles are formed from the surface membrane of platelets by an exocytotic budding process, and are released into the extracellular space [24]. These PDMPs range in size from 0.02 to 0.1 μm and have both pro- and anticoagulant activities [60]. The microparticles bind to leukocytes, endothelial cells, and subendothelial matrix [47], and subsequently enhance adhesion of monocytes [5] and granulocytes [15].

14.2.3.3 Antibacterial Proteins

Antibacterial proteins are important components of the innate immune system that are found in many organisms and produced by various types of cells. These proteins bind to and disrupt bacterial membranes. Platelets store antibacterial proteins in α-granules and the proteins are released after cell activation [34]. These molecules are referred to as thrombocidins. Thrombocidin 1 and thrombocidin 2, which are related to the CXC-chemokine family, are lethal to various bacterial species such as *Staphylococcus aureus* [34].

14.2.3.4 Serotonin

Serotonin (5-HT) is a monoamine neurotransmitter that is biochemically derived from tryptophan and is stored in platelet-dense granules and mast cell granules. 5-HT affects the activation, migration, phagocytosis, cytokine production, and apoptosis of T cells, B cells, monocytes, and dendritic cells [10, 12, 25, 26, 32, 35, 52, 61].

14.3 Platelet Interactions with Leukocytes and the Endothelium

14.3.1 Interaction of Platelets with the Endothelium

Under normal physiological conditions, platelets circulating in blood do not interact with nonactivated endothelium. On activation, endothelial cells express adhesion molecules such as von Willebrand factor, P-selectin, and E-selectin that mediate platelet rolling (Fig. 14.1) [6]. Platelet–endothelial cell interactions induce full activation of platelets and surface expression of P-selectin, leading to firm adhesion of platelets on the endothelium.

Table 14.3 Influence of platelets on the function of leukocytes

	Increase	Decrease
Neutrophil	Adhesion	
	Migration	
	Mediator release	
T helper cell	Adhesion	Proliferation
	Migration	Mediator release
	Proliferation	
T cytotoxic cell	Adhesion	
	Migration	
	Proliferation	
	Mediator release	
	Cytotoxic activity	
B cell	Adhesion	
	Migration	
	Proliferation	
	Isotype switching	
	Germinal center formation	
	Antibody production	
Natural killer cell	Adhesion	Cytotoxic activity
	Migration	
Monocyte/macrophage	Adhesion	Mediator release
	Migration	
	Mediator release	
	Differentiation to macrophages	
	Proteolysis	
Dendritic cell	Maturation	Maturation
	Mediator release	Phagocytic activity

14.3.2 Interaction of Platelets with Leukocytes

Platelets affect the function of leukocytes, including neutrophils, T cells, natural killer cells, B cells, monocytes, macrophages, and dendritic cells via direct cell–cell contacts and/or via soluble mediators (Table 14.3). Cell–cell contacts between platelets and leukocytes can occur in two ways: (1) leukocyte adhesion to activated platelets that are adherent to the endothelium or the extracellular matrix, and (2) intravascular formation of platelet–leukocyte complexes [6]. In both cases, the interaction between platelet P-selectin and leukocyte PSGL-1 is the first step of a complex formation, leading to activation of integrins on the leukocyte surface and firm adhesion of leukocytes to activated platelets (Fig. 14.2).

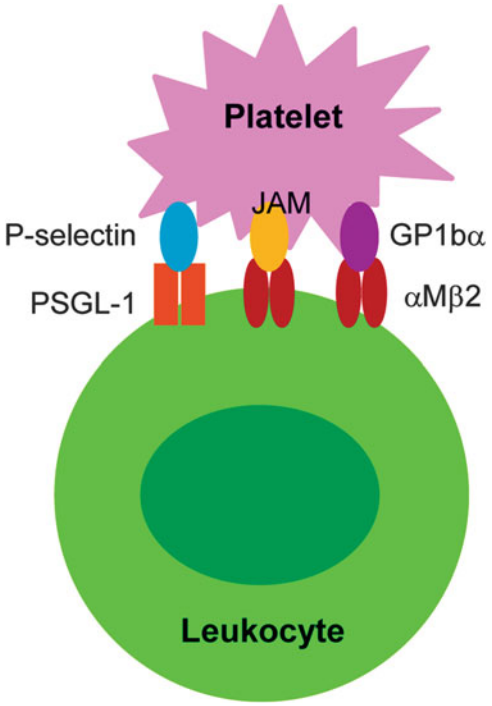

Fig. 14.2 Interaction of platelets with leukocytes via adhesion molecules. *JAM* junctional adhesion molecule

14.4 Role of Platelets in the Pathogenesis of Inflammatory Diseases and Conditions

14.4.1 Allergic Skin Diseases

14.4.1.1 Platelet Activation in Atopic Dermatitis

Platelets are increasingly recognized as important in allergic skin inflammation. Atopic dermatitis is a recurrent pruritic eczematous skin disease (see Chap. 22). In patients with atopic dermatitis, scratching due to itch often results in excoriation and subsequent platelet aggregation at the inflamed skin lesion, implying that blood platelets may be activated compared with those in healthy individuals. Serum levels of PF-4 and β-TG, and plasma levels of PDMPs and soluble P-selectin, which are widely known as platelet activation markers, are significantly increased in patients with atopic dermatitis [30, 56–58]. Plasma levels of serotonin are also elevated in patients with atopic dermatitis [52]. Interestingly, the levels of these mediators correlate with disease severity. These findings suggest that platelets circulate in an activated state in patients with atopic dermatitis and are involved in the pathogenesis of this disease.

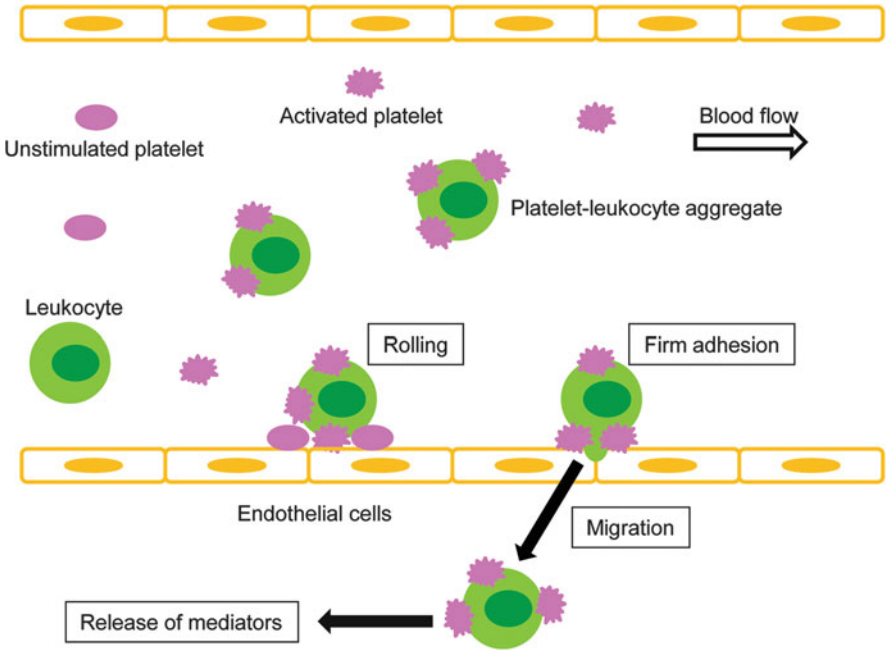

Fig. 14.3 Recruitment of platelets from circulating blood to inflamed sites

14.4.1.2 Role of Platelet P-Selectin in Allergic Skin Diseases

In animal models of allergic dermatitis, platelets have been shown to be important for recruitment of leukocytes into inflamed skin through formation of platelet–leukocyte aggregates via P-selectin on platelets in blood [20, 55, 57, 58]. The platelet–leukocyte aggregates are formed in circulating blood, roll along the endothelium, and transmigrate into subendothelial tissue (Fig. 14.3). Platelet P-selectin also contributes to the process of antigen sensitization [42]. These findings suggest that platelet P-selectin is important for development of cutaneous allergic reactions.

14.4.1.3 Role of Platelet-Derived Soluble Mediators in Allergic Skin Diseases

After platelets reach inflamed skin tissue by formation of platelet–leukocyte complexes, platelets can release soluble mediators including chemokines at sites of inflammation [55, 57, 58]. Platelet-derived soluble mediators can recruit leukocytes to inflamed skin and stimulate keratinocytes, endothelial cells, and other platelets, leading to aggravation of the inflammatory response [31]. Platelets are also stimulated via FcεRI and initiate contact hypersensitivity responses by releasing 5-HT [44]. Furthermore, platelets contain numerous potential chemical mediators of

pruritus such as histamine, 5-HT, interleukin-1β, transforming growth factor-β (TGF-β), platelet-activating factor, and prostaglandin E2 [37]. Thus, platelets may induce pruritus in lesions of allergic skin diseases.

14.4.2 Bacterial Infection

Platelets are involved in antibacterial host defense through interactions with gram-positive bacteria, gram-negative bacteria, and spirochetes. Platelets can recognize pathogens via TLRs [51], bind and capture bacteria, and directly kill the bacteria by releasing microbicidal proteins from α-granules [34]. Platelets can also eliminate bacterial pathogens by aggregating around the bacteria [50].

14.4.3 Psoriasis

Psoriasis is a chronic relapsing inflammatory disease of the skin characterized by erythrosquamatous plaques (see Chap. 23). A high incidence of occlusive vascular diseases such as cardiovascular events, thrombophlebitis, cerebrovascular accident, and pulmonary embolism has been found in patients with severe psoriasis [19, 62]. Other studies have shown that platelet aggregation in vitro elicited by thrombin or adenosine diphosphate is significantly increased in psoriatic patients compared with healthy individuals, and that this elevated platelet aggregation is significantly decreased after improvement of psoriatic skin lesions [7, 22]. Platelet activation markers such as β-TG and PF-4 in blood are also significantly elevated in patients with psoriasis [56, 59]. These findings suggest that blood platelets may be activated in psoriasis. Interestingly, Ludwig et al. [43] showed that activated platelets increase rolling of leukocytes in murine skin and that expression of P-selectin on blood platelets is elevated in patients with psoriasis in parallel with disease severity. These findings suggest that platelet P-selectin is associated with leukocyte rolling on the endothelium in psoriasis.

14.4.4 Regulation of Inflammatory Processes

Platelets have a large quantity of TGF-β in α-granules [4]. Serum levels of TGF-β are decreased in patients with immune thrombocytopenia and increased after restoration of normal platelet counts by therapy [1, 2]. Patients with immune thrombocytopenia also have deficiencies in $CD4^+CD25^+FoxP3^+$ regulatory T (Treg) cells, but the number and function of Treg cells are enhanced following an increase in the platelet count in blood [40, 53]. Therefore, platelet-derived TGF-β may contribute to Treg cell differentiation [50]. In addition, in vitro studies show

that PF-4 and PDMPs induce differentiation of Treg cells [41] and regulatory macrophages [48], respectively. Treg cells are increased in inflamed skin and were found to contribute to control of inflammatory responses in a mouse model of atopic dermatitis [16]. Therefore, platelet-derived mediators may be involved in regulation of skin inflammatory processes.

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Chapter 15
Adipose Tissues

Satoshi Nakamizo and Gyohei Egawa

Abstract The innate immune system provides rapid protection from foreign pathogens. However, under certain conditions of metabolic dysfunction, components of the innate immune system are activated in the absence of external pathogens, leading to pathologic consequences. Recent studies have revealed that obesity is associated with significant increases of M1 macrophages in adipose tissues that produce pro-inflammatory cytokines such as TNF-α and IL-1β. In addition, many reports have demonstrated that adipose tissues are metabolically active and produce inflammatory mediators, known as adipokines. The latter include TNF-α, MCP-1/CCL2, plasminogen activator inhibitor-1 (PAI-1), IL-6, leptin, and adiponectin. These findings support the theory that obesity itself is a low-grade chronic inflammatory state. Indeed, the association between psoriasis and obesity has been the subject of recent evidence-based reviews. Overall, adipose tissue inflammation in obesity demonstrates that the immune system and metabolism are highly integrated.

Keywords Adipose tissue • Obesity • Subcutaneous adipose tissue • Macrophage • Psoriasis • Adipokine • Leptin • Adiponectin

15.1 Introduction

Subcutaneous adipose tissues are distributed throughout the body, with the exception of the eyelids, clitoris, penis, pinna, and scrotum. They act as padding, an energy reserve, as well as insulation for thermoregulation [29]. In addition, adipose tissues act as an endocrine organ and produce some peptide hormones, cytokines, and paracrine transmitters [29]. Moreover, it is now apparent that obesity induces inflammatory changes in adipose tissues and may be involved in the pathogenesis of psoriasis. In this section, we review the newly defined functions of subcutaneous adipose tissues.

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15.2 Obesity and Adipose Tissue Inflammation

15.2.1 Obesity and Pathophysiology of the Skin

When energy intake exceeds expenditure, the excess energy is stored in adipocytes, leading to obesity. Obesity may alter some skin functions. The epidermal barrier is reported to be impaired in obesity, so that obese individuals display increased transepidermal water loss and dry skin [15]. Obese individuals have large skin folds and sweat more profusely when overheated [53]. Obesity inhibits lymphatic flow [53] and alters collagen formation [4]. The delayed-type hypersensitivity response is increased in obese individuals and decreases with weight reduction [44], which may be related to an alteration in the production of cytokines by adipocytes.

15.2.2 Macrophage Accumulation in Adipose Tissues

Adipose tissues are composed of not only adipocytes, but contain a variety of cell types, which are termed the stromal vascular fraction (SVF). This fraction includes mesenchymal stem cells, nerve cells, vascular endothelial cells, T cells, B cells, and macrophages. In 2003, pioneering studies by Xu et al. [52] and Weisberg et al. [51] demonstrated that obesity is associated with significant increases of macrophages in the SVF. Flow cytometric analysis revealed that macrophages account for approximately 40 % of the SVF in obese rodents, whereas it accounts for only 10 % in lean littermates. Weisberg et al. examined the gene expression profile of adipose tissue from multiple obese mouse models and reported that macrophage-related genes were upregulated in obese animals [51].

Recruitment of macrophages into adipose tissues is an early event in obesity-induced adipose inflammation. The monocyte chemoattractant protein-1 (MCP-1)/CCL2, one of the major chemoattractants for macrophages via CCR2, is secreted primarily by macrophages and vascular endothelial cells, and also by adipocytes [49]. Adipose tissue macrophages (ATMs) express CCR2 and recruit additional monocytes/macrophages, promoting a feedforward process [49, 50]. Indeed, CCR2-deficient mice display reduced ATM contents, reduced pro-inflammatory cytokines, and improved systemic insulin sensitivity relative to body weight-matched wild-type controls [50]. Similar effects were observed when obese wild-type mice were treated with a CCR2 antagonist [50]. Conversely, transgenic mice overexpressing MCP-1/CCL2 in adipocytes exhibited increased numbers of ATMs, hepatic steatosis, and insulin resistance [18].

15.2.3 Heterogeneity of ATMs

ATMs that reside in lean adipose tissue differ from those in obese adipose tissue (Fig. 15.1). Classically activated macrophages, termed M1 macrophages, are generally stimulated by T-helper (Th)-1–type cytokines such as interferon (IFN)-γ or bacterial by-products. M1 macrophages are pro-inflammatory, secreting cytokines such as TNF-α and IL-1β, and have high phagocytic and bactericidal potential [13]. M1 macrophages are generally recruited from the circulation in a CCR2-

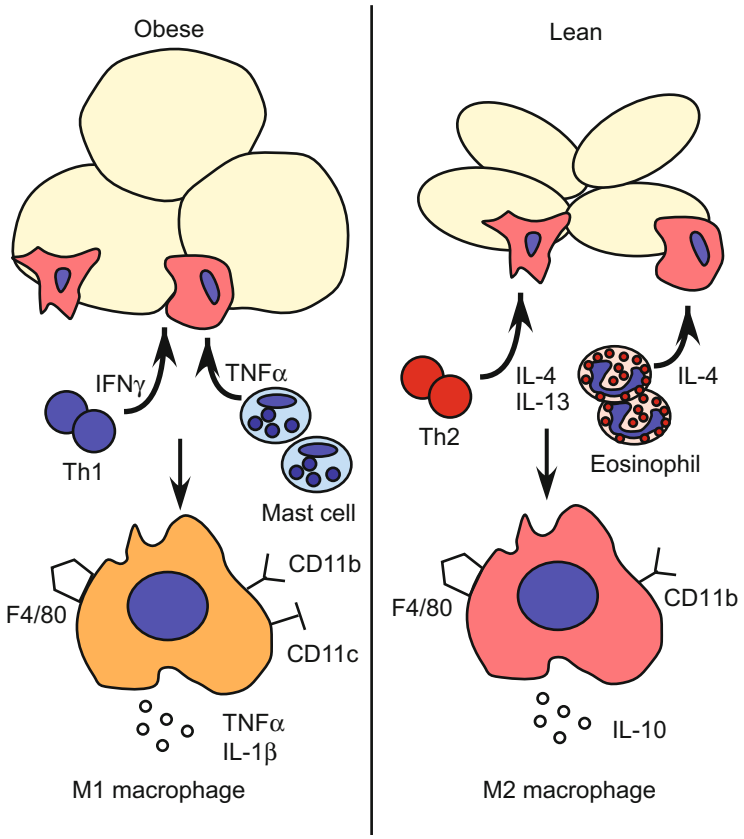

Fig. 15.1 Role of the immune system in obese versus lean adipose tissue. In obese adipose tissue, Th1-type cytokines such as IFN-γ stimulate M1 macrophage polarization. Mast cells are also increased in obese adipose tissues, contributing to insulin resistance. M1 macrophages produce pro-inflammatory cytokines such as TNF-α and IL-1β. In contrast, in lean adipose tissue, Th2 cells produce anti-inflammatory cytokines such as IL-4 and 13, which promote alternatively activated M2 macrophage polarization. M2 polarization is also induced by eosinophils via IL-4. M2 macrophages secrete other anti-inflammatory signals such as IL-10, which maintains insulin sensitivity in lean adipose tissue. Macrophages are bone-marrow–derived myeloid cells. Hence, both M1 and M2 macrophages express the myeloid cell surface markers F4/80 and CD11b. However, only the M1 population expresses the marker CD11c

dependent manner, so it is likely that their accumulation in adipose tissues is possibly due to increased entry from the circulation. In contrast, Th-2–type cytokines, such as IL-4, 10, and 13, promote alternatively activated M2 macrophages. M2 macrophages have antiparasitic functions, secrete anti-inflammatory cytokines such as IL-10, and function in tissue repair and remodeling [14]. M2 macrophages are thought to be derived from replication of tissue-resident macrophages; hence it is possible, but unproven, that their increase in adipose tissue is due to accelerated local multiplication.

The local environment of adipose tissues may influence the ratio of M1/M2 macrophages. M2-predominant ATMs are typically seen in normal lean individuals. On the other hand, obesity promotes infiltration of macrophages and induces a shift in the ATM balance towards the M1 phenotype. In fact, obesity shifts the ATM M1/M2 ratio from 1:4 in normal mice to 1:1.2 in obese mice [23]. This shift is evident in the expression level of CD11c on ATMs, a marker for M1 macrophages [24]. Flow cytometric analysis revealed that >90 % of recruited monocytes become $CD11c^+$ M1 macrophages in obese adipose tissues [24].

15.2.4 Adipocyte-Resident Tregs

As discussed above, obesity is a kind of chronic, low-grade inflammation of adipose tissue that promotes insulin resistance and type-2 diabetes. These findings raise the question of how adipocyte inflammation can escape the powerful mechanisms of cells and molecules normally responsible for guarding against a runaway immune response. Feuerer et al. answered this question in a recent report in which they observed that $CD4^+$ $Foxp3^+$ regulatory T cells (Tregs) with a unique phenotype were highly enriched in the abdominal fat of normal mice, but their numbers were strikingly and specifically reduced in the abdominal fat of insulin-resistant obese mice [12]. Loss-of-function and gain-of-function experiments revealed that these Tregs influenced the inflammatory state of adipose tissues and, thus, insulin resistance. Cytokines differentially synthesized by fat-resident regulatory and conventional T cells directly affected the synthesis of inflammatory mediators and glucose uptake by cultured adipocytes [12]. These observations suggest that harnessing the anti-inflammatory properties of Tregs to inhibit the mechanisms seen in metabolic syndrome may have therapeutic potential.

15.3 Inflammatory Mediators from Adipose Tissues

In 1964, D. Ogston and G. M. McAndrew reported increased fibrinogen levels in plasma in obese subjects [31]. This was the first report to describe a correlation between inflammation and obesity. Nowadays, the hypothesis that adipose tissues have an endocrine function [1] is strongly supported by the findings that adipocytes

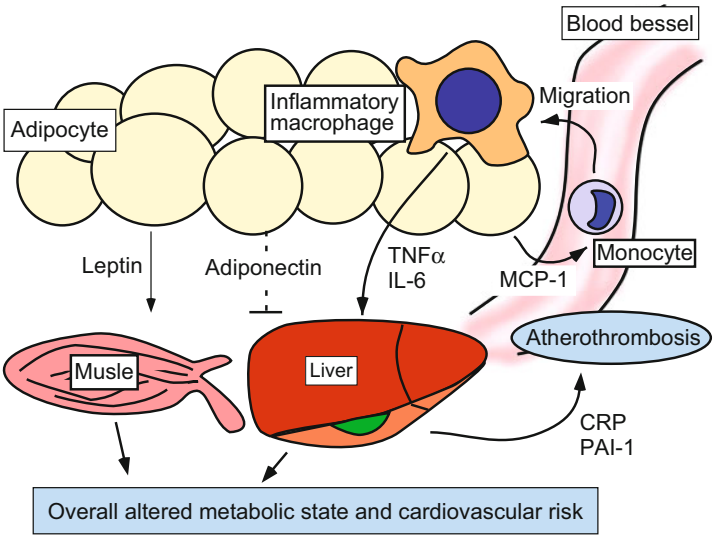

Fig. 15.2 Adipose tissue as an endocrine and paracrine organ. Obesity is associated with increased serum adipokine levels. TNF-α and IL-6, as well as leptin, induce insulin resistance in skeletal muscles and liver synthesis of pro-clotting factor that result in increasing risk of cardiovascular failure. In contrast, adiponectin mediates protective effects in obesity-related metabolic and vascular diseases. PAI-1 impairs fibrinolysis and enhances the risk of atherothrombosis

secrete a variety of mediators, termed adipokines, which are involved in the inflammatory network (Fig. 15.2). These mediators include adiponectin, leptin, PAI-1, IL-6, and TNF-α [54]. Interestingly, although a higher expression level of IL-6 was found in visceral fat as compared with subcutaneous fat, no significant regional variation has been reported in TNF-α production. Notably, not only increased cytokine production but also impaired cytokine catabolism seem to be determinants of obesity-related inflammatory status [28].

15.3.1 Adiponectin

Adiponectin is an anti-inflammatory cytokine produced mainly by adipocytes. Low serum levels of adiponectin have been reported in several chronic diseases such as obesity and psoriasis [3]. In patients affected by inflammatory diseases, high levels of cytokines such as TNF-α and IL-6 may reduce adiponectin production [3]. Adiponectin levels are inversely correlated with serum CRP levels in obese individuals. Moreover, reduced levels of adiponectin are associated with insulin resistance, impaired endothelium-dependent vasodilatation, impaired ischemia-induced neovascularization, and diastolic heart failure. Thus, adiponectin mediates protective effects in obesity-related metabolic and vascular diseases [3].

15.3.2 Leptin

Leptin is one of the main adipose-derived cytokines. Leptin has been investigated primarily for its role in controlling energy homeostasis by regulating appetite [33]. Leptin is also important for cell-mediated immunity. Previous studies have shown that $CD4^+$ T cells are hyporeactive in leptin-deficient mice [22]. Congenital leptin deficiency in humans results in lower frequency of blood $CD4^+$ T cells as well as impaired T-cell proliferation and production of cytokines such as IFN-γ, and this hyporesponsiveness can be restored by exogenous leptin [11]. Leptin appears to enhance Th1 and suppresses Th2 immune responses. In vitro, leptin acts on naive T cells, increasing their IL-2 secretion and proliferation, as well as increasing IFN-γ production by memory T cells [22]. Leptin is required for the induction and progression of autoimmune encephalomyelitis in mice [25], and it was recently shown that leptin inhibits the proliferation of regulatory T cells [10]. Leptin also activates macrophages by promoting IL-1β, IL-6, TNF-α, and IL-12 production [26]. Thus, elevated leptin levels may lead to enhanced Th1 type immune responses due to diminished regulatory T cell activity.

15.3.3 Resistin

Resistin was originally discovered in mouse adipocytes and assigned a key role in the induction of murine insulin resistance [45]. Human adipocytes, however, do not produce resistin [30], but resistin is produced by cells in the SVF, particularly by macrophages [9]. Resistin production is increased in the subcutaneous adipose tissues of obese individuals compared with lean individuals [38], but there is only a weak correlation between body mass index (BMI) and serum resistin levels [46]. Resistin is also produced by peripheral blood monocytes, and is particularly enhanced during their differentiation into macrophages [21, 32]. Resistin stimulates macrophages to produce inflammatory cytokines such as TNF-α, IL-1β, IL-6, CXCL8, and IL-12 [40], and conversely, TNF-α, IL-1β, IL-6, and lipopolysaccharides stimulate resistin production [21], suggesting a vicious cycle-type role of resistin in pathogenesis.

15.3.4 PAI-1

Another remarkable obesity-related abnormality is the increased circulating level of PAI-1 [2], the most important inhibitor of plasminogen activation. PAI-1 is correlated with all components of the insulin-resistance syndrome, and its level significantly decreases with weight loss [27]. Thus, the increased PAI-1 levels observed in obesity, by impairing fibrinolysis, enhance the risk of atherothrombosis. In

addition, obesity and metabolic syndrome appear to be associated with other hemostasis alterations, such as enhanced platelet activity [2].

15.3.5 IL-6

Adipose tissue also produces a large amount of IL-6 [29]. The metabolic role of IL-6 is not fully understood; mice deficient in IL-6 develop mature-onset obesity and carbohydrate and lipid dysmetabolism [48]. On the other hand, mice chronically exposed to IL-6 develop insulin resistance [20]. Thus, this cytokine is likely a critical metabolic modulator. Obesity is associated with increased secretion of IL-6, and therefore higher hepatic release of acute-phase reactants, especially CRP [54]. CRP levels are higher in obese than in nonobese individuals, directly correlating with the waist-to-hip ratio even after adjustment for BMI [47]. CRP also directly correlates with other cardiovascular risk factors and increased cardiovascular risk in the absence of acute inflammation.

15.3.6 TNF-α

Not only ATMs but also adipocytes produce TNF-α. In obese subjects, a 7.5-fold increase in TNF-α secretion from adipose tissues was observed compared to lean subjects [19]. TNF-α has manifold effects on inflammation and metabolism. In psoriasis patients, TNF-α exerts pro-inflammatory activity on the synovial tissue and endothelium [35], contributing to oxidative stress and leucocyte recruitment into atherosclerotic plaques. Moreover, it induces insulin resistance in skeletal muscles, liver synthesis of pro-clotting factor and lipoprotein lipase inhibition, and increases serum levels of atherogenic lipoproteins [37]. Thus, TNF-α contributes significantly to atherosclerotic lesion development, especially by promoting the expression of adhesion molecules on endothelial cells, the recruitment and activation of inflammatory cells, and the initiation of the inflammatory cascade inside the arterial wall [36, 41].

15.4 Obesity and Psoriasis

15.4.1 Role of Obesity in Psoriasis Patients

The association between psoriasis and obesity was the subject of a recent evidence-based review [6]. A key question is whether obesity causes or is a consequence of psoriasis. Childhood-onset obesity may particularly predispose an individual to both psoriasis [5] and psoriatic arthropathy [42], which suggests a genetic link.

Perhaps the best evidence that obesity can cause psoriasis is the fact that bariatric surgery can produce a rapid remission of psoriasis. In addition, there is increasing evidence that progressive weight loss can induce significant improvements in the severity of psoriasis [16]. However, it could be argued that a lack of physical activity, due either to the cosmetic impact of psoriasis or to the locomotor effect of psoriatic arthropathy, might predispose an individual to obesity [39].

15.4.2 Adipokines in Psoriasis Patients

As discussed above, ATMs produce TNF-α, as well as other cytokines involved in the pathogenesis of psoriasis such as IL-1, IL-6, IL-17, and IFN-γ [6, 43]. These adipokines, as well as leptin, are recruited and stimulated in obesity and may have an autocrine and paracrine effect on nearby skin [6] (Fig. 15.3).

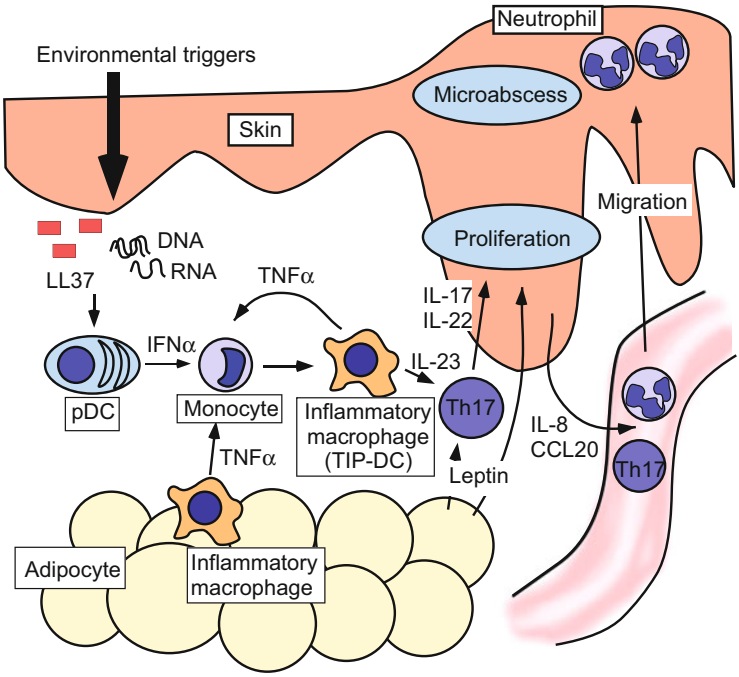

Fig. 15.3 Obesity and psoriasis. This schema depicts the association between psoriatic skin lesion and adipokines. Environmental stimuli trigger keratinocytes to produce self-DNA/RNA and LL37 complexes, which stimulate the synthesis of IFN-α by plasmacytoid DCs (pDC). IFN-α, in association with TNF-α, activates inflammatory macrophages. In obese individuals, TNF-α production is likely to be enhanced by ATMs and adipose tissues. Activated inflammatory macrophages produce multiple cytokines that promote differentiation and expansion of Th17 cells. Th17 cytokines induce keratinocytes to produce CCL20 and IL-8, chemoattractants for CCR6-expressing T cells and neutrophils, thus promoting the accumulation of these cells in the psoriatic skin. IL-22 and leptin enhance keratinocyte proliferation

Leptin, produced by adipocytes, decreases regulatory T cells and is involved in inflammatory processes stimulating cytokine release [22], as well as its more established role in appetite suppression and metabolic control [33]. In fact, leptin levels have been shown to correlate with the severity of psoriasis [7]. On the other hand, elevated levels of resistin from ATMs lead to insulin resistance and upregulate the production of inflammatory cytokines including TNF-α[17]. Resistin levels are also increased in patients with psoriasis, correlating with obesity and increased severity of psoriasis [17]. In contrast, low serum levels of adiponectin have been observed in obese psoriatic patients as compared with the nonobese psoriatic patients [8, 34].

Thus, there is some indirect evidence that the immunological and metabolic alterations associated with obesity may be linked with the pathophysiology of psoriasis.

15.5 Conclusions

On a histological level, adipose tissue inflammation in obesity is associated with macrophage accumulation. It is interesting to note that many cellular and biochemical components of the immune system that normally protect the host from foreign pathogens, such as macrophages and Toll-like receptors, also play a pathologic role in obesity-related inflammation. Overall, adipose tissue inflammation in obesity demonstrates the fact that the immune system and the metabolism are highly integrated.

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Part II
Immune Systems in the Skin

Chapter 16
Innate Immunity

Takashi Satoh and Naotomo Kambe

Abstract Innate immunity senses the presence of a pathogen by recognizing molecules typical to a microbe but not shared by host cells, termed pathogen-associated molecular patterns (PAMPs). In addition, danger-associated molecular pattern (DAMPs) molecules also initiate a noninfectious inflammatory response through the innate immune system. Many nonimmune cells resident in the skin, as well as immune cells, are endowed with an array of pattern recognition receptors (PRRs) that orchestrate self-defense against invading pathogens by detecting the presence of pathogenic microorganisms. The major PRRs are Toll-like receptors (TLRs), nucleotide-binding oligomerization domain, leucine-rich repeat containing, or Nod-like receptors (NLRs), retinoic acid-inducible gene I-like receptors (RLRs), and C-type lectin receptors (CLRs). Recognition of PAMPs and DAMPs by those receptors initiates several different responses to eliminate the pathogen, such as the production of type I interferon and inflammatory cytokines. Antimicrobial peptides, produced by keratinocytes and neutrophils that have migrated to skin, also play an important role in innate immunity due to their antimicrobial activity. Dysregulation of innate receptors or antimicrobial peptides leads to increased susceptibility to infection and inflammatory diseases in the skin.

Keywords PRR • TLR • NLR • RLR • CLR • AMP • PAMPs • DAMPs

16.1 Introduction

Vertebrates evolved adaptive immunity for self-defense, whereas innate immunity protects both plants and animals against invading pathogens. Adaptive immunity uses a process of somatic gene rearrangement to generate an enormous repertoire of

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Table 16.1 Pattern recognition receptors (PRRs) and their proposed lignads

Group	PRR	PAMPs and DAMPs	Source of PAMPs and DAMPs
TLRs	TLR1/2	Triacyl lipopeptides	Bacteria
	TLR2	Peptidoglycan	Gram (+) bacteria, e.g., *S. aureus*
		Glycolipids	Mycobacteria
		Phosphilipomannan	*C. albicans*
	TLR3	dsRNA	dsRNA viruses
	TLR4	Lipopolysaccharide	Gram (−) bacteria
	TLR5	Flagellin	Flagellated bacteria, e.g. *Salmonella typhimurium*
	TLR6/2	Diacyl lipopeptides	Mycoplasma
	TLR7 and TLR8	ssRNA	Viruses, e.g., influenza, HIV-1
	TLR9	Unmethylated CpG DNA	Bacteria
NLRs	NOD1	iE-DAP	Bacteria, e.g., *H. pylori*, *Shigella flexneri*, *Listeria monocytogenes*, *E. coli*, *P. aeruginosa*
	NOD2	MDP	Bacteria, e.g., *S. aureus*, *S. pneumonia*, *Shigella fexneri*, *Listeria monocytogenes*, *M. tuberculosis*
	NLRP1	Anthrax lethal toxin	*Bacillus anthracis*
	NLRP3	MDP, DNA, RNA, toxins	Bacteria, e.g., *L. monocytogenes*, *S. aureus*
		ATP, MSU, cholesterol	Endogenous
		Silica, asbestos, alum	exogenous
	NLRC4	Flagellin	Flagellated bacteria, e.g., *S. tyhimurum*, *L. pneumonia*, *P. aeruginosa*, *S. flexneri*, *L. monocytogenes*
AIM2	AIM2	dsDNA	*Francisella tularensis*, *L. monocytogenes*, *vaccinia virus*
RLRs	RIG-I	Short dsRNA, ssRNA	Viruses, e.g., HCV, VSV, *reovirus*
	MDA5	Long dsRNA	Viruses, e.g., *picornavirus*, *vaccinia virus*
CLRs	Dectin-1	β-glucan	Fungi, e.g., *C. albicans*, Mycobacteria
	Dectin-2	High mannose, α-mannans	*C. albicans*, *C. neoformans*, *S. cerevisiae*, *M. tuberculosis*

antigen receptors that are capable of fine distinctions between closely related molecules. In contrast, innate immunity relies on a limited number of germline-encoded receptors and secreted proteins that recognize features common to many pathogens (Table 16.1). The recognition of pathogens by innate immunity is, however, essential for both the initiation of adaptive immune responses and the generation of long-lasting adaptive immunity [1].

Innate immunity provides the first line of host defense, which can immediately respond to pathogens. Innate immunity was previously considered as a physical barrier provided by mucous membranes and ciliary action in the lungs and nasal passages, and the stratum corneum and keratinocytes' tight junctions in the skin, which do not require specific recognition of microbes. Over the past two decades, however, there has been rapid progress in our understanding of immune responses to microbial components, which are evolutionally conserved between species. Currently, chemical and cellular barriers that specifically recognize microbes are regarded as the main component of innate immunity; chemical barriers include specialized soluble molecules that possess antimicrobial activity, whereas cellular barriers are comprised of an array of cells with sensitive receptors that detect microbial components.

Interestingly, the fundamental mechanisms of innate immunity and its basic intracellular signaling are conserved between plants and animals. In plants, for example, the innate-immunity–mediated necrotic cell death of infected cells and the subsequent release of danger signals in the local site can effectively block the proliferation and spreading of pathogens [2], whereas activation of mammalian innate immunity can cause lytic cell death, which can lead to efficient clearance of intracellular bacteria [3].

16.2 Pattern Recognition Receptors

Innate immunity senses the presence of a pathogen by recognizing molecules typical to a microbe but not shared by host cells, termed pathogen-associated molecular patterns (PAMPs). The major pattern recognition receptors (PRRs) of the innate immune system are Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD), leucine-rich repeat (LRR) containing or Nod-like receptors (NLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), and C-type lectin receptors (CLRs) [4]. Recognition of PAMPs by these receptors initiates several different mechanisms, such as the release of type I interferon and inflammatory cytokines, to eliminate the pathogen.

An array of PRRs are present on immune cells resident in skin, such as epidermal Langerhans cells (LCs), dermal dendritic cells (DCs), and mast cells, as well as nonimmune cells, such as keratinocytes, melanocytes, vascular endothelial cells, fibroblasts, and adipocytes. This wide distribution of PRRs in nonimmune cells in the skin is suitable for immediate self-defense response because skin is continuously exposed to microorganisms and also a target of an array of viral, bacterial, and fungal microbes, such as HPV, *Streptococcus aureus*, and *Candida albicans*, respectively. Therefore, the capacity of nonimmune cells, such as keratinocytes, to induce an immediate innate antimicrobial response is crucial.

TLRs recognize pathogens at the cell surface (TLRs 1, 2, 4–6, 10) or within the endosome (TLRs 3, 7–9), CLRs recognize fungal components at the cell surface, and NLRs and RLRs act as intracellular surveillance molecules [4, 5]. To date,

10 functional human TLRs [6] and 23 members of the human NLR protein family have been reported [7]. The RLR family contains retinoic-acid–inducible gene I (RIG-I) and melanoma-differentiation–associated gene 5 (MDA5), which sense cytosolic double-stranded RNA (dsRNA). The CLR family has a central role in immunity to fungal pathogens.

16.3 Toll-Like Receptors

Each TLR recognizes a distinct PAMP by forming a homo- or heterodimer. TLR2 can form a heterodimer with either TLR1 or TLR6 to recognize bacterial tri- or diacyl lipopeptides (LP), respectively [8], and TLR2 homodimer recognizes peptidoglycan (PGN) (Fig. 16.1). The other representative TLR-targeted molecules are dsRNA, which appears during the replication cycle of most viruses and is recognized by TLR3; lipopolysaccharide (LPS) from gram-negative bacteria, recognized

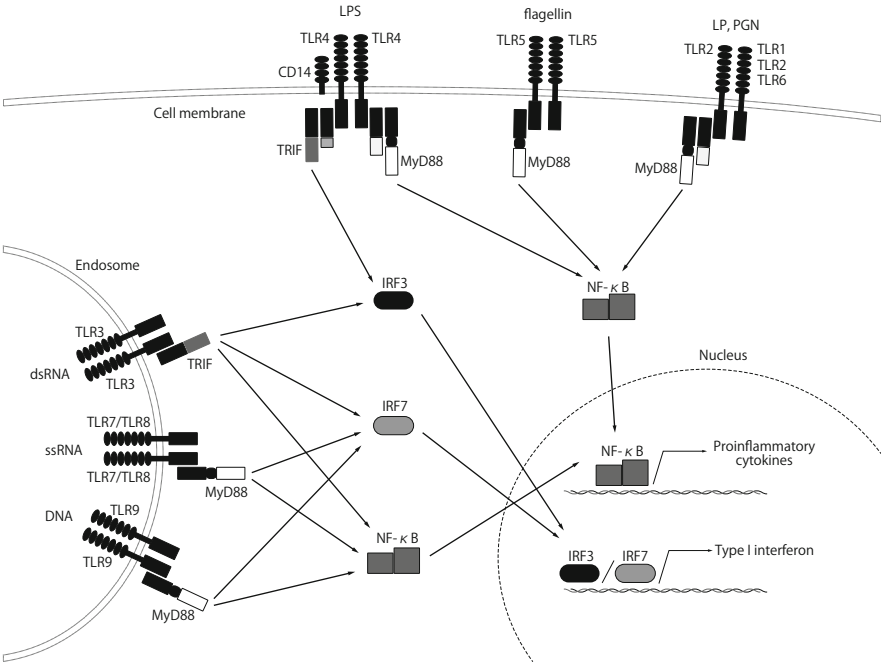

Fig. 16.1 TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 are located in the plasma membrane and recognize mainly bacterial PAMPs, whereas TLR3, TLR7, TLR8, and TLR9 are located in the endosomal membrane and recognize mainly viral nucleic acids, such as dsRNA and ssRNA. The TLR signaling pathways are channeled through the adapter molecules MyD88 and/or TRIF. These adapter molecules activate NF-κB, resulting in the transcription of pro-inflammatory cytokines, whereas activation of endosomal TLRs and TLR4 leads to activation of IRF3 and IRF7, resulting in the production of type I interferon

by TLR4 along with CD14; bacterial flagellin, recognized by TLR5; viral single-stranded RNA (ssRNA) by TLR 7/8; and unmethylated CpG motifs, which are abundant in microbial DNA, recognized by TLR9 [8].

All TLRs are type I transmembrane receptors that have ectodomains containing LRR that are involved in recognition of PAMPs, and transmembrane domains and intracellular Toll/interleukin-1 receptor (TIR) domains are required for downstream signal transduction. Once a TLR is activated, the TIR domain of the TLR interacts with an adapter molecule, such as MyD88 (myeloid differentiation factor-88) and TRIF (Toll/interleukin 1 receptor domain-containing adaptor-inducing interferon β) [4]. MyD88 is used by all TLRs except TLR3, and activates the transcription factor NF-κB and mitogen-activated protein kinases (MAPKs). The MyD88-dependent pathway leads to the production of inflammatory cytokines such as TNF-α, IL-1, IL-6, and IL-12, chemokines including IL-8 and MIP2, costimulatory molecules such as CD80 and CD86, and adhesion molecules such as ICAM-1. TLR3 and TLR4 trigger the MyD88-independent TRIF pathway that leads to activation of the transcription factors interferon regulatory factor 3 and 7 (IRF3/7) and NF-κB, which promotes type I interferon (interferon (IFN)-α/β) and inflammatory cytokine expression [4].

In human skin, TLRs are expressed in different cell types, from the epidermis to adipose tissue, with great variation in expression level and functionality. For instance, keratinocytes express functional TLRs 1–3 and 5–6 [9], and play a dynamic role in host defense beyond their role as a physical barrier. There is some controversy about the expression of TLR4 [10, 11], TLR7, and TLR9 [9, 12] in keratinocytes. Activation of TLR2 on keratinocytes by *S. aureus* results in IL-8 production, whereas activation of TLR3 by dsRNA leads to type I IFN production [13]. Epidermal LCs express all TLRs, particularly TLRs 1–3, 5, 6, and 10 [14], and produce IL-6, IL-8, and TNF-α after TLR2/3 stimulation [14]. Other cells, such as dermal monocytes/macrophages, dermal DCs, T and B cells, and mast cells, vascular endothelial cells, and skin stromal cells such as fibroblasts and adipocytes are known to express TLRs [15].

16.4 NLRs and the Inflammasome

The NLRs are all located in the cytoplasm and sense foreign or endogenous PAMPs or danger-associated molecular patterns (DAMPs), many of which are released from dying cells and following tissue injury, through an incompletely understood mechanism [16]. NLRs contain a centrally located NOD and an LRR domain near their carboxyl terminus. The LRR domain of NLRs is thought to play a role in the recognition of PAMPs and DAMPs, just as the LRR domain of TLRs does. The NOD is thought to be involved in the induction of conformational changes and self-oligomerization necessary for NLR function [16]. The NLRs are considered to be a very ancient family of innate immune receptors because the plant resistance (R) proteins responsible for defense against plant pathogens are NLR homologues. NLRs are grouped into subfamilies according to their additional domains. Typical

additional domains present in NLRs are the caspase activation and recruitment domains (CARDs) and the pyrin domains (PYDs). These domains are involved in protein interactions, allowing for the recruitment of downstream effector molecules.

Widely studied members of the NLR family are NOD1 and NOD2. These receptors recognize components of bacterial cell walls; specifically, NOD1 recognizes γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP) [17] and NOD2 senses muramyl dipeptide (MDP) [18, 19]. Recognition of microbial products by NOD1/2 activates receptor-interacting serine-threonine kinase 2 (RIP2) via cellular inhibitors of apoptosis 1 and 2 (cIAP1 and 2), subsequently leading to ubiquitination of NF-κB essential modulator (NEMO) and activation of the pro-inflammatory NF-κB pathway [20]. The expression of NOD1/2 was first described in antigen-presenting cells such as monocytes/macrophages, but recent studies have revealed the functional expression of NOD1/2 in human keratinocytes [21, 22].

Another well-studied group of NLR family genes is NLRP1, NLRP3, and NLRC4, which form a multiprotein complex, termed the inflammasome. A variety of ligands can activate these receptors, particularly NLRP3 (Table 16.1). In addition to PAMPs such as ssRNA R837, the pore-forming toxin hemolysin derived from *S. aureus* and nigericin, many DAMPs, such as environmental pollutant crystals including silica and asbestos, vaccine adjuvants such as aluminum salt (alum) and endogenous metabolic stresses such as high glucose, cholesterol, β-amyloid, monosodium urate crystals (MSU), and extracellular ATP, can activate NLRP3 [23–33]. How these different stimuli with various molecular patterns can be recognized by only NLRP3 is largely unknown. K^+ efflux due to cell membrane disruption or lysosomal destabilization after crystal phagocytosis has been speculated to produce reactive oxygen species (ROS) that damage mitochondria, resulting in the activation of NLRP3 [34]. This activation leads to signaling through the adaptor protein ASC to activate pro-caspase-1, which then cleaves pro-IL-1β and pro-IL-18 into their active forms, resulting in inflammation (Fig. 16.2). NLRP3 activation is also known to cause necrotic cell death in hematopoietic cells and the subsequent release of danger signals, similar to the hyperresponse caused by R-proteins in plants [2, 3, 35]. NLRP3 is mainly expressed in hematopoietic cells such as LCs, DCs, and neutrophils in the skin, but also in other cell types, such as skin keratinocytes and mast cells [36, 37].

Absent in melanoma 2 (AIM2), a member of the PYHIN family (also called the HIN-200 family) and a cytosolic microbial dsDNA receptor, forms an inflammasome with ASC to trigger caspase-1 activation [38, 39]. AIM2 is expressed in LCs in normal epidermis and functionally expressed in keratinocytes under inflammatory conditions [40].

16.5 Other PRRs

RLRs contain three cytoplasmic RNA helicases that are critical for host antiviral responses [41]. RIG-I and MDA-5 sense dsRNA, leading to production of type I interferon. LGP2 contains an RNA binding domain, but lacks the CARD domains,

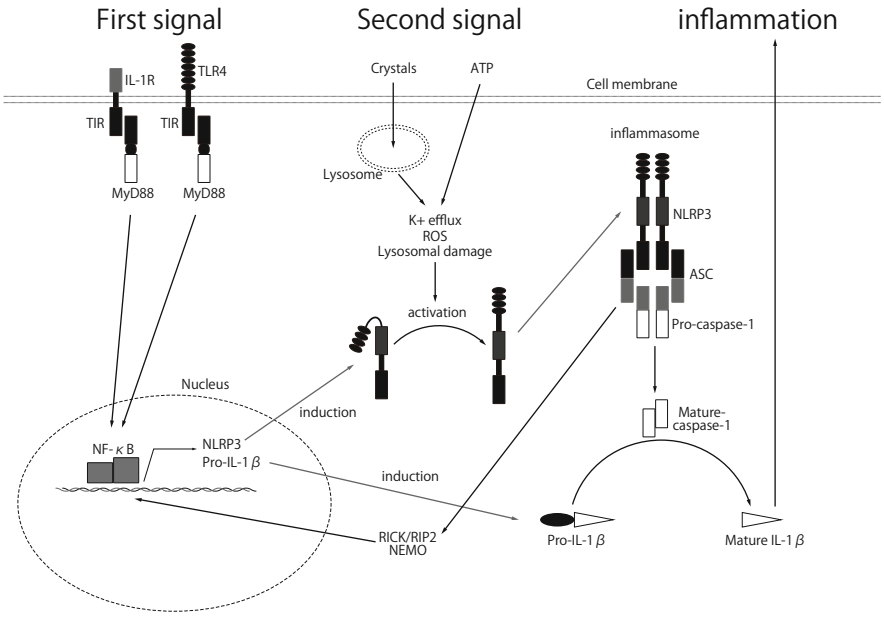

Fig. 16.2 The assembly of the NLRP3 inflammasome is known to require two distinct signals. (1) First signal: stimulation of PRRs, such as TLR4 and IL-1, activates the MyD88-dependent pathway via TIR. Activation of TIR results in NF-κB activation, leading to the upregulation of pro-IL-1β and, most importantly, the expression of NLRP3. (2) Second signal: NLRP3 can be activated by endogenous and exogenous stimuli, such as ATP and silica. The mechanism by which these ligands activate NLRP3 is largely unknown, but the involvement of potassium efflux, lysosomal damage, mitochondria damage, and reactive oxygen species (ROS) have been suggested. Upon activation of NLRP3, homotypic interactions are formed between the PYD domains of NLRP3 and ASC. Subsequently, the CARD domain of ASC molecules recruits pro-caspase–1, and active caspase-1 is produced, resulting in processing the pro-form of IL-1β into the active form of IL-1β. Additionally, the assembly of the NLRP3 inflammasome leads to the activation of RIP2, resulting in the activation of NF-κB and the production of pro-inflammatory cytokines through the ubiquitination of NEMO

and thus acts as a negative feedback regulator of RIG-I and MDA-5. In human keratinocytes, RIG-I is induced by IFN-γ and tumor necrosis factor (TNF)-α stimulation [42].

The CLRs family has a central role in immunity to fungal pathogens [43]. This family includes Dectin-1, Dectin-2, mannose receptor, C-type lectin receptor DC-SIGN, and Mincle. CLRs are described in Chap. 17.

16.6 Antimicrobial Peptides

Pathogenic microorganisms in the skin can be killed by antimicrobial peptides (AMPs), such as defensins and cathelicidins, which are constitutively produced by keratinocytes and induced after an inflammatory response, and direct activation of PRRs by microbial components [44]. They are short cationic peptides that disrupt the cell membranes of bacteria, fungi, and the membrane envelopes of some viruses within minutes. AMPs are an ancient, evolutionarily conserved class of antimicrobial peptides made by many eukaryotic organisms, including mammals, insects, and plants. AMPs have pleiotropic functions, not only to kill microbes but also to control host physiological functions such as inflammation, angiogenesis, and wound healing.

The two major subfamilies of AMPs in humans are cathelicidins and human β-defensins (hBDs) [45] (Table 16.2). The expression levels of hBD-2, hBD-3, and human cathelicidin in keratinocytes are very low at the steady state and upregulated during infection, inflammation, and wound healing [46, 47], whereas hBD-1 is constitutively expressed in keratinocytes [48]. Most AMPs have an overall net positive charge that ensures their accumulation and subsequent penetration of the negatively charged microbial cell walls [49]. In addition to keratinocytes, eccrine cells, sebocytes, neutrophils, eosinophils, mast cells, platelets, and T cells produce AMPs [50].

16.7 Innate Immunity and Skin Diseases

Atopic dermatitis (AD): AD patients have a greater susceptibility to bacterial, fungal, and viral infections, such as *S. aureus*, *C. albicans*, and herpes simplex. Associations between genetic variants in TLR2 and severe AD [51–53] and reduced TLR2 expression in keratinocytes and circulating monocytes from patients with AD [54, 55] have been reported. TLR2 recognizes lipopeptides and lipopteichoic acid from *S. aureus* [56] and cell wall polysaccharide mannnan from *C. albicans* [57]. In addition, LL-37 (processed human cathelicidin hCAP-18) and hBD-2 are significantly decreased in skin lesions in AD patients [58], resulting in *S. aureus*

Table 16.2 Antimicrobial peptides (AMPs) and their sources

AMP	Examples	Structure	Cells
α-defensin	α-defensin 1-4, HD-5,6	β-sheet	Neutrophils, epithelial cells (GI)
β-defensin	hBD 1-4	β-sheet	Neutrophils, epithelial cells (keratinocytes, respiratory, GI)
Chathelicidin	hCAP-18/LL-37	α-helix	Neutrophils, epithelial cells (keratinocytes, respiratory, GI), mast cells

HD human defensin, *GI* gastrointestinal

colonization. Reduced LL-37 expression in AD also renders patients susceptible to eczema herpeticum caused by herpes simplex proliferation [59].

Psoriasis: Abundant LL-37 expressed by keratinocytes in psoriatic lesions is thought to make a critical contribution to disease pathogenesis. The overexpressed LL-37 binds to extracellular self-DNA and forms aggregated structures, and is delivered to endosomes in plasmacytoid DC where it triggers TLR9 activation, resulting in type I interferon production [60]. Furthermore, rare development of infectious skin diseases in psoriasis is thought to be caused by the increased expression of LL-37 and hBD-2/3 in psoriatic lesions [61, 62].

Acne Vulgaris: Lipid components from *Propionibacterium acnes* trigger TLR2 expression in monocytes surrounding sebaceous follicles in acne lesions, leading to the production of IL-8 and IL-12 [63]. IL-1β in acne lesions has been reported to be produced by monocytes responding to *P. acnes* via the NLRP3 inflammasome-mediated pathway [64].

Rosacea: TLR2 is highly expressed in the skin of rosacea patients [65], resulting in high cathelicidin and KLK5 expression, which drive disease development [66].

Blau syndrome: This is a rare autosomal dominant disorder characterized by early onset granulomatous skin rash, symmetric arthritis, and recurrent uveitis with onset below 4 years of age [67]. Blau syndrome is caused by a gain of function mutation in NOD2 [68].

Cryopyrin-associated periodic syndrome (CAPS): This is a group of rare autosomal dominant autoinflammatory diseases caused by gain of function mutations in NLRP3 that lead to excessive IL-lβ production [69]. CAPS generally occurs in early childhood presenting with a variety of symptoms, such as periodic fever, urticarial skin rash, arthritis, aseptic meningitis, sensorineural hearing loss, and optic neuritis.

16.8 Summary

Many innate immune receptors and antimicrobial peptides contribute to the first line of defense against many pathogens in the skin, and disruption of these systems can lead to infectious and inflammatory diseases. The exact mechanisms of microbial recognition and the subsequent responses are not fully understood, and further research in this field is needed.

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Chapter 17
C-Type Lectin Receptors

Nobuo Kanazawa

Abstract C-type lectins, originally defined as proteins binding carbohydrates in a Ca^{2+}-dependent manner, form a large family containing soluble and membrane-bound proteins. Among them, those expressed on phagocytes and working as pathogen pattern-recognition receptors were designated as C-type lectin receptors (CLRs), in accordance with Toll-like receptors (TLRs), NOD-like receptors (NLRs), and RIG-I–like receptors (RLRs). Most of the genes for CLRs are clustered in human chromosome 12 close to the natural killer gene complex. Similar to the killer lectin-like receptors whose genes are clustered in this complex, most of the CLRs induce activating or regulatory signal cascades in response to distinct pathogen- or self-derived components, through the immunoreceptor tyrosine-based activating or inhibitory motif, respectively. In this chapter, some representative CLRs are picked up and their structural features leading to the functional consequences are discussed, especially on the signaling cascades and pathogen interactions, including some impacts on cutaneous pathophysiology. These CLRs should provide targets to develop effective vaccination and therapeutics for distinct infectious and autoimmune/inflammatory diseases.

Keywords CARD9 • CLEC • CLR • DC • FcRγ • ITAM • LC • pDC • Syk

17.1 Overview

C-type lectin (CTL) was originally defined as a protein that binds carbohydrate in a Ca^{2+}-dependent manner [105]. CTLs form a large family containing soluble and membrane-bound proteins and the family members share a common domain structure named the carbohydrate recognition domain (CRD), which consists of 18 highly conserved amino acid residues including two folds of disulfide bonds formed by four cysteine residues [20]. However, not all of the structurally related CRDs have been shown to bind Ca^{2+} or carbohydrates, and the term C-type lectin-like domain (CTLD) has been designated for the domain without Ca^{2+}-dependent

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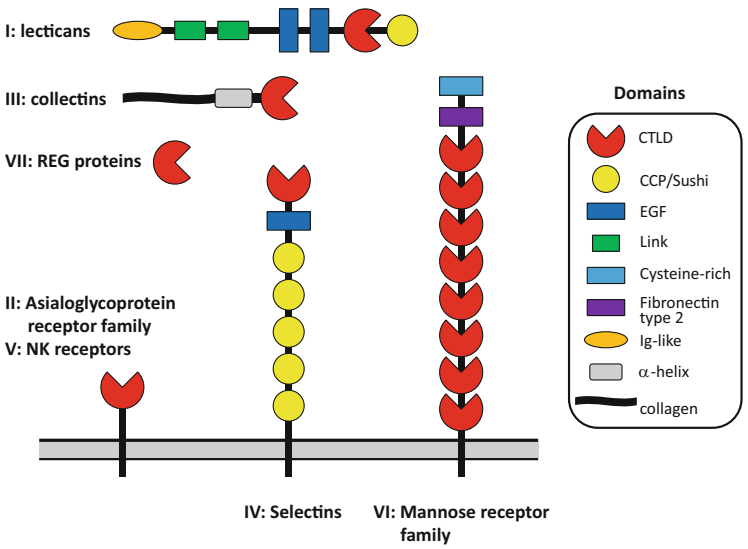

Fig. 17.1 Domain structure for the traditional seven (I–VII) groups of vertebrate CTLs. Domain names are listed in the box

sugar-binding capacity, whose amino acid sequence shows incomplete conservation of the common residues in the CRD [22]. It should be noted that the term CTLD is also used for the broad definition containing all CTLs irrespective of carbohydrate recognition [116].

Based on the domain structure, vertebrate CTLs were divided into 7 (I–VII) or, more recently, 17 (I–XVII) groups including the traditional seven groups [58, 116]. Among them, group I (lecticans: aggrecan, brevican, versican, and neurocan), III (collectins: mannose-binding protein and pulmonary surfactant proteins), and VII (Reg proteins) are soluble, whereas group II (asialoglycoprotein receptor family), IV (selectins), V (natural killer receptor family), and VI (mannose receptor family) are bound to the cell membrane (Fig. 17.1).

The natural killer (NK) receptor family (group V) contains a series of type II transmembrane proteins with a single CTLD expressed on NK cells, whose genes are clustered in the NK gene complex (NKC) in chromosome 12 [113]. They are collectively called killer lectin-like receptors (KLRs) and are equipped to regulate the killer activity of their expressing cells. Among them, heterodimers of CD94 and activating/inhibitory NKG2 receptors, as well as the activating NKG2D homodimer, recognize major histocompatibility complex (MHC) class I or related molecules, to distinguish target cells appropriately. The CTLDs included in these receptors have no capacity to bind carbohydrate.

Phagocytes, such as macrophages and dendritic cells (DCs), also express various kinds of CTL receptors on their cell surface for antigen capture. The mannose receptor (MR) family (group VI) contains type I transmembrane proteins with multiple CTLDs, such as the MR (CD206) and DEC-205 (CD205) [27]. They

commonly consist of an N-terminal cysteine-rich domain and a fibronectin type II domain as well as eight or ten CTLDs. In contrast, the asialoglycoprotein receptor family (group II) contains type II transmembrane proteins with a single CTLD, such as DC-specific ICAM3-grabbing nonintegrin (DC-SIGN, CD209), dectin-1, dectin-2, DC immunoreceptor (DCIR), and macrophage-inducible C-type lectin (Mincle) [4, 5, 9, 33, 69]. Notably, expression of some receptors is specific and they can be markers for distinct DC subsets; langerin (CD207) on Langerhans cells (LCs) and blood DC antigen (BDCA)-2 (CD303) on plasmacytoid DCs (pDCs) [25, 99]. Most of the genes for these group II receptors are also clustered in or next to the NKC (Fig. 17.2) [55]. In contrast to KLRs, most receptors of both groups contain fully conserved CRDs binding carbohydrates, and are considered to work as pattern recognition receptors (PRRs) recognizing various pathogen-associated molecular patterns (PAMPs). Especially, dectin-1 is critical for recognition of fungal β-glucan and has a collaborative effect with Toll-like receptor (TLR) 2 on yeast-induced activation signals, whereas DC-SIGN is involved in the recognition of a variety of microorganisms including viruses, bacteria, fungi, and parasites, and suppresses the immune activation signals induced by these pathogens [13, 32, 100]. For these pathogen-recognizing CTL receptors, "CLRs" have been designated analogous to other PRRs, such as TLRs, NOD-like receptors (NLRs), and RIG-I–like receptors (RLRs) [37]. Notably, most of the CLRs are linked to the signaling cascade through the immunoreceptor tyrosine-based activating or inhibitory motif (ITAM or ITIM, respectively), which was first identified in paired KLRs [38, 102]. Hemi-ITAM in dectin-1 and ITAM of Fc receptor γ chain (FcRγ) associated with dectin-2, BDCA-2, and mincle recruit spleen tyrosine kinase (Syk) leading to activating signals,

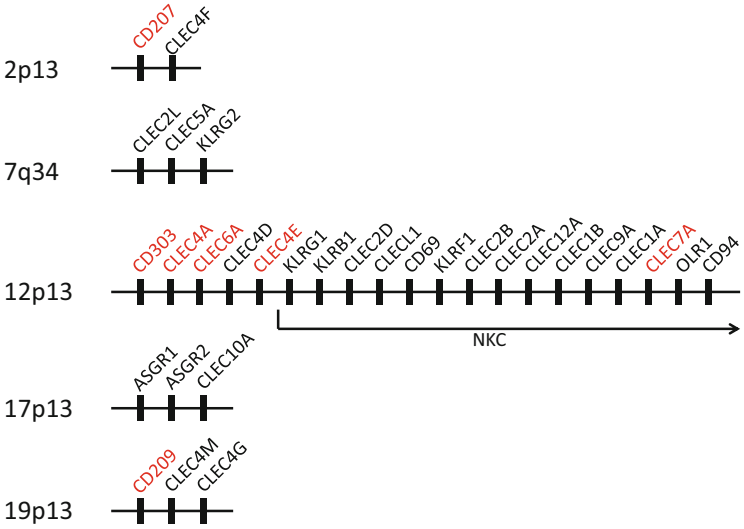

Fig. 17.2 Clustering of CLR genes. Names of the genes for CLRs discussed in this chapter are shown in *red*

whereas ITIM in DCIR recruits Src homology two domain-containing tyrosine phosphatases (SHPs) exhibiting inhibitory effects (Fig. 17.3) [57, 74]. In addition, some of them are also able to bind endogenous self-molecules and are involved in some pathophysiological aspects. For example, DC-SIGN have a role in cellular trafficking, DC-SIGN and dectin-1 can mediate an interaction between DC and T cells, and dectin-2 is involved in ultraviolet (UV)-induced tolerance [2, 5, 33, 35]. Furthermore, dectin-1 and DCIR are involved in the development of autoimmunity in mice, and SNPs in *CLEC4A* and *CLEC4E* encoding DCIR and mincle, respectively, are reportedly associated with autoimmune arthritis [31, 47, 65, 108, 115].

In this chapter, some representative CLRs are picked up and their structural features leading to the functional consequences are discussed, especially on the signaling cascades and pathogen interactions, including some impacts on cutaneous pathophysiology. Regarding the nomenclature, genes for the CLRs have formally been designated by the symbol "CLEC (C-type lectin domain containing)", which is approved by the Human Genome Organization Gene Nomenclature Committee (HGNC) (http://www.genenames.org/genefamilies/CLEC).

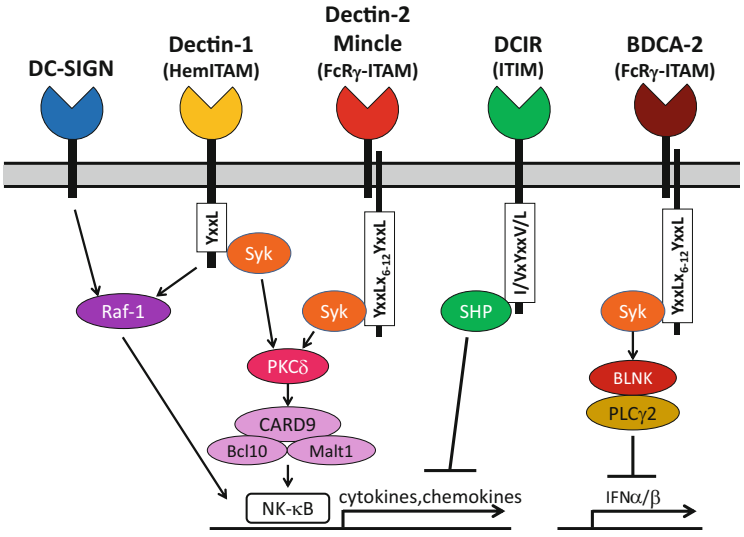

Fig. 17.3 Representative signaling cascades through distinct intracellular motifs of CLRs (HemITAM and ITIM) or ITAM of the associating FcRγ

17.2 Selected CLRs

17.2.1 Langerin (CD207, CLEC4K)

Langerhans cells (LCs) are immature DCs resident in the epidermis (see Chap. 4). As sentinels, they form a honeycomb-like network and spread dendrites to the skin surface through epidermal tight junctions. Once they capture and internalize antigens, they modulate expression of adhesion molecules and chemokine receptors to migrate through dermal lymphatics into the draining lymph node and there, after maturation, they present processed antigens to the specific T cells. LCs have been defined electron-microscopically by the presence of Birbeck granules (BGs) with a "tennis-racket"-like appearance, and immunohistochemically with BG-specific Lag antibody [12, 56].

Langerin has been designated as the first surface LC marker recognized by the LC-specific monoclonal antibody (mAb) DCGM4, and subsequently isolated by the expression cloning strategy [98, 99]. Recent structural analysis has revealed that langerin stands as a stable homotrimer via the coiled-coil interaction of its neck region [28]. Langerin has no signaling motif other than the proline-rich domain for internalization in its intracellular domain. However, the presence of such an intracellular domain provides a possibility that langerin interacts with other proteins to modulate or mobilize cellular machanisms required for defense against pathogens, such as interfering viral TLR signaling [100].

As predicted by the presence of EPN (glutamate–proline–asparagine) motif in its CRD, langerin can bind mannose, fucose, and N-acetylglucosamine structures and this binding leads to internalization and transfer of the antigen into BGs, which consist of superimposed and zippered plasma membrane [92, 99]. Langerin deficiency by the W264R point mutation in humans abolished the BG formation without apparent immunodeficiency [103]. Notably, langerin expression is not specific in LCs but is also detected in a part of dermal DCs in mice [14, 39, 75]. By analysis of langerin-DTR mice, in which langerin-expressing cells were transiently depleted by diphteria toxin administration, distinct ontogeny of langerin$^+$ dermal DCs from migrating LCs have been revealed.

Langerin recognizes human immunodeficiency virus (HIV)-1 through high-mannose structures in its envelope glycoprotein gp120 [19]. In contrast to dermal/submucosal DCs, which transmit HIV-1 to T cells through DC-SIGN, epidermal/mucosal LCs clear HIV-1 and prevent its infection to T cells through langerin without inflammation. Immunoelectron-microscopic analysis showed that HIV-1 was captured by langerin and internalized into BGs, to be finally degraded. Therefore, langerin on epidermal LCs is considered a natural barrier to HIV-1 infection. As inhibition of langerin caused HIV-1 infection in LCs and its subsequent transmission to T cells, anti-HIV therapeutics should not interfere with langerin expression or functions.

Langerin also recognizes *Candida* and *Malassezia* species through cell wall mannose structures and β-glucans [18, 96]. *Mycobacterium* species are also

recognized and the langerin binding to their components such as lipoarabinomannan (LAM) leads to its internalization into BGs and efficient loading to CD1a for presentation to T cells [49]. Therefore, BGs might be an organelle specialized to load glycolipid antigens to CD1a.

17.2.2 DC-SIGN (CD209, CLEC4L)

Although DC-SIGN has been designated by its pivotal role on DC-T cell interaction, it is well-known as a PRR for a variety of pathogens. Indeed, it was first cloned from placenta as a HIV-1 gp120-binding protein independent of CD4 [17]. DC-SIGN stands as a tetramer and contains dileucine (LL) and YxxL internalization motif in its intracellular domain [90]. Its binding to gp120 was inhibited by mannan, D-mannose, and L-fucose and, after binding, gp120 was immediately internalized [17]. DC-SIGN does not allow HIV-1 entry into DCs, but promotes its efficient infection in *trans* of cells expressing CD4 and chemokine receptors [34]. DC-SIGN also functions for capturing dengue virus, a mosquito-mediated flavivirus that causes hemorrhagic fever, and is indispensable for its infection to DCs [73, 95]. Interestingly, the cytoplasmic tail of DC-SIGN is not essential for, but enhances dengue virus infection to DCs. DC-SIGN is also involved in infection of other viruses such as Ebola, cytomegaloviruses, human C-type hepatitis virus (HCV), measles, and severe acute respiratory syndrome (SARS) coronavirus, bacteria such as *Helicobacter pylori* and *Klebsiela Pneumonae*, fungi such as *Candida albicans*, and parasites such as *Leishmania pifanoi* and *Schistosoma mansoni* [74, 101].

DC-SIGN further works for capturing and internalizing *Mycobacterium* species (*M. tuberculosis* and BCG) through the mannose-capped mycobacterial cell wall LAM (ManLAM) [36]. In immature DCs, internalized mycobacteria or ManLAM are transported into lysosome, where they are colocalized with LAMP1. Notably, targeting DC-SIGN with soluble ManLAM inhibits mycobacteria- or lipopolysaccharide (LPS)-induced DC maturation and induces IL-10 production, suggesting that DC-SIGN signaling interferes with the TLR signaling to cause an anti-inflammatory effect. Such an effect has been shown to be mediated by a serine/threonine kinase Raf1 signaling, which does not require the cytoplasmic YxxL motif of DC-SIGN but involves formation of a distinct protein complex (LSP1, KSR, CNK, LARG, and RhoA) with DC-SIGN [42]. Raf1 signaling does not induce DC activation but modulates TLR-mediated nuclear factor (NF)-κB activation, resulting in increased and prolonged transcription of IL-10 and some other cytokines. By analysis of bronchoalveolar lavage cells, alveolar macrophages from tuberculosis patients specifically expressed DC-SIGN, and its expression was induced by *M. tuberculosis* independently of TLR4, IL-4, and IL-13 [97]. Indeed, accumulation of *M. tuberculosis* in DC-SIGN-expressing alveolar macrophages was immunohistochemically shown.

By the case-control studies, a single nucleotide polymorphism (SNP) in the promoter region of DC-SIGN, −336 C has reportedly been involved in a risk for parenteral HIV-1 infection in European Americans [68]. It has also been reported that −336 SNP is associated with severity of dengue diseases, including severe dengue fever and dengue hemorrhagic fever [86]. In tuberculosis, association of −871 SNP has further been reported by analysis of a South African population living in the areas showing a high incidence rate of tuberculosis [6]. −871G and −336A are associated with protection against tuberculosis, and their combination showed a higher frequency in non-African populations, possibly as a result of genetic adaptation to a longer history of exposure to tuberculosis.

Recently, it has been reported that the anti-inflammatory capacity of intravenous immunoglobulin (Ig) is recapitulated in mice expressing human DC-SIGN, by transfer of bone-marrow–derived macrophages or DCs treated with the Fc fragment of IgG containing glycans terminating in sialic acids (IgG-sFc) [1]. Treatment with IgG-sFc induced IL-33 production in macrophages/DCs and caused expansion of IL-4–producing basophils in their administered mice, which in turn increased expression of an inhibitory Fc receptor, FcγRIIB, on effector macrophages. Such a novel IgG-sFc–induced DC-SIGN–mediated Th2/regulatory pathway might be an endogenous mechanism for immunological homeostasis and provide a line of evidence for therapeutic effects of intravenous Ig administration on various inflammatory diseases such as Kawasaki disease, autoimmune dermatomyositis/vasculitis/neuropathy, myasthenia gravis, pemphigus vulgaris, and severe drug eruptions [88].

17.2.3 Dectin-1 (CLEC7A)

Dectin-1 was originally reported as a DC-specific lectin that had been cloned from a cDNA library of murine XS52 cells after subtraction of J774 macrophage library [5]. At first, dectin-1 was considered a costimulatory molecule because soluble recombinant dectin-1 stimulated T-cell proliferation. However, subsequently, dectin-1 was identified as a β-glucan receptor on macrophages with a different binding site from that for T cells [13]. Dectin-1 is capable of binding a major yeast cell wall component zymosan and a variety of β-glucans from fungi and plants, however, it does not recognize carbohydrates with other linkages. Although the *CLEC7A* gene is located within the NKC, dectin-1 expression is not observed in NK cells, but in myeloid cells including monocytes, DC, macrophages, and neutrophils [55, 114].

Dectin-1 has a cooperative role with TLR2 on zymosan-induced macrophage activation [32]. Dectin-1 and TLR2 are colocalized on the surface of zymosan-treated macrophages and the zymosan-induced activation signals require dectin-1 as well as TLR2. Notably, the YxxL hemi-ITAM (hemITAM) in the cytoplasmic tail of dectin-1 is shown to be essential for this activation. Upon zymosan-binding, this hemITAM directly recruits and activates spleen tyrosine kinase (Syk), probably

dependent on the receptor dimerization, which induces NF-κB activation through interaction with caspase recruitment domain (CARD) nine coupled with the Bcl10–Malt1 complex [46, 82]. Recently, protein kinase (PK) C-δ was identified to link Syk activation and CARD9 signaling [93]. This Syk activation also induces IL-1β secretion, through pro-IL–1β transcription and processing, mediated by the CARD9-Bcl10–Malt1 and the Malt1-ASC–caspase 8 complex, respectively [45]. The latter complex is called noncanonical caspase 8-dependent inflammasome. Additionally, zymosan-induced dectin-1–dependent signals reportedly include phospholipase (PL) Cγ2-dependent Ca^{2+} influx leading to activation of mitogen-activated protein kinases (MAPKs) as well as NF-κB [109].

Furthermore, NLRP3 inflammasome is activated through dectin-1–Syk signaling in *Mycobacterium abscessus* (Mabc)-activated human macrophages, whereas both TLR2 and dectin-1 are required for Mabc-induced IL-1β secretion [63]. A cytoplasmic scaffold protein p62/SQSTM1 mediates this dectin 1-Syk–NLRP3 pathway, at least partly through production of reactive oxygen species (ROS).

Finally, Raf1 signaling, which regulates NF-κB activation independently of Syk, is also involved in the dectin-1 activation, as observed in case of the DC-SIGN activation [43]. Among Syk-induced cytokines, expressions of IL-1β, IL-6, IL-10, and IL-12 are enhanced but the IL-23 expression is impaired by Raf1 signaling, and, therefore, Th1 responses become dominant by dectin-1 activation. Notably, although both soluble monomer and particulate polymer of β-glucan can bind dectin-1, only the polymer can activate dectin-1 signaling by forming a so-called "phagocytic synapse," by not only clustering the receptor but also excluding inhibitory receptors with tyrosine phosphatase activities, CD45 and CD148 [40].

Interestingly, ZAP70-deficient SKG mice reportedly developed autoimmune arthritis only in the presence of dectin-1 agonist, indicating a role of dectin-1 signaling as a trigger for manifestation of subclinical autoimmune phenotype [115]. Furthermore, zymosan-mediated dectin-1 signaling induced IL-23 production and exacerbated autoimmunity through Th1/Th17 responses in TLR2-deficient mice, whereas zymosan-mediated TLR2 signaling activated $Foxp3^{+}$ regulatory T (Treg) cells through induction of IL-10 and Raldh2, an essential enzyme in retinoic acid metabolism [66]. Thus, antifungal dectin-1 signaling is considered rather aggressive and regulated by the simultaneous TLR2 signaling to prevent autoimmunity. Notably, dectn-1–deficient mice were shown to be impaired in zymosan recognition and increased in susceptibility to *Candida albicans* infection, whereas, in another report, they were rather more susceptible to *Pneumocystis carini* infection [83, 94].

In 2009, a nonsense mutation (Y238X) in the *CLEC7A* gene was identified in a Dutch family with chronic mucocutaneous candidiasis (OMIM#613108) [29]. The mutation was homozygous in three sisters with vulvovaginal candidiasis and/or onychomycosis and heterozygous in their parents with onychomycosis. The mutated dectin-1, which lacks C-terminal 10 amino acids, showed defective β-glucan binding and induction of cytokine productions after stimulation with β-glucan or *Candida albicans*. However, fungal phagocytosis and killing were shown to be unaffected and, therefore, invasive fungal infection was not observed

in the patients. The allele frequency of the Y238X variant is reportedly 0.069 in the healthy Dutch population and identified carriers are all heterozygous, and the variant has never been found in the Chinese population [29]. In addition, the same variant was reported to be a risk factor of increased susceptibility to invasive aspergillosis in hematopoietic stem cell transplantation, with the highest risk in cases where the variant is present in both donors and recipients [16].

17.2.4 Dectin-2 (CLEC6A)

Dectin-2 was reported as another DC-specific lectin that had been isolated by subtraction cloning from murine XS52 cells, whereas its human homologue was originally cloned by the database search of human chromosomal sequences homologous to the exon sequences of murine DC immunoactivating receptor (DCAR) [4, 55]. DCAR was identified as the putative activating partner of DC immunoreceptor (DCIR), and the overall amino acid sequence of murine DCAR is highly homologous (61% identity) to murine dectin-2 [54]. Although the gene for dectin-2 (*CLEC6A*) is clustered with those for DCIR (*CLEC4A*) and murine DCAR (*Clec4b*) and located next to the NKC, dectin-2 expression is observed in various myeloid cells including monocytes, macrophages, neutrophils, and DC subsets such as LCs [55].

Indeed, murine DCAR is the first reported DC/macrophage CLR that associates with an $YxxLx_{6–12}YxxL$ ITAM-bearing adaptor molecule, the γ chain of FcR (FcRγ), whereas the direct human orthologue of DCAR is absent and the functional significance of this molecule is still unknown [57]. Similar to murine DCAR, dectin-2 with a short cytoplasmic domain associates with FcRγ, a type I membrane protein with a short ectodomain that contains ITAM intracellularly and provides a docking site for Syk [85]. Dectin-2 stimulation activates the CARD9-Bcl10–Malt1 pathway through PKCδ and induces various cytokines and chemokines mediated by NF-κB activation [80, 93]. Additionally, dectin-2 signaling activates a MAPKs pathway through PLCγ2, as is the case with dectin-1 [41]. Most importantly, through Malt1-mediated selective c-Rel NF-κB subunit activation, dectin-2, but not dectin-1, signaling leads to Th17 responses [44].

As predicted by the presence of EPN motif in its CRD, dectin-2 binds structures with high mannose [70]. Actually, a number of pathogens including fungi such as *Candida albicans*, *Saccharomyces cerevisiae*, *Aspergilus fumigatus*, *Cryptococcus neoformans*, *Paracoccidioides brasiliensis*, *Microsporum audouinii*, and *Trichophyton rubrum*, as well as parasites such as *Histoplasma capsulatum* and *Schistosoma mansoni*, and *Mycobacterium tuberculosis* are recognized by dectin-2 [57]. Notably, the yeast and hyphal forms of *Candida albicans* induce different responses through dectin-2 [11, 85]. Most impressively, it has been shown by analyses of dectin-2–deficient mice that dectin-2 is essential for protective Th17 response to intravenous infection with *Candida albicans* [84].

By stimulation of murine bone-marrow–derived DCs with soluble schistosomal egg antigens following pretreatment with TLR ligands, NLRP3-dependent IL-1β secretion was increased through dectin-2-FcRγ-Syk signaling and ROS production [79]. Furthermore, house dust mites are also recognized by dectin-2 and induce eicosanoid production, leading to the Th2 responses [7, 8].

Interestingly, murine dectin-2 is reportedly involved in ultraviolet (UV)-induced immune regulation [2]. Although injection of soluble ectodomain of dectin-2 (sDec2) has no effect on induction of contact hypersensitivity (CHS), it inhibits UV-mediated suppression of CHS and tolerance induction. SDec2-binding $CD4^+$ $CD25^+$ T cells were shown to produce IL-4, IL-10, and TGFβ upon hapten stimulation and to transfer the UV-induced tolerance.

17.2.5 DCIR (CLEC4A) and DCAR (Clec4b)

DCIR was originally identified as a molecule homologous to hepatic asialoglycoprotein receptors and macrophage lectin, by selecting sequences in DC libraries following the database search of human genomic sequences using the tblastn algorithm [9]. DCIR is unique among known DC/macrophage CLRs because of the presence of I/VxxYxxV/L immunoreceptor tyrosine-based inhibitory motif (ITIM) in its cytoplasmic portion. As the ITIM-related signaling capacity of DCIR, SH2 domain-containing tyrosine phosphatase (SHP)-1 and SHP-2 can be recruited to human DCIR upon stimulation [48, 78]. Furthermore, using murine B cells expressing a chimeric receptor containing a cytoplasmic portion of DCIR and ectodomain of FcγRIIB (DCIR-FcR), coligation of DCIR-FcR and B cell receptor (BCR) showed DCIR-ITIM-dependent inhibitory effects on BCR-mediated calcium influx and tyrosine phosphorylation of cellular proteins [52]. On the other hand, murine DCAR was identified as a molecule whose CRD shows 91% amino acid identity with that of DCIR [53]. DCAR interacts with FcRγ to stand efficiently on the cell surface and ligation of the chimeric DCAR-FcR induced activation signals into its expressing cells dependent on the ITAM in FcRγ. Thus, DCIR and DCAR are equipped as structurally and functionally paired immunoreceptors in mice, although the biological significance of their pairing has been undefined [55].

Among in vitro generated human DC subsets, a higher expression of DCIR was observed in $CD14^+$-derived dermal-type DCs than in $CD1a^+$-derived LC-type DCs [9]. In monocyte-derived DCs (moDCs), its expression did not change during differentiation from monocytes to immature DC, but remarkably decreased after stimulation with LPS or CD40 ligand. Notably, DCIR expression was detected not only in myeloid DCs, but also in monocytes, macrophages, B cells, neutrophils, and plasmacytoid DCs (pDCs) [55, 71]. By reverse transcription (RT)-PCR, the only reliable method to distinguish expression of DCIR and DCAR, DCAR was rather limitedly observed, strongly in spleen and lung and weakly in skin and lymph nodes, whereas DCIR was detected ubiquitously. Both increased during the bone-marrow–derived DC development, but increase of the DCAR expression preceded

that of DCIR [53]. Notably, murine DC-specific mAb 33D1 recognized DCIR2, which can be a specific marker for $CD8^-$ DCs located in the red pulp and marginal zone of the spleen [23].

Despite the presence of an EPS (glutamate–proline–serine residues) motif conserved in DCIR and DCAR, which is postulated to recognize galactose residues [21], their recognizing carbohydrates have not been clarified. Notably, DCIR reportedly participates in capturing HIV-1 and its transmission to $CD4^+$ T cells [60]. HIV-1 captured by surface DCIR is stored as intact virion and replicates de novo intracellularly in infected DCs and, subsequently, is transmitted to $CD4^+$ T cells through induced DCIR expression on them, where HIV-1 replication is enhanced [61, 62]. These processes have been shown to be mediated by the ITIM signaling. Furthermore, DCIR can act as an attachment factor for HCV on pDCs [30]. Notably, the binding of HCV to DCIR on pDCs induced specific inhibition of TLR9-induced IFNα production, in contrast to the case of DCIR activation on DCs by anti-DCIR monoclonal antibody (mAb), which induced specific inhibition of TLR8-induced IL-12 and TNFα production [30, 71, 72].

As predicted by the presence of a tyrosine-based internalization motif overlapping with the ITIM, targeting DCIR on DCs with antigen-conjugated Abs induced clathrin-dependent receptor internalization, its trafficking into lysosomal compartments, and efficient antigen cross-presentation to $CD8^+$ T cells [9, 59]. In contrast, targeting murine DCIR2 induced class II presentation to $CD4^+$ T cells, including stimulation of $Foxp3^+$ Treg cells [23, 112].

The most impressive results on in vivo significance of DCIR have been obtained by analyses of DCIR2-deficient mice. They spontaneously developed sialadenitis and enthesisis and exacerbated collagen-induced arthritis with marked expansion of DCs [31]. Furthermore, in humans, by the case-control study, a SNP in the *CLEC4A* gene was shown to be associated with the susceptibility to anticyclic citrulinated peptide Ab (ACPA)-negative rheumatoid arthritis (RA) [47, 65].

17.2.6 BDCA-2 (CD303, CLEC4C)

Among BDCAs, which were designated for a series of antigens recognized by mAbs raised against human $CD4^+$ $lineage^-$ blood DCs, BDCA-2 is unique by its restricted expression on $CD11c^-$ CD123 (IL-3 receptor)^{bright} pDCs in fresh human blood without expression on immature moDCs or $CD34^+$-derived DCs [24]. Although murine dectin-2 was once proposed as the murine homologue of human BDCA-2, its proper murine orthologue is still undefined and murine DCAR, which lacks a human orthologue, is rather considered closely related to human BDCA-2 on structural and functional aspects [74].

In DC subpopulations, BDCA-2 expression was detected strongly in pDCs and very weakly in immature moDCs, and its expression level was downregulated after culture with IL-3 in pDCs and after maturation with LPS in moDCs [3, 25, 26].

In tonsil, BDCA-2 expression was observed on $CD123^+$ pDCs that were primarily present in T-cell areas but not in the germinal center.

Similar to DCAR and dectin-2, BDCA-2 associates with FcRγ and cross-linking of BDCA-2 on pDCs results in intracellular Ca^{2+} mobilization depending on Src-family protein tyrosine kinases and on tyrosine phosphorylation of cellular proteins [15, 25]. However, unlike other FcRγ-coupled CLRs, BDCA-2 signaling does not lead to NF-κB activation through the Syk–PKCδ–CARD9 pathway, but passes through the Syk–BLNK–PLCγ2 pathway [81]. Indeed, contrary to its predicted immunoactivating property, ligation of BDCA-2 suppressed the production of type I IFN and the secretion of TNF-related apoptosis-inducing ligand (TRAIL) in pDCs induced by various TLR agonists [25, 76]. Furthermore, inhibition of type I IFN production enhances IL-12 production in pDCs to polarize immune responses towards Th1 [26]. BDCA-2-mediated suppression of type I IFN production by CpG oligonucleotides or by influenza virus suggests that viruses target BDCA-2 for immune escape. On the other hand, the same phenomenon induced by antidouble-stranded DNA mAb plus plasmid DNA or by sera from patients of systemic lupus erythematosus (SLE) suggests that targeting BDCA-2 is beneficial for treatment of SLE.

As predicted by the presence of the triglutamate (EEE) late endosomal sorting motif in the cytoplasmic portion of BDCA-2, it was reported that targeting BDCA-2 with mAb induced internalization of the Ab complexes through phosphorylation of actin, tubulin, and clathrin, resulting in antigen presentation to $CD4^+$ T cells [25]. However, more recently, BDCA-2 signaling has rather been shown to inhibit antigen processing and presentation to T cells by pDCs, and, furthermore, to suppress the induction of costimulatory molecules on CpG-stimulated pDCs [51].

Although BDCA-2 contains the EPN motif in its CRD similar to dectin-2, it has recently been reported that BDCA-2 recognizes asialo-galactosyl-oligosaccharides with terminal β-galactose residues and that pDCs bind to subsets of $CD14^+$ monocytes, moDCs, and several tumor cell lines via recognition of asialo-galactosyl-oligosaccharides by BDCA-2 [77]. Accordingly, these cells can introduce the suppression signal into their binding pDCs through BDCA-2, especially on type I IFN secretion. Furthermore, BDCA-2 can bind gp120 of HIV-1 and HCV glycoprotein E2, both of which can also suppress the type I IFN secretion by pDCs [30, 67].

17.2.7 Mincle (CLEC4E)

Mincle was originally identified from murine peritoneal macrophages by subtraction cloning as a molecule specifically induced by activation of NF-IL6 transcription factor [69]. Mincle is expressed on monocytes, macrophages, neutrophils, myeloid DCs, and subsets of B cells, but not on T cells, pDCs, and NK cells [104].

Mincle associates with FcRγ dependently on the positively charged arginine residue in the transmembrane portion of mincle, as is the case with murine DCAR

but is not the case with dectin-2 [110]. Mincle-FcRγ activation induces signaling through Syk–PKCδ–CARD9–NF–κB and MAPK pathways, resulting in the induction of various cytokines and chemokines.

As predicted by the presence of the EPN motif in its CRD, mincle can bind carbohydrates containing α-mannose or other structures, and fungal, mycobacterial, and necrotic cell components have been identified as ligands for mincle [50, 106, 111]. The *Candida albicans* cell wall component was one of the first identified ligands and mincle was shown to mediate the protective antifungal immunity [106]. *Malassezia* is also recognized by mincle and induces cytokine and chemokine production and inflammatory cell recruitment through mincle [111]. Mincle also recognizes *Fonsecaea pedrosoi*, which was revealed to cause chromomycosis in the skin due to insufficient costimulation of TLR on mincle signaling [91].

Mincle also recognizes mycobacterial trehalose dimycolate (TDM), which is an immunomodulatory glycolipid component and has been called mycobacterial cord factor, and mediates TDM or synthetic trehalose dibehenate-induced inflammatory responses including production of cytokines and nitric oxide, NLRP3 inflammasome activation, Th1 and Th17 activation, and granuloma formation [50, 64, 87, 89, 107]. However, in mincle-deficient mice, variable inflammatory responses were observed dependent on the species of infected mycobacteria, probably because they contained different combinations of PAMPs [10, 64]. Interestingly, mycobacteria-induced granuloma formation was unaffected irrespective of mycobacteria species.

Also interesting is that the significance of mincle expression was reportedly different in monocytes and neutrophils. Mincle expression was associated with cytokine production in monocytes, whereas it was rather associated with fungal uptake and killing in neutrophils [104]. Furthermore, the expression of mincle was reciprocally detected between monocytes and neutrophils in individuals.

Mincle also works as a sensor for necrosis by recognizing spliceosome-associated protein (SAP) 130 released from necrotic cells [110]. Notably, mincle has been shown to use a different binding site for SAP130 from that used for carbohydrate recognition. Although it is predictable that mincle is involved in the regulation of autoimmunity, an autoimmune phenotype of mincle-deficient mice has never been reported. In contrast, it has recently been reported that a SNP in the *CLEC4E* gene is associated with ACPA-positive RA [108].

17.3 Concluding Remarks

As described in this chapter, critical roles of the representative CLRs have been revealed in the responses for viral, fungal, and mycobacterial infections, as well as in the maintenance of immunological homeostasis (Table 17.1). As a surface barrier to environmental stimuli including invasive pathogens, skin provides the first battlefield and dermatologists should prepare more and better ways to combat pathogens and keep homeostasis. CLRs should certainly provide targets to develop effective vaccination and therapeutics for distinct infectious and autoimmune/inflammatory diseases.

Table 17.1 Summary of the characteristics of selected CLRs

	CD	CLEC	Tail Motif	Signaling Enzymes	CTD Motif	Interacting Pathogens	Functions
Langerin	CD207	CLEC4K	P-rich	None	EPN	HIV-1 (gp120), *Candida*, *Malassezia*, *Mycobacteria* (LAM)	BG formation, HIV-1 degradation, glycolipid antigen loading to CD1a
DC-SIGN	CD209	CLEC4L	LL, YxxL	Raf-1		HIV-1 (gp120), dengue, ebola, CMV, HCV, measles, SARS, *Helicobacter pilori*, *Klebsiela Pneumonae*, *Leishmania pifanoi*, *Schistosoma mansoni*, *Candida albicans*, *Mycobacteria* (ManLAM), IgG-sFc	In *trans* HIV-1 infection, interfere with TLR signal for pathogen immune escape (susceptibility to HIV-1 infection and tuberculosis), IVIG anti-inflammatory effect
Dectin-1		CLEC7A	HemITAM	Syk, PLCγ2, Raf-1		*Candida albicans*, *Pneumocystis carini*, *Mycobacterium abscessus*	Prevention of mucocutaneous candidiasis, Exacerbation of autoimmunity
Dectin-2		CLEC6A	FcRγ-ITAM	Syk, PLCγ2	EPN	*Candida albicans*, *Saccharomyces cerevisiae*, *Aspergilus fumigatus*, *Cryptococcus neoformans*, *Paracoccidioides brasiliensis*, *Microsporum audouinii*, *Trichophyton rubrum*, *Histoplasma capsulatum*, *Schistosoma mansoni*, *Mycobacterium tuberculosis*, house dust mite, $CD4^+$ $CD25^+$ T cells	Th17 response to *Candida albicans* infection, house dust mite-induced Th2 responses, UV-induced tolerance
DCIR		CLEC4A	ITIM, Y-internalization	SHP-1, SHP-2	EPS	HIV-1, HCV	HIV-1 infection, susceptibility to ACPA-negative RA
BDCA-2	CD303	CLEC4C	FcRγ-ITAM, EEE	Syk	EPN	HIV-1 (gp120), HCV, monocytes, moDCs, tumor cell lines	Suppression of type I IFN production by pDCs
Mincle		CLEC4E	FcRγ-ITAM	Syk	EPN	*Candida albicans*, *Malassezia*, *Fonsecaea pedrosoi*, *Mycobacteria* (cord factor, TDM), necrotic cells (SAP130)	Antifungal and mycobacterial immunity, susceptibility to ACPA-positive RA

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Chapter 18
Emergence of Virulent Staphylococci Overriding Innate Immunity of Skin in Communities

Junzo Hisatsune and Motoyuki Sugai

Abstract More than half a century has passed since MRSA emerged in the world, and the characteristic nature of MRSA keeps changing. Initially healthcare-associated MRSA prevailed in hospital and healthcare facilities, and thereafter community-associated MRSA emerged and spread in communities. Reports of severe case infection by CA-MRSA are gradually increasing in Japan. Now MRSA can be classified into 11 types according to its staphylococcal cassette chromosome (SCC*mec*) type. Together with the variety of virulence gene repertoire, clinically important MRSA is the group with diverse genotypes. In this chapter, we focused on CA-MRSA and summarized its history, diversity, and virulence factors.

Keywords Community-associated MRSA • Healthcare-associated MRSA • PVL • ACME • PSMs • Skin • SSTI

18.1 Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) was first reported in 1961 and thereafter it has been known as a leading etiological agent of nosocomial infection. MRSA produces extra-low–affinity penicillin binding protein PBP2′ in addition to PBP 1-4 and becomes resistant to virtually all ß-lactam antibiotics including oxacillin, narrow spectrum semisynthetic penicillin, and cefems [1, 2]. This resistance trait is acquired through obtaining a mobile cassette chromosome SCC*mec* containing *mecA*, gene for PBP2′ [3]. Studies of SCC*mec* revealed it has a sequence variety that has been used for epidemiological typing of MRSA [4]. For MRSA typing, other typing methods such as multilocus sequence typing (MLST) [5], spa-typing (polymorphism of gene for cell wall protein, Protein A) [6], accessory

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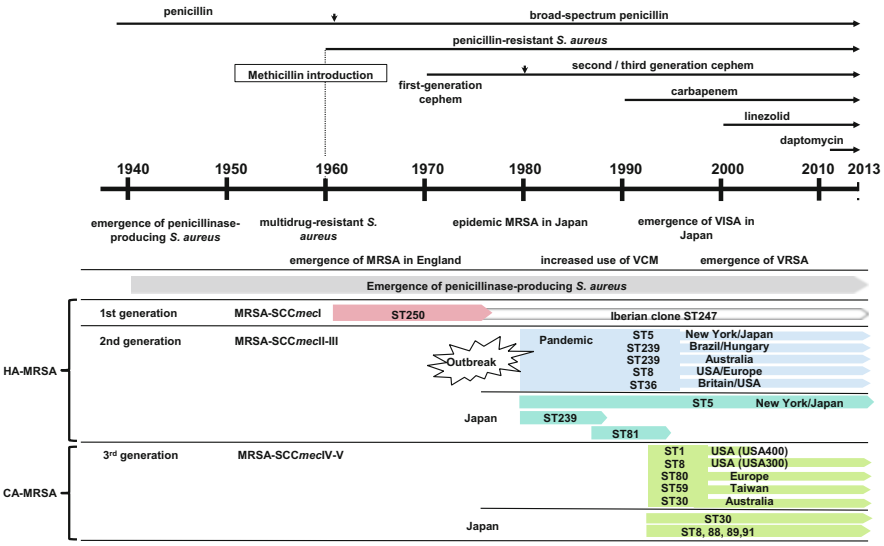


Fig. 18.1 History of methicillin-resistant *Staphylococcus aureus*

gene regulator (*agr*) typing [7], coagulase typing [8, 9], and virulence gene typing have been used in combination (Fig. 18.1).

Looking back at the history of ß-lactam resistance of *S. aureus*, we notice we have experienced emergence of three generations of MRSA. The first generation appeared just after introduction of methicillin into the community in England in the 1960s [10]. This is now regarded as the Archaic MRSA clone (ST250) [11, 12]. This type spread in Europe and part of the United States, but did not become a pandemic clone. It gradually faded out from Europe by the end of the 1980s and a possible descendant can be found in outbreaks in Spain [13]. In the late 1970s, new outbreaks occurred in the United States and the isolates eventually spread on a global level causing pandemic [14]. We call these isolates second-generation MRSA. In Japan, MRSA isolation started to increase in the 1980s and ST5 is called the NY/Japan clone, which is dominant in healthcare facilities in Japan as well as in the United States. The isolation of MRSA belonging to the two generations, classical and second generation MRSAs, is the only one limited to hospitals and healthcare facilities, and such MRSA is called hospital-acquired or healthcare-associated MRSA (HA-MRSA) [15]. The reason why we started these names is because we started experiencing the emergence of a third generation of MRSA with a different nature [16]. In the 1990s, we started to isolate MRSA from the community and it was called community-acquired MRSA (CA-MRSA) [17–19].

Recently, news that MRSA from pig farms was spreading in communities and causing infection surprised society [20]. A certain clonal group ST398 of MRSA, once the inhabitant of humans, crossed into livestock, where it acquired drug resistance and jumped back to humans. These strains are now called livestock-

associated MRSA (LA-MRSA), which is now regarded as another problematic new population in MRSA.

18.2 Emergence of CA-MRSA

In August 1999, the CDC in the United States released a weekly report MMWR with the title of "Four Pediatric Deaths from Community-Acquired Methicillin-Resistant *Staphylococcus aureus*—Minnesota and North Dakota, 1997–1999" [21]. It reported four cases of fatal MRSA infections. The patients were neonates and infants (12 months to 7 years old). Two cases developed necrotic hemorrhagic pneumoniae and all cases manifested serious septicemia. Those patients did not have any risk factors for MRSA infection. The pattern of drug resistance and pulsed-field gel electrophoresis indicated the isolates were different from those of nosocomial MRSA. By this time, several sporadic cases have been reported in United States. In March 2003, *Science* posted a short report "Resistant Staph Finds New Niches" describing "MRSA has been an important cause of infections mainly in hospitals and nursing homes, where it's often resistant to all antibiotics except vancomycin, considered the last resort" [22]. Epidemiologists started seeing more cases of MRSA among people who had no specific connection to hospitals-for instance, among Native Americans, athletic teams, intravenous drug users, and schoolchildren [23–25]. Initially, some researchers speculated that those "community-acquired" microbes might have "escaped" from hospitals. But a genetic comparison of community-and hospital-acquired MRSA strains by Patrick Schlievert of the University of Minnesota, Twin Cities, and colleagues, published in January, suggests that the community variety arose independently and not long ago, when a wild strain picked up a so-called cassette chromosome, a mobile genetic element, containing a gene for methicillin resistance. At that time, several outbreaks of furuncles/carbuncles turned up on hands, legs, chests, buttocks, and genitalia among prisoners and gays had drawn the attention of doctors on the US West Coast [26]. Similar strains were also isolated in Europe and the strong virulence has been focused although the incidence ratio is very low. Most focused elapse of disease starts with skin soft tissue infection and shifting to hemorrhagic necrotic pneumonia [27, 28]. Most isolates produced a leukotoxin called Panton Valentine Leukocidin (PVL) and PVL has been regarded as one of the important virulence factors produced by CA-MRSA [27]. This toxin was discovered from *S. aureus* causing skin soft tissue infection by Philip Noel Panton and Francis Valentine in 1983 and designated after their names [29, 30]. Not all CA-MRSA produce PVL but PVL is regarded as an important virulence factor of CA-MRSA [31, 32].

In the United States, lineages such as USA300 and USA400 started to cause outbreaks in healthcare facilities suggesting that these CA-MRSA started to invade nosocomial settings and become established [33, 34]. In the near future, classification of MRSA by the site of isolation will be useless. In Japan, there was no sign of increase of CA-MRSA isolation from such severe fatal infection but very recently

our laboratory started to receive a number of CA-MRSA from severe SSTI and fatal infections suggesting that the isolation of CA-MRSA is gradually increasing.

18.3 Clinical Background of CA-MRSA Infection and Bacteriology

Classification of MRSA into HA-MRSA or CA-MRSA depends on the clinical background of the patient where the isolate derived from and bacteriology of the isolate. Normally CA-MRSA is isolated from healthy personnel unrelated to previous history of medical treatment [28]. More precisely, isolates from the patient such as (1) outpatient or inpatient within 48 h post-admission, (2) no history of isolation of MRSA, (3) no history of operation, renal transfusion, hospitalization, (4) no history of indwelling of catheter or medical devices are regarded as CA-MRSA. Also CA-MRSA possesses several bacteriological characteristics as shown in Fig. 18.2. In Japan, HA-MRSA carries SCC*mec* type II or III and often manifests multidrug resistance: HA-MRSA is resistant to not only ß-lactams but macrolides and aminoglycosides [35]. On the other hand, CA-MRSA carries SCC*mec* type IV or V, which is a relatively smaller sized element and susceptible to other antimicrobial agents [36]. But USA300 is an exception: it shows resistance to fluoroquinolones and mupirocin as well [37]. USA300 is a multidrug resistant CA-MRSA now prevalent worldwide and in Japan the isolation started after 2007 [38].

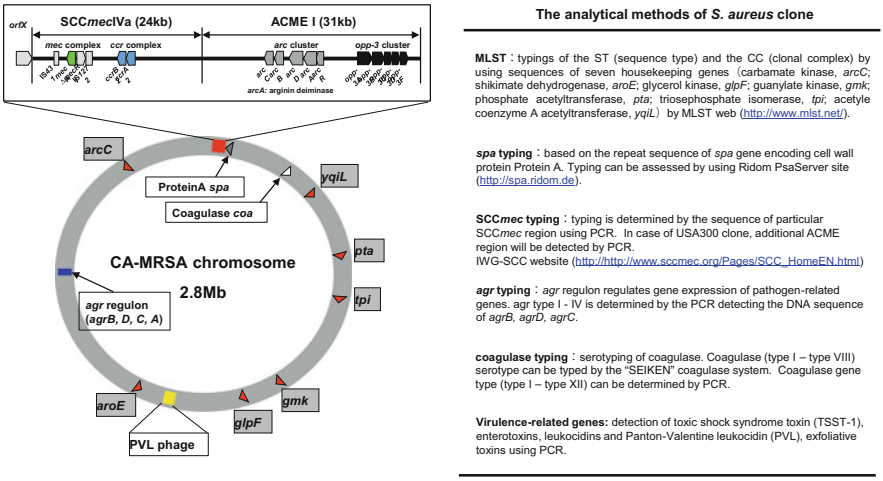
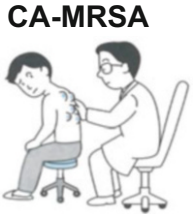


Fig. 18.2 Genotyping methods of MRSA. *Left*, schematic genome map of CA-MRSA. *Grey squares* indicate housekeeping genes. *Right*, the genetic typing methods to analyze *S. aureus* clone

18.4 Genetic Diversity of CA-MRSA

Upon acquiring SCC*mec*, *S. aureus* becomes MRSA [39]. SCC*mec* is a mobile genetic element flanked by *att* sites of about 15 bp nucleotide sequences in both ends. SCC*mec* is integrated into the genome through recombination at the *att* sequence present in the 3′ terminal of *orfX* [40, 41]. SCC*mec* can be classified into 11 groups, I–XI [41, 42]. As shown in Fig. 18.3, SCC*mec* is composed of the *ccr* gene complex (*ccr*: cassette chromosome recombinase involved in site-specific recombination of SCC*mec*) and *mec* gene complex. Typing is stipulated by the International Working group on the Staphylococcal Cassette Chromosome elements: IWG-SCC (http://www.sccmec.org) [43, 44]. The *ccr* gene complex is composed of either *ccrAccrB* or *ccrC* alone. *ccrAccrB* is classified into four different types (AB1, AB2, AB3, AB4) according to sequence difference [45]. The *mec* gene complex is composed of *mecA* and upstream two regulatory genes, *mecR1* and *mecI*, and is classified into three types, classes A–C [11, 46]. In class A, *mecR1* and *mecI* are present just upstream of *mecA* and IS*431* downstream of *mecA*. In class B, *mecR1* is truncated by insertion of IS*1273*. In class C, *mecR1* is truncated by IS*431* in the same (C1) or opposite (C2) direction to the preceding IS*431* forming a transposon.

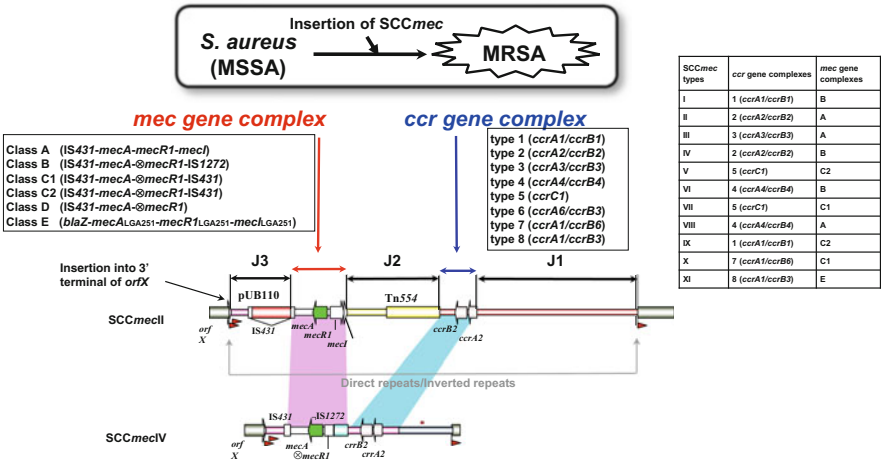
Emergence of MRSA started with Archaic clone (ST250/SCC*mec* I) [47, 48]. Then the second generation pandemic clones including the New York/Japan clone (ST5/SCC*mec* II) [35], pediatric clone (ST5/SCC*mec* IVa) [49], Berlin



background of patient	• inpatients • elderly • lost anatomical barrier • immunocompromised	• no risk factor for MRSA infection • all age (more common in children) • normal anatomical barrier and immunity
disease	sepsis, pneumoniae, enteritis	skin and soft tissue infections
antibiotic susceptibility	multiple resistant	resistant to β-lactam (susceptible to other antimicrobials
SCC*mec* typing	SCC*mec* I, II, III (mainly type II in Japan)	SCC*mec* IV, V
MLST	ST5, ST247, ST239	ST8, ST30, ST80, ST59
Virulence factors	toxic shock syndrome toxin etc.	PVL (rare in Japan), exfoliative toxin, PSMs↑

Fig. 18.3 Comparative characteristics of HA-MRSA and CA-MRSA (Partially modified from Ref. [2])

clone (ST45/SCC*mec* I) [15, 50, 51], Iberian clone (ST247/SCC*mec* I) [48, 51], Brazilian clone (ST239/SCC*mec* IIIa) [52, 53], EMRSA-15 (ST22/SCC*mec* IV) [54, 55], EMRSA-16 (ST36/SCC*mec* II) [49, 56], and others (Fig. 18.4). CA-MRSA appeared worldwide around 1999 as PVL-producing virulent MRSA. Representative PVL-positive CA-MRSA include USA400 (ST1/SCC*mec* IVa) [57] and USA300 (ST8/SCC*mec* IVa) prevalent in the United States [37], European clone (ST80/SCC*mec* IV) [58], pandemic clone (ST30/SCC*mec* IVa or IVc) [59], the clone prevalent in Taiwan and United States (ST59/SCC*mec* V) [60, 61], new European clone (ST22/SCC*mec* IV or V, in Japan IVa) [62, 63], and Bengal Bay clone (ST772/SCC*mec* V) prevalent in India and Malaysia [64, 65]. In Japan, the ratio of isolation of PVL-positive CA-MRSA is extremely low, 3–5 % compared to that of the United States [66]. CA-MRSA in Japan is largely PVL-negative. Recently, ST8/SCC*mec* IV distinct from the USA300 clone has been frequently isolated from pneumoniae, sepsis, musculoskeletal abscess, and disseminated severe infection [67, 68]. This clone carries *spj* encoding new cell wall protein CWASP/J in SCC*mec* in addition to *tst* and *sec* although this does not possess *pvl* and ACME genes (Fig. 18.5) [67, 69]. More classical CA-MRSA belonging to ST88, ST89, and ST91 produces exfoliative toxin B and is involved in impetigo and staphylococcal scalded skin syndrome in neonate/infants [35]. Genetical relations of representative clonal complexes of HA-MRSA, CA-MRSA, and recently emerged LA-MRSA are depicted in Fig. 18.6. Table 18.1 summarizes the genetic diversity of the CA-MRSA clone in the world.



SCC*mec* types	*ccr* gene complexes	*mec* gene complexes
I	1 (*ccrA1/ccrB1*)	B
II	2 (*ccrA2/ccrB2*)	A
III	3 (*ccrA3/ccrB3*)	A
IV	2 (*ccrA2/ccrB2*)	B
V	5 (*ccrC1*)	C2
VI	4 (*ccrA4/ccrB4*)	B
VII	5 (*ccrC1*)	C1
VIII	4 (*ccrA4/ccrB4*)	A
IX	1 (*ccrA1/ccrB1*)	C2
X	7 (*ccrA1/ccrB6*)	C1
XI	8 (*ccrA1/ccrB3*)	E

Fig. 18.4 Structure and typing of SCC*mec*. Methicillin-susceptible *S. aureus* will be converted to MRSA upon acquiring SCC*mec*, a genomic island encoding *mecA* and other genetic elements. SCC*mec* carries several elements including (1) the *mec* gene complex (*mec*); (2) the *ccr* gene complex (*ccr*); (3) regions other than *mec* and *ccr* designated as "joining regions" (J1, J2, and J3); (4) characteristic nucleotide sequences, inverted repeats, and direct repeats at both ends; and (5) the integration site sequence (ISS) for SCC. The SCC*mec* is located at the 3′ end of *orfX* in chromosome

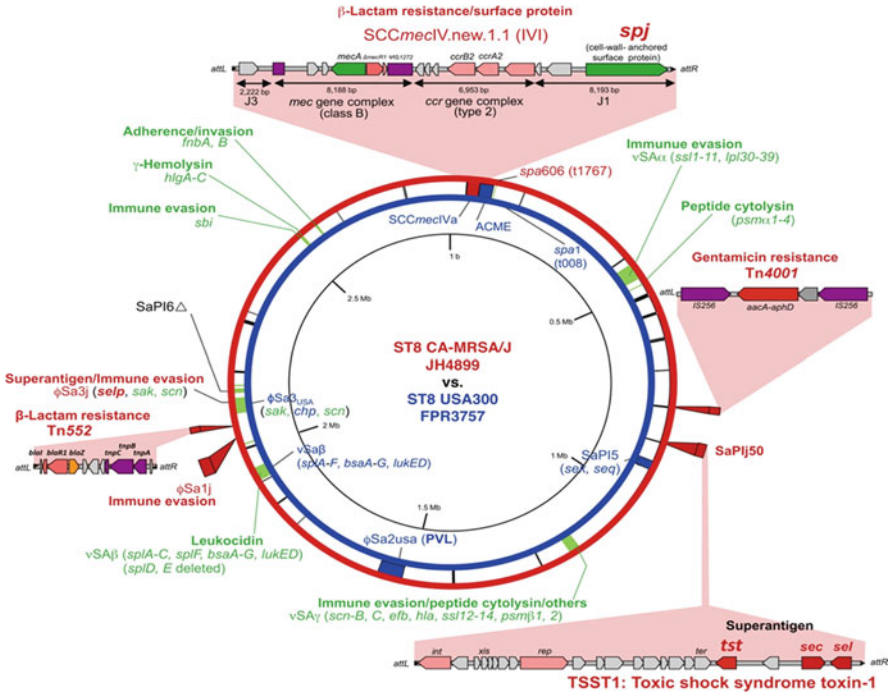


Fig. 18.5 Genomic comparison of ST8/SCC*mec*IVl and USA300. Genomic information of JH4899 (ST8/SCC*mec*IVl) and USA300 (ST8/SCC*mec*IVa) are presented in circular maps (outside, JH4899; inside, USA300). Note a novel cell wall protein CWASP/J encoded by the *spj* in SCC*mec*IVl. JH4899 lacked phiSA2usa (carrying a PVL gene) and ACME, which are present in USA300. (Part of figure was modified from Ref. [68])

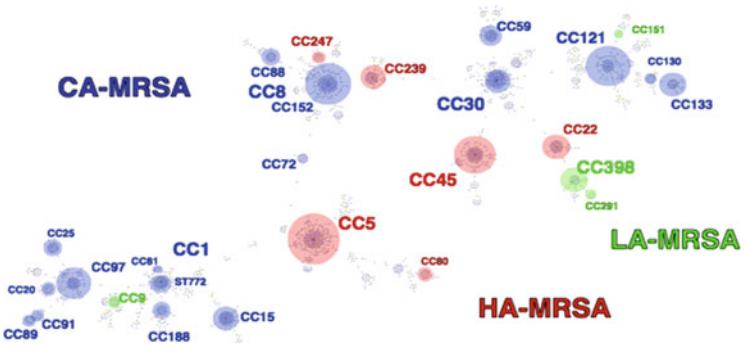


Fig. 18.6 Clonal diversity of MRSA by e-BURST analysis. eBURST analysis using MLST indicated relations of HA-MRSA, *red*; CA-MRSA, *blue*; and LA-MRSA, *green*

Table 18.1 Diversity of CA-MRSA clones

ST	*arcC*	*aroE*	*glpF*	*gmk*	*pta*	*tpi*	*yqiL*	*spa*	SCC*mec*	Regional clones
1	1	1	1	1	1	1	1	131	IV	USA300, CMRSA7, WA-1
5	1	4	1	4	12	1	10	2	I, II, IV, V, VI	Geradine, Pediatric
6	12	4	1	4	12	1	3	Unknown	IV	–
8	3	3	1	1	4	4	3	1	IV, V, VI	USA300, CMRSA10, WA-12
22	7	6	1	5	8	8	6	113	IV, V	–
30	2	2	2	2	6	3	2	19	IV, V	SWP, OSPC, WSPP, USA1100
45	10	14	8	6	10	3	2	73	IV, V, VI	–
59	19	23	15	2	19	20	15	17	IV, V	USA1000, Taiwan
72	1	4	1	8	4	4	3	193	IV, V	USA700
75	36	3	43	34	39	52	49	Unknown	IV, V	WA-8, WA-79
78	22	1	14	23	12	53	31	Unknown	IV	–
80	1	3	1	14	11	51	10	70	IV	European CA-MRSA
88	22	1	14	23	12	4	31	9	IV, V, VI	–
91	1	26	28	18	18	54	50	416	II, IV, V	–
93	6	64	44	2	43	55	51	1143	IV, V	Queensland
97	3	1	1	1	1	5	3	105	IV, V	–
121	6	5	6	2	7	14	5	312	V	–
152	46	75	49	44	13	68	60	207	V	Balkan
377	46	75	49	50	13	68	60	207	V	Balkan
772	1	1	1	1	22	1	1	692	V	Bengal Bay, WA-60

Partially modified from Ref. [9]

ST sequence typing, *arcC – yqiL* alle number of seven housekeeping genes, *spa* Protein A genotype, SCC*mec* SCC*mec* type, *CMRSA* Canadian epidemic clone, *WA* Western Australian clone, *SWP* South West Pacific clone, *OSPC* Oceanic Southwest Pacific clone, *WSPP* Western Samoan Phage patten

18.5 Virulence Factors of CA-MRSA

When bacteria attach and adhere to the surface of a host, and invade into the body, the host will recognize the bacteria as a foreign body and start exerting an eliminating system, which is called immunity. *S. aureus* produces a variety of virulence factors including cell surface proteins and secreted protein toxins, and the degree of production and their repertoire vary according to clone types (Fig. 18.7). The bacteria also possess evasion tools to escape from the host immune system [70, 71]. -HA-MRSA is associated with inpatients in hospitals/health care facilities [72]. Therefore it might be relatively easy for them to go inside the host body inasmuch as all they have to do is to use the anatomical barrier niche such as catheter tubing to invade the blood circulation and start disrupting the acquired immunity system using superantigens. On the other hand, CA-MRSA infects healthy bodies and it needs to go through a tight anatomical barrier to reach soft tissue. Therefore CA-MRSA needs a variety of virulence factors necessary to overcome the innate immune barrier system. Some important virulence factors for CA-MRSA are listed as follows.

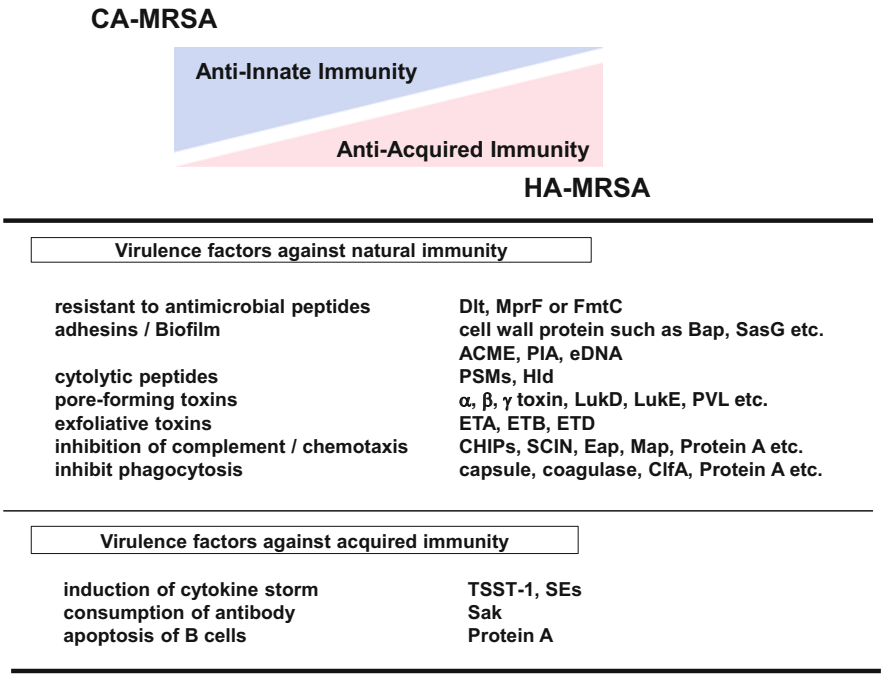


Fig. 18.7 Comparative characterization of virulence factors of HA-MRSA and CA-MRSA

18.5.1 Leukocidin

Several types of leukocidins are known including PVL that selectively lyse leukocytes and its variants LukED and LukMFPV, and Luk that lyses rabbit red blood cells [73, 74]. PVL production is reportedly associated with furunculosis [75]. Genes for PVL are tandemly encoded as *lukS-PV* and *lukF-PV* forming an operon and the genes are embedded in the lysogenic phage genome between the lytic enzyme gene and *att* site of the phage [74]. This phage can mobilize genes for PVL and several PVL-transducing phages have been reported such as ΦPVL and ΦSTL [76, 77]. PVL is composed of two components, LukS-PV and LukF-PV, and these components attack and integrate into the cell membrane of white blood cells (neutrophils, granulocytes, monocytes, macrophages, etc.) to form pores made of hexamer–octamer, which induce necrosis in the end [74, 78, 79]. Under low concentration, PVL induces apoptosis or the generation of inflammatory factors such as IL-8 and LTB4 [80, 81]. PVL is suggested to be involved in the onset of skin and soft tissue infection such as furunculosis and carbuncle [82]. PVL-producing *S. aureus* often causes invasive infectious diseases such as deep-seated subcutaneous abscess, osteomyelitis, necrotic pneumoniae, and sepsis [25]. PVL is one of the leading virulence factors involved in CA-MRSA infection, but some cases by PVL-nonproducing CA-MRSA exist. In the case of severe invasive CA-MRSA infection, a PVL-producing strain must be taken into account for the etiological agent.

18.5.2 Cytolytic Peptides

Cytolytic peptides are a complex of peptides that are produced by virtually all MRSA isolates. They are called PSMs (phenol soluble modulin-like peptide) based on their solubility to phenol [83]. Each peptide is N-terminally formulated. Various groups are known including δ hemolysin composed of 26 amino acids, four types of PSMαs, PSMα1-4 composed of 20–25 amino acids, and two types of PSMβs, PSMβ1-2 composed of 44 amino acids. CA-MRSA is reported to produce more abundant PSMs than HA-MRSA [25, 84]. PSMs are suggested to induce lysis of neutrophils, production and disruption of biofilms, and are implicated in pathogenesis of CA-MRSA infection [83]. In HA-MRSA, a gene for PSM-*mec*, 20 amino acids peptide, is encoded in the J2 region of SCC*mec* II, III, and VIII [85]. A recent study indicated a transcript of PSM-*mec* acts as a functional *psm-mec* RNA suppressing other pathogenic genes on the genome and renders HA-MRSA less virulent [86, 87]. In support of this, a point mutation was found in the promoter region of *psm-mec* and this mutation canceled the suppression of genes for PSMαs and induced high expression of PSMαs in highly virulent HA-MRSA (SCC*mec* II).

18.5.3 Arginine Catabolic Mobile Element (ACME)

ACME was first discovered in CA-MRSA USA300 (ST8/SCC*mec*Va) as the complex of SCC*mec*Va (ca. 34 kb) and ACME (ca 31 kb), but later found in ST5/SCC*mec* II and IV, ST59/SCC*mec*IVa, ST97/SCC*mec* V, ST1/SCC*mec* Iva, and ST239/SCC*mec* III, and coagulase negative staphylococci such as *Staphylococcus epidermidis*, *S. haemolyticus*, and *S. capitis* [37, 88–90]. ACME is a mobile element of a gene cluster composed of two clusters, the arginine–metabolism gene (*arc*) cluster (*arcA, arcB, arcD, arcA, arcR*) and the quorum-sensing gene cluster (*opp-3A, opp-3B, opp-3C, opp-3D, opp-3E*) [37]. There are three types I–III in ACME: ACME-I possesses clusters of *arc* and *opp*; ACME II possesses only the *arc* cluster; ACME-III possesses only the *opp* cluster [37, 91, 92]. Involvement of ACME to adherence to skin and mucosal surface, and horizontal transfer of the bacteria have been suggested but detailed mechanisms remain elusive. The *arc* cluster is involved in the metabolic pathway of arginine (arginine deiminase system, ADS) and ornithine, ammonia, carbon dioxide, and ATP are generated as the metabolites of L-arginine [93]. The generation of ammonia by ADS neutralizes the acidic condition of skin thereby enabling colonization of *S. aureus* on the skin surface. The *Opp-3* cluster is implicated in the uptake of peptides as nutrient and in regulation of growth through quorum-sensing [94].

18.5.4 Attachment and Adherence to Host (Biofilm)

S. aureus uses two mechanisms for their adherence to host: nonspecific binding by using hydrophobic and/or electrostatic interaction with plastic or metal surface; and specific binding to extracellular matrix and/or serum protein [95]. Bacterial proteins used for such binding are collectively called microbial surface components recognizing adhesive matrix molecules (MSCRAMM). MSCRAMM includes proteins such as Protein A (Spa), fibronectin-binding protein (FnBPA/B), collagen-binding protein (Cna), fibrinogen-binding protein (ClfA/B), Sdr protein, and others, and they are believed to be involved in initial attachment of the bacteria to the tissue [96]. Subsequent adhesion and biofilm formation are necessary for the bacteria to establish infection in the host [97]. Polysaccharide intercellular adhesion (PIA) is the major such factor, and genes involved in synthesis and production of PIA are encoded on *icaA, icaD, icaB,* and *icaC* as an operon [98–100].

18.5.5 Protection from Antimicrobial Peptides

Antimicrobial peptides, defensins and LL-37, are produced by platelets, neutrophils, and keratinocytes and play an important role in innate immunity [101]. They

show antimicrobial activity through destroying the lipid bilayer of the bacteria and protect bacterial infection [102]. Dlt protein modifies teichoic acids with D-Ala and neutralizes surface negative-charge and protects the challenge of positively charged antimicrobial peptides [103, 104]. MprF modifies phsophatidylglycerol with L-lysine and works similarly to protect the bacteria from antimicrobial peptides [105, 106].

18.5.6 Escape from Phagocytes

The capsule is a cell surface polysaccharide and a representative of the antiphagocytotic agent of bacteria [107]. The *S. aureus* capsule can be classified into 11 serotypes [108, 109]. Among them types 5 and 8 are microcapsule-prevalent in most clinical isolates [110]. CA-MRSA mainly possesses type 8 and HA-MRSA type 5. The freeform of Protein A binds to the Fc portion of the antibody binding to the bacterial cell surface and interrupts opsonization [111, 112]. Cell wall protein ClfA/B induces the deposit of fibrinogen on the cell surface to protect bacterial cells from macrophages [113]. Extracellular coagulase forms a complex with prothrombin, and the complex acts as active staphylothrombin to convert fibrinogen to fibrin [114, 115]. Producing bacteria are covered by a fibrin clot and escape from phagocytosis of the macrophages [116].

18.5.7 Escape from Complement Activation and Chemotaxis

CHIPS, Eap, and SCIN are extracellular proteins secreted by *S. aureus*. CHIP (chemotaxis inhibitory protein of *S. aureus*) binds to receptors for formyl peptide and C5a on the cell membrane of neutrophils thereby inhibiting chemotaxis of neutrophils [117, 118]. Eap binds to ICAM-1 on endothelial cells coating the inner surface of blood vessels and inhibits recognition and binding of neutrophil to endothelial cells for extravascular migration [119, 120]. SCIN (staphylococcal complement inhibitor) acts on C3 convertase and inhibits complement activation [121].

18.6 Conclusion

MRSA infection is one of the leading bacterial infectious diseases posing a public concern. As we described in this chapter, CA-MRSA has various genotypes and appears to keep evolving through exogenously acquiring genes and endogenous mutations to cope with environmental changes. Recently there has been a gradual increase of isolation of CA-MRSA in medical facilities including those from severe

cases. In a clinical setting, typing of MRSA for risk assessment is important. However, detailed characterization and analysis for such purpose requires time and manpower. The recent advent of a new generation sequencer enabled us to characterize resistance genes, virulence genes, genotypings, and so on in high resolution and fidelity in a short time. Concomitantly, fundamental data describing the potential risk of each clinical isolate using meta genome data and experimental data are required. Further studies on clinical and basic MRSA research may be necessary to control nosocomial infection, improve prognosis, and decrease medical cost.

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Chapter 19
Viral Infection

Tatsuyoshi Kawamura

Abstract The skin plays a central role in host defense against a broad array of potentially pathogenic microbes, including viruses. Over the past decade, remarkable progress has been made towards understanding the innate immune responses, especially to viral detection. To recognize the invading viruses, various types of cells resident in the skin express many different pattern-recognition receptors (PRRs) such as C-type lectin receptors (CLRs), Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), and cytosolic DNA sensors, that can sense the pathogen-associated molecular patterns (PAMPs) of the viruses. Viral recognition by the innate immune system is more challenging than recognition of other pathogen classes, because any given viral protein is unlikely to be shared among diverse viruses. The cutaneous antiviral innate immunity constitutes the first line of host defense that limits the virus dissemination from the skin, and also plays an important role in the activation of adaptive immune response, which represents the second line of defense. More recently, the third immunity "intrinsic immunity" has emerged, providing an immediate and direct antiviral defense mediated by host intrinsic restriction factors. This chapter summarizes the current knowledge of the host antiviral immune systems in the skin, highlighting the innate immunity against skin-associated viruses, and describes how viral components are recognized by cutaneous immune systems.

Keywords C-type lectins (CLRs) • Toll-like receptors (TLRs) • NOD-like receptors (NLRs) • RIG-I like receptors (RLRs) • Antimicrobial peptide (AMPs) • Intracellular DNA sensors • Intrinsic antiviral immunity

Abbreviations

AIDS	Acquired immune deficiency syndrome
AIM2	Absent in melanoma 2

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AMPs	Antimicrobial peptides
AP-1	Activator protein 1
APCs	Antigen-presenting cells
APOBEC3	Apolipoprotein B mRNA-editing enzyme-catalytic, polypeptide-like 3
ASC	Apoptosis-associated speck-like protein containing a caspase recruitment domain
ATRX	Alphathalassemia/mental retardation syndrome X-linked
BMDC	Bone-marrow–derived DC
CARD	Caspase activation and recruitment domain
CCR5	CC chemokine receptor 5
cGAS	Cyclic GMP–AMP synthase
CLEC	C-type lectin-like receptor
CLR	C-type lectin receptor
CMV	Cytomegalovirus
DAI	DNA-dependent activator of IFN-regulatory factors
DAMP	Danger-associated molecular pattern
DC	Dendritic cell
DCIR	DC immunoreceptor
DC-SIGN	DC-specific intercellular adhesion molecule-3–grabbing nonintegrin
DNA-PKcs	DNA-dependent protein kinase
ds	Double-stranded
EBERs	Nonpolyadenylated noncoding RNA that forms stem-loop structure by intermolecular base-pairing
EBV	Epstein–Barr virus
ER	Endoplasmic reticulum
GM-CSF	Granulocyte macrophage colony-stimulating factor
HBD	Human β defensing
hDaxx	Human death domain-associated protein
HIV	Human immunodeficiency virus
HMGB1	High mobility group box-1
HNP	Human neutrophil peptides
HPV	Human papilloma virus
HSV	Herpes simplex virus
IFN	Interferon
IL	Interleukin
IRF	Interferon regulatory factor
KSHV	Kaposi's sarcoma-associated herpesvirus
LARG	Leukemia-associated Rho guanine nucleotide exchange factor
LCs	Langerhans cells
LTR	Long terminal repeat
MAP	Mitogen-activated protein
MAVS	Mitochondrial antiviral signaling protein

MDA5	Melanoma differentiation-associated gene 5
MDP	Muramyl dipeptide
MITA	Mediator of IRF3 activation
MyD88	Myeloid differentiation protein 88
ND10	Nuclear domain 10
NF-κB	Nuclear factor-κB
NLR	NOD-like receptor
NLRP3	NACHT LRR and PYD domain-containing protein 3
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
pDC	Plasmacytoid dendritic cells
PKR	Protein kinase R
PML	Promyelocytic leukemia protein
PRR	Pathogen recognition receptor
RIG-I	Retinoic acid inducible gene I
RLR	RIG-like receptor
RNAPII	RNA polymerase II
ROS	Reactive oxygen species
RT	Reverse transcriptase
SAMHD1	SAM domain and HD domain-containing protein 1
si	Short hairpin
Sp100	Speckled protein of 100 kDa
ss	Single-stranded
STAT	Signal transducer and activator of transcription
STDs	Sexually transmitted diseases
STING	Stimulator of IFN genes
SYK	Spleen tyrosin kinase
TBK1	TANK-binding kinase 1
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor α
TRAF	TNF receptor-associated factor
TREX1	Three primerepair exonuclease 1
TRIF	Toll/IL-1 receptor domain-containing adaptor inducing IFN
TRIM5α	Tripartite motif 5α
VSV	Vesicular stomatitis virus
VV	Vaccinia virus
VZV	Varicella zoster virus
WNV	West Nile virus

19.1 Introduction

The skin, as the interface between the body and the environment, provides the first line of host defense against a broad array of viral infections. In primary viral invasion *via* the skin, the outcome is the result of a race between the ability of a virus to replicate inside the skin and the capacity to mount an effective cutaneous immune response. The innate immunity constitutes the very first line of host defense that limits the virus dissemination from the local skin sites of infection, and also plays an important role in the activation of adaptive immune response, which represents the second line of defense. Over the past decade, remarkable progress has been made towards understanding the innate immune responses, especially to viral detection. More recently, the host' third immunity "intrinsic immunity" has emerged. Unlike the innate and adaptive immune systems, intrinsic immunity provides an immediate and direct antiviral defense mediated by intrinsic restriction factors, which are mostly pre-existent in certain cell types. Understanding the mechanisms of cutaneous innate and intrinsic immune responses may have important implications for the design of preventive and therapeutic vaccines.

As the body's most exposed interface with the environment, the skin is constantly challenged by potentially pathogenic microbes, including viruses. There are many viral skin infections, which range from the common to the rare, from the intractable to the self-healing, and from those causing just local infection in the skin to those with associated systemic diseases. Representative viruses transmitted *via* skin or associated with cutaneous symptoms are listed in Table 19.1. To recognize the viruses, the cells resident in the skin, including keratinocytes (KCs), Langerhans cells (LCs), melanocytes, mast cells (MCs), dermal dendritic cells (DCs), macrophages, and fibroblasts, express many differentpattern-recognition receptors (PRRs) that can detect the pathogen-associated molecular patterns (PAMPs) of the invading viruses, which in turn activate antiviral interferon and pro-inflammatory responses. The skin cells also provide an early warning system by releasing stored and inducible cytokines as "alarmin" or danger-associated molecular patterns (DAMPs).

This chapter summarizes the current knowledge of the host antiviral immune systems in the skin and the recent advances regarding cutaneous antiviral immunity, highlighting the innate immunity against the viruses that are transmitted *via* skin or associated with cutaneous symptoms.

19.2 Antiviral Innate Immunity

19.2.1 Viral Recognition by Skin Cells

In the skin, the PAMPs of invading pathogens are recognized by host innate receptors known as PRRs located both at the skin cell surface and within the skin

Table 19.1 Representative viruses transmitted *via* skin or associated with cutaneous symptoms

dsDNA viruses	
Poxviridae	Molluscum contagiosum virus
Herpesviridae	α: human herpesvirus 1, 2, 3 (HSV-1, HSV-2, VZV)
	β: human herpesvirus 5, 6, 7 (HCMV, HHV-6, HHV-7)
	γ: human herpesvirus 4, 8 (EBV, KSHV)
Papillomaviridae	Human papillomavirus (HPV) 1, 2, 3, 4, 5, 6, 7, 8, 10, 11, 57, 60
Smallpox	Vaccinia virus (VV)
ssDNA viruses	
Parvoviridae	Human parvovirus B19
DNA and RNA reverse transcribing virus	
Retroviridae	Human T-lymphotropic virus 1 (HTLV-1)
Lentivirus	Human immunodeficiency virus 1 (HIV-1)
Negative stranded ssRNA viruses	
Rhabdoviridae	Rabies virus
Paramyxoviridae	Measles virus
Positive stranded ssRNA viruses	
Picornaviridae	Human enterovirus A, Coxsackie virus A
Flaviviridae	Dengue virus (DV), Japanese encephalitis virus (JEV), West Nile virus (WNV)
Togaviridae	Rubella virus

cells (see also Chaps. 17, 18, and 19). Viral recognition by the innate immune system is more challenging than recognition of other pathogen classes, because any given viral protein is unlikely to be shared among diverse viruses [1, 2]. However, remarkable progress has been made over the past few years towards understanding the contribution of PRRs, such as C-type lectins (CLRs), Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-I like receptors (RLRs), and other cytosolic PRRs, to viral detection.

In general, the detection of viral PAMPs *via* PRRs initiates two major innate immune signaling cascades: the first involves the activation of the transcription factors interferon regulatory factor 3 (IRF3) and/or IRF7, nuclear factor kappa B (NF-κB) and activator protein 1 (AP-1), which cooperate to induce the transcription of type I interferons (IFNs, e.g., IFN-α and IFN-β), chemokines and pro-inflammatory cytokines (Figs. 19.1 and 19.3). Through the secretion of type I IFNs, the response can be amplified and spread to surrounding uninfected skin cells and thereby activate hundreds of interferon-stimulated genes (ISGs), most of which encode products with profound antiviral effects, such as the degradation of viral nucleic acids or inhibition of viral gene expression [3, 4]. The second signaling pathway results in the formation of an inflammasome complex, which activates caspase-1, a protease which processes pro-interleukin (IL) 1-β and pro-IL–18 to generate active cytokines ready for secretion (Fig. 19.2). In general, TLRs and RLRs are involved in the expression of type I IFNs or pro-inflammatory cytokines

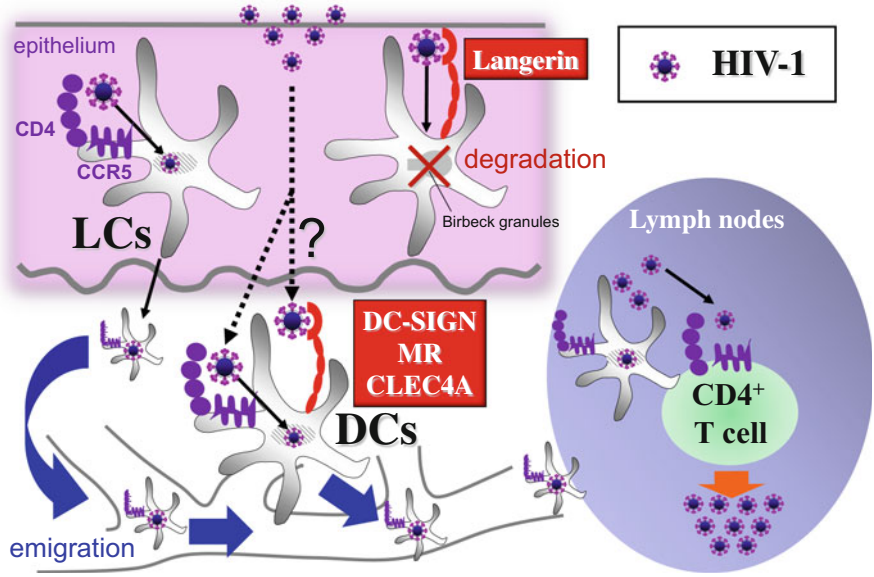

Fig. 19.1 CLRs-mediated HIV-1 recognition and initial biologic events in sexual transmission of HIV. Langerhans cells (LCs) within epithelium of genital mucosa or skin are infected with invading HIV-1 *via* CD4 and CCR5, and then emigrated to the draining lymph nodes, where they replicate the virions and transmit the virus to CD4+ T cells, leading to vigorous production of new HIV virions by T cells (*cis*-infection). HIV-1 bound to langerin is internalized within birbeck granules, where the virus is degraded. DC-SIGN, MR, and CLEC4A promote *cis*-infection of DCs and also facilitate *trans*-infection to CD4+ T cells

and chemokines, whereas viral detection by NLRs leads to caspase-1–mediated processing of IL-1β. The importance of viral recognition *via* these PRRs and subsequent cytokine production is illustrated by the extreme susceptibility to percutaneous viral infection of mice lacking the PRRs and the cytokines.

19.2.2 C-Type Lectin Receptors (CLRs)

CLRs play a pivotal role in the immune system as pattern-recognition and antigen-uptake receptors. CLRs are an important family of PRRs and involved in the recognition of certain skin-associated viruses, which express high-mannose structures (see Chap. 18). The cutaneous innate immune cells can bind skin-associated viruses *via* CLRs, such as DC-specific ICAM-3–grabbing nonintegrin (DC-SIGN; CD209) [5–7], langerin (CD207) [8, 9], Mannose receptor (MR; CD206) [10, 11], DEC-205 (CD205) [12], C-type lectin-like receptor (CLEC) 4A (also known as DC immunoreceptor; DCIR) [13, 14], CLEC 5A [15, 16], and CLEC9A (also known as DNGR-1) [17, 18] (Table 19.2). These CLRs recognize mannose, fucose, and

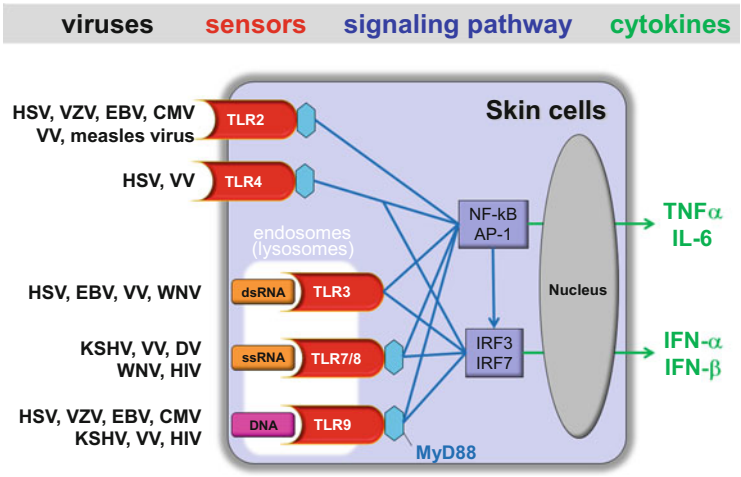

Fig. 19.2 TLRs-mediated viral recognition and signaling pathways in skin cells. Upon ligand stimulation by the indicated skin-associated viruses, TLRs, except the TLR3, recruit the adaptor MyD88. This allows NF-κB to translocate into the nucleus and simultaneously activates the MAP kinase pathway triggering the activation of AP-1. Together, NF-κB and AP-1 induce the expression of pro-inflammatory cytokines. Upon viral ligand stimulation, TLR7 and TLR9 associate with MyD88 leading to nuclear translocation of IRF7. Stimulation of TLR3 and TLR4 permits nuclear translocation of IRF3. IRF3 or IRF7, along with NF-kB and AP-1, cooperate to induce the expression of type I IFNs, IFN-β, and IFN-α, respectively

glycosylated structures of the viruses *via* conserved carbohydrate recognition domains. CLRs have endocytic activity, mediate internalization of the viral ligand or the virus itself, and can direct cargo into intracellular compartments that allow for antigen processing/presentation and the induction of specific gene expression profiles to the viruses, either by modulating TLR signaling or by directly inducing gene expression (see Chap. 18). Although most CLRs do not act as "self-sufficient" PRRs for the viruses, the few CLRs trigger distinct signaling pathways *via* a number of kinases, including spleen tyrosin kinase (SYK) and Src kinase, that modulate the induction of specific cytokines [19, 20]. In addition, recent studies have suggested that several CLRs, such as DC-SIGN and CLEC4A, induce signaling pathways that modulate TLR-induced gene expression at the transcriptional or posttranscriptional level [19, 21]. For example, the interaction of DC-SIGN with mannose-containing virus, such as human immunodeficiency virus (HIV-1) and measles virus, affects TLR4-mediated immune responses by DCs [22]. The crosstalk between TLR4 and DC-SIGN depends on the prior activation of NF-κB by TLR signaling and is therefore not limited to TLR4, but also includes triggering of other NF-κB–inducing PRRs, such as TLR3 and TLR5 [22].

CLRs not only act as beneficial to the host, but also contribute viral spread and pathogenicity. Most studies for CLRs in cutaneous viral infection have long been focused on their roles in the sexual transmission of HIV-1. HIV-1 can bind various CLRs expressed by cutaneous innate immune cells (Table 19.2), which also express

Table 19.2 C-type lectin receptors and related skin-associated viruses

CLRs	Viral Ligands	Expression
DC-SIGN (CD209)	HIV-1, CMV, DV, measles virus	Myeloid DCs, macrophages
Langerin (CD207)	HIV-1, measles virus	Langerhans cells (dermal DCs)
Mannose receptor (CD206)	HIV-1, DV	Myeloid DCs, macrophages
DEC-205 (CD205)	HIV-1	Myeloid DCs
CLEC4A (DCIR)	HIV-1	Myeloid DCs, macrophages, pDCs
CLEC5A	DV, JEV	Monocytes, macrophages
CLEC9A (DNGR-1)	HIV-1	Myeloid DCs

CD4 and coreceptors (CCR5 and CXCR4), required for HIV-1infection (Figs. 19.1 and 19.4) [19, 20]. There are two major mechanisms of HIV-1 transmission between cells; one is *cis*-infection, where target cells are infected with progeny virions, which are released by productively infected cells, *via* CD4 and coreceptors, and the other is *trans*-infection, where target cells are infected with virions, which were captured by the neighboring donor cells without productive infection, *via* the CLRs-mediated virological synapse or the exosome-like pathway [19]. Of note, *cis* and *trans* pathways *via* CLRs are not mutually exclusive, because, in the *cis*-infection, CLRs can facilitate de novo infection of donor cells. Indeed, DC-SIGN, MR, and CLEC4A promote *cis*-infection of innate immune cells through increased interactions between HIV-1 glycoprotein envelope gp120 and CD4, but also facilitate viral capture and CD4+ T-lymphocyte *trans*-infection by mediating viral endocytosis into nondegradative endosomes permitting the intracellular storage of intact virions [19, 20]. DC-SIGN–mediated signaling is even more complex, as HIV-1 requires signaling by DC-SIGN for the replication and the generation of full-length viral transcripts in DCs [23]. In contrast, Langerin impairs infection of LCs by HIV-1 *via* subsequent internalization within birbeck granules, where the virus is degraded (Fig. 19.1) [8]. Nevertheless, there is a saturation of langerin at higher virus concentrations that overwhelms the protective mechanism of action so that langerin ultimately is unable to completely prevent LC infection [8] and CD4/CCR5-mediated *cis*-infection of LCs is considered to be a major pathway involved in the sexual transmission of HIV-1 (Fig. 19.1) [24–26]. In fact, LCs can be productively infected by HIV-1 ex vivo and transmit the virus to cocultured CD4+ T cells [27–29]. Importantly, consistent with the preferential sexual transmission of R5 HIV-1 (CCR5-utilizing HIV), LCs are preferentially infected by R5 HIV, but not X4 HIV (CXCR4-utilizing HIV) [27, 28, 30]. Moreover, it has been observed that up to 90% of initially infected target cells were LCs in Rhesus macaques exposed to intravaginal simian immunodeficiency virus (SIV), and topical application of CCR5 inhibitors completely protected Rhesus macaques from intravaginal exposure of SHIV, a chimeric simian/human immunodeficiency virus, whereas the CLR-inhibitor, mannan, could not [31–33].

19.2.3 Toll-Like Receptors (TLRs)

Several endosomal members of the TLRs family such as TLRs-3, -7, -8, and -9 can recognize nucleic acids of both DNA and RNA viruses (Table 19.1). Viral double-stranded (ds) RNA (dsRNA), single-stranded (ss) RNA (ssRNA), and unmethylated CpG-rich motif in DNA can be recognized by TLR3, TLR7/TLR8, and TLR9, respectively, and all of these nucleic acid-sensing TLRs induce the nuclear translocation of IRF3 and/or IRF7, leading to the expression of type I IFNs (Fig. 19.2) (see also Chap. 17) [1]. The viral ligand stimulation of TLR7/TLR8 and TLR9 recruits the adaptor MyD88, allows NF-κB to translocate into the nucleus, and simultaneously activates the MAP kinase pathway triggering the activation of AP-1. Together, NF-κB and AP-1 induce the expression of pro-inflammatory cytokines. Because viral particles are generally endocytosed and degraded in late endosomes or lysosomes, viral DNA and RNA are released into these intracellular acidic compartments, allowing viral nucleic acids to be in close contact with endosomal TLRs. Alternatively, nucleic acids present in the cytoplasm may be engulfed by an autophagosome, which subsequently fuse with the endosome, and are recognized by the TLRs [34].

dsRNA that is produced by most viruses during their replicative cycle is often considered as a PAMP [35]. RNA viruses are important producers of dsRNA, however, only a few RNA viruses have been shown to induce a TLR3-dependent innate immune response [36]. During West Nile virus (WNV), a mosquito-transmitted flavivirus infection, TLR3-mediated inflammatory responses by viral dsRNA contribute to the development of lethal encephalitis by facilitating virus entry into the brain [37]. TLR3 is hypothesized to enable recognition of a DNA virus because virtually all viruses produce dsRNA at some point during replication [38]. Indeed, in several skin-associated DNA virus infections, TLR3 has been shown to play a role in the antiviral response (Fig. 19.2). For example, it has been demonstrated that herpes simplex virus types 1 (HSV-1) activates keratinocytes to produce type I IFN in a TLR3-dependent manner [39] and TLR3 is required to control HSV-1 spreading to the central nervous system [40]. Activation of TLR3 by viral dsRNA induces not only protective effects against viral infection but also effects that contribute to viral pathogenesis. In Epstein–Barr virus (EBV)-associated hemophagocytic lymphohistiocytosis and infectious mononucleosis, TLR3 contributes to the sudden release of inflammatory cytokines, as the release of EBV-encoded small RNA (EBAR) from EBV-infected cells, which are giving rise to dsRNA-like molecules, activate immune cells via TLR3 signaling to produce type I IFNs and TNF-α [41]. New insights have recently emerged regarding the TLR3-mediated recognition of viral dsRNA; Class A scavenger receptors bind to extracellular viral dsRNA, which is released into the extracellular space after lysis of infected cells and mediate uptake and presentation of dsRNA to TLR3 in the endosome [42]. As well as TLR3, a recent study revealed that TLR4 stimulation also promotes activation of IRF3 and signal to NFκB [43]. Indeed,

TLR4 has been shown to be important for the induction of antiviral cytokines in the response to HSV and vaccinia virus (VV) (Fig. 19.2) [2].

TLR7 and TLR9 detect viral RNA and DNA, respectively, in the endosomal lumen of virus-infected cells and TLR7- and TLR9-dependent signaling pathways require MyD88 and IRF7 to induce type I IFNs, especially IFN-α (see Chap. 17). TLR7/8 can be stimulated by several skin-associated viruses, including Kaposi's sarcoma-associated herpesvirus (KSHV), VV, Dengue virus (DV), West Nile virus (WNV), and HIV-1, and TLR9 can sense all herpes viruses, VV, and HIV-1 (Fig. 19.2) [44]. Most skin cells are able to produce type I IFNs in response to viruses. However, it is of note that for these endosomal TLRs, induction of type I IFNs generally occurs in plasmacytoid DCs (pDCs), "professional IFN producers," [44, 45] which are essentially absent in normal skin but have been described in lesions of inflammatory diseases of the skin (e.g., psoriasis vulgaris, contact dermatitis, and lupus erythematosus) [46]. Intriguingly, in mice models, after *i.v.* inoculation of HSV, TLR9-dependent recognition of CpG motifs in HSV DNA mediates IFN-α secretion by pDCs, whereas pDCs have a negligible impact on local type I IFN production after vaginal or cutaneous HSV infection [47]. Nevertheless, it is still possible that pDCs may play some roles in human anti-HSV immune responses, because pDCs have been shown to infiltrate the reactivated lesions of genital herpes abundantly and persist for months after healing [48]. HSV recognition by TLR9 is considered to be mediated through an endocytic pathway, but does not require HSV viral replication [49], suggesting that the interaction between HSV glycoproteins and cell surface receptors may induce HSV internalization and subsequent release of viral DNA in the endosomal compartment, allowing the interaction with TLR9 [36]. TLR7 has been shown to be involved in recognition of WNV following intradermal infection, and TLR7 activation by ssRNA of WNV promotes migration of epidermal LCs [50, 51]. In addition, deficiency in TLR7 dramatically impairs recognition of ssRNA and immune cell homing to infected target cells during infection with WNV, resulting in increased viremia and susceptibility to lethal WNV infection [52]. TLR7 has also proved to be relevant to episodic reactivation of latent Kaposi's sarcoma-associated herpesvirus (KSHV) [53] and HIV pathogenesis through type I IFN production by pDCs [54]. A recent study has demonstrated that HIV-1 evolves a strategy to turn TLR8 signaling in DCs to its own advantage [23]. TLR8 triggering by HIV-1 ssRNA activated NF-κB, which was required for transcription initiation of the integrated HIV-1 genome by RNA polymerase II (RNAPII), and transcription elongation by RNAPII resulted in full-length HIV-1 transcripts and productive DC infection. Indeed, inhibition of TLR8-induced signaling prevented productive HIV-1 infection of DCs and subsequent transmission of HIV-1 to T cells. Thus, HIV-1 subverts the innate signaling pathways by TLR8 for its replication in DCs and subsequent transmission to T cells. Additionally, VV DNA can induce large amounts of type I IFNs in cells after recognition by TLR8 [55].

Recent studies have revealed that TLR2 can be stimulated by several skin-associated viruses, including HSV-1 and HSV-2 [56, 57], varicella-zoster virus (VZV) [58], human cytomegalovirus (HCMV) [59, 60], mouse cytomegalovirus

(MCMV) [61, 62], measles virus [63], and VV [62, 64] (Fig. 19.2). In most of these cases, the recognition by TLR2, in combination with TLR1 or TLR6, recruits the adaptor MyD88, resulting in translocation of NF-κB into the nucleus and triggering the activation of AP-1, which cooperate to induce the expression of pro-inflammatory cytokines, thus acting as beneficial to the host and mounting an effective innate or adaptive immune response to viruses. For example, VZV and measles virus have been demonstrated to activate human monocytes to produce pro-inflammatory cytokines such as IL-6 in a TLR2-dependent manner [58, 63]. In addition, TLR2 and CD14 expressed on permissive human fibroblast cell strains recognize viral envelope proteins of HCMV, leading to induction of inflammatory cytokines and innate immune activation [60]. Surprisingly, although it is well known that bacterial ligands for TLR2 are unable to drive type I IFN production (see Chap. 17), VV and MCMV have recently been demonstrated to be able to induce type I IFNs *via* TLR2 [62], thus acting as beneficial to the host. Note that this specialized response requires TLR2 internalization and is mediated by a particular cell type (Ly6C, hi, inflammatory monocytes), which has been recently found to recruit to skin in allergic inflammation [65]. On the contrary, viral interaction with TLR2 can be detrimental to its host. HSV-1 recognition by TLR2 induces the expression of inflammatory cytokines in the brain that cause lethal encephalitis [57]. TLR2 activation by measles virus not only induces pro-inflammatory cytokines but also upregulates surface expression of CD150, the receptor for measles virus, indicating that activation of TLR2-dependent signals might contribute viral spread and pathogenicity [63].

19.2.4 NOD-Like Receptors (NLRs)

The cytoplasmic PRRs, nucleotide oligomerization domain (NOD)-like receptors (NLRs), have been recently implicated to recognize viral PAMPs. NLRs that contain a caspase activation and recruitment domain (CARD) are part of the NLRC subfamily including NOD2, whereas NLRs that possess a PYD form the NLRP (also known as NALP) subfamily including NALP3 (see Chap. 17). NLRP3 is essentially involved in the activation of the inflammasome, whereas NOD2 plays a role in the activation of NF-κB and mitogen-activated protein (MAP) kinase pathways. Using a variety of cell types including DCs, macrophages, fibroblasts, and melanoma cells, a critical role for the NLRP3 inflammasome has been demonstrated during the infection of various skin-associated viruses, including HSV, VZV, WNV, Japanese encephalitis virus (JEV), measles virus, and HIV-1 (Fig. 19.3) [44, 66–70]. Interestingly, susceptibility to HIV infection in human patients was associated with several polymorphisms in the *Nlrp3* gene [71]. Because NALP3 is unlikely to bind directly to viral nucleic acids, NLRP3 inflammasome is considered to be activated through an indirect mechanism. One possibility is that viral RNA is sensed by endosomal TLRs or cytoplasmic RLRs (see Chap. 20.2.4), such as retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-

associated gene 5 (MDA5), leading to the priming of NLRP3 inflammasome for activation and the upregulation of pro-IL–1β through NF-κB–mediated signaling pathways (Fig. 19.3) [72]. In addition, RIG-I has been shown to interact directly with the adaptor protein, apoptosis-associated Speck-like protein containing a CARD (ASC) and regulates inflammasome activation, leading to production of IL-1β. RIG-I can mediate IL-1β production in response to ssRNA virus, vesicular stomatitis virus, by activating the caspase-1–dependent inflammasome, which is required to form a complex containing RIG-I and the adaptor protein ASC in an NLRP3-independent manner [73]. Another group also reported that RIG-I interacts with ASC, and regulates inflammasome activation and NLRP3 transcript levels [74], suggesting that RIG-I may prime and activate the inflammasome via the NLRP3-dependent and -independent pathway (Fig. 19.3). Intriguingly, the encephalomyocarditis virus sensed by MDA did require NALP3 for inflammasome activation [73]. The role of RLRs in inflammasome activation is further complicated because type I IFNs, which can be regulated by RLRs, have also been shown to regulate NLRP3 activation [75].

In addition to stimulus from viral ssRNA, dsRNA, and dsDNA, virus-mediated NLRP3 activation requires another signal to induce the production of mature IL-1β by a wide variety of DAMPs including (i) ion leakage from intracellular organelles (e.g., the Golgi) into the cytosol; (ii) the generation of reactive oxygen species resulting from PRR signaling, endoplasmic reticulum (ER), and mitochondrial stress, or virus-induced damage to endosomes, (iii) the P2x7 ion channel is opened in response to extracellular ATP from damaged or necrotic cells allowing for potassium efflux; (iv) during entry, virus infection damages endosomes and releases proteases such as cathepsin B into the cytosol; (v) pore-forming toxins; and (vi) uric acid crystals [36, 72, 76] (See Chap. 17). Indeed, several viral infections have been shown to cause these varieties of DAMPs, which are then recognized by NLRP3, which in turn, recruits the adaptor ASC, and then ASC interacts with the pro-caspsase–1 which becomes activated and allows mature IL-1β production [72].

AIM2 (absent in melanoma 2) has recently been shown to bind DNA and engage the caspase-1–activating adaptor protein ASC to form a caspase-1–activating inflammasome (Fig. 19.3). Knockdown of AIM2 abrogates caspase-1 activation in response to dsDNA of VV and MCMV [77, 78], indicating that AIM2 plays a role in sensing DNA viruses. In addition, another DNA receptor, IFN-γ–inducible protein 16 (IFI16) has been reported to induce ASC-dependent inflammasome activation in a variety of cell types, including DCs, macrophages, and fibroblasts, during infection with DNA viruses such as HSV-1, EBV, and KSHV [77]. NOD2 was also shown to sense several viruses, including Rous sarcoma virus, and mediate host defense *via* IRF3 and/or mitochondrial antiviral signaling (MAVS) [79]. Recently, it has been shown that the NLR family member NLRX1 interacts with MAVS protein to regulate the interferon and NF-κB pathway negatively [80, 81]. Although conflicting reports have been presented with regard to the implication of NLRX1 as a negative regulator of MAVS-dependent cytosolic antiviral responses [84], NLRs may modulate the host antiviral apparatus either positively or negatively.

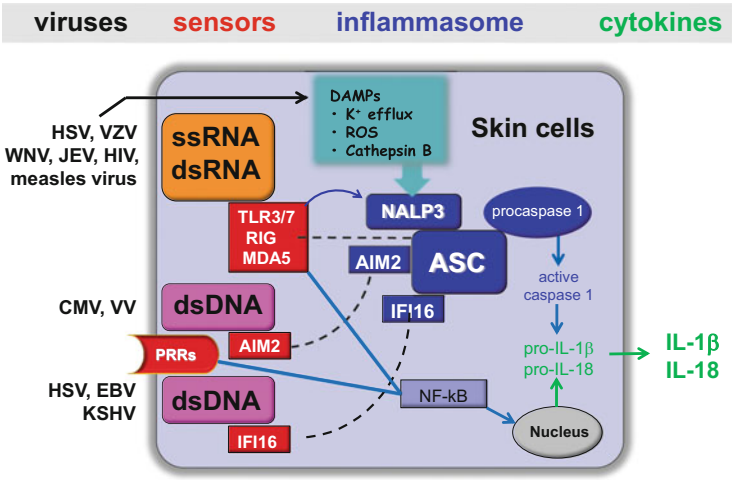

Fig. 19.3 Viral recognition and IL-1β/IL-18 production via inflammasome by skin cells. During the indicated skin-associated virus infections, viral ssRNA or dsRNA is recognized by endosomal TLRs (TLR3/7) or cytoplasmic RLRs (RIG-I or MDA5), leading to the priming of NLRP3 inflammasome for activation and the upregulation of pro-IL–1β/pro-IL–18 through NF-κB–mediated signaling pathways. Following the priming, the NLRP3 inflammasome is assembled and activated in response to virus-induced DAMPs. AIM2 or IFI16 in the cytosol bind viral dsDNA and also engage the adaptor protein ASC to form a caspase-1–activating inflammasome. The AIM2/IFI16 inflammasome requires another signal for IL-1β/IL-18 production, which comes from the virus that triggers other PRR-dependent signaling pathways and allows pro-IL–1β production through NF-κB–mediated signaling

19.2.5 RIG-I–Like Receptors (RLRs)

The viral dsRNA in the cytosol can be recognized by an additional family of cytosolic receptors, RLRs, including RIG-I, MDA5, and LGP2 (laboratory of genetics and physiology 2) (Fig. 19.4) (see Chap. 17) [78, 79]. RLRs are expressed in most skin cell types and are greatly increased with type I IFNs exposure after virus infection. RIG-I and MDA5 contain two CARDs in tandem at their amino terminus that are important for their signaling functions. In general, infection by RNA viruses leads to the generation of long dsRNA in the cytosol that is structurally different from host cellular RNA, which is single-stranded with short and often imperfectly matched stem loops [80]. RIG-I is preferentially involved in the detection of short dsRNA (up to 1 kb) or stem-loop RNA species bearing 5′-triphosphate (5′ ppp) ends, which serve in part to define a nonself RNA PAMP, whereas MDA-5 is more sensitive to long dsRNA (more than 2 kb) containing branched structures [36, 79, 80]. Although RIG-I had initially been implicated in the recognition of ssRNAs with 5′-triphosphate groups, recent studies have demonstrated that pure ssRNA with a 5′-triphosphate group is unable to activate RIG-I [81, 82]. Viral RNA recognition by RIG-I and/or MDA5 leads to the production of type I IFNs via the adaptor protein: mitochondrial antiviral signaling protein

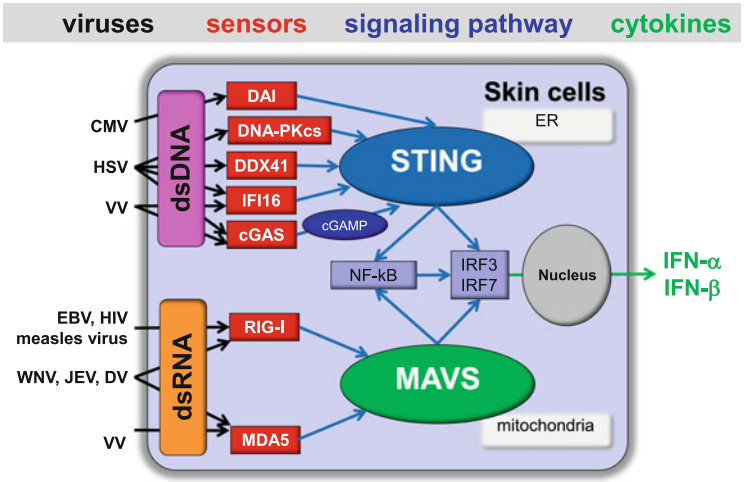

Fig. 19.4 RLRs- and cytosolic DNA sensor-mediated viral recognition and type I IFN production by skin cells. During the indicated skin-associated virus infections, viral dsRNA is recognized by cytoplasmic RLRs (e.g., RIG-I or MDA), leading to the production of type I IFNs via the adaptor protein MAVS and the transcription factors NFκB, IRF3, and IRF7. DNA introduced by invading DNA viruses and/or damaged host cells are released and can meet sensors in the cytosol of skin cells. Multiple DNA sensors, such as DAI, DNA-PKcs, DDX41, cGAS, and IFI16, are considered to activate STING located at the endoplasmic reticulum (ER), culminating in the activation of transcription factors NFκB, IRF3, and IRF7, which then lead to induction of type I IFNs

(MAVS) and the transcription factors, namely NFκB, IRF3, and IRF7 (Fig. 19.3) [78, 80]. RIG-I and MDA-5 recognize different viruses; RIG-I is essential for immune defense against some skin-associated viruses such as EBV, measles virus, and HIV, whereas MDA-5 is required for IFNs induction by HSV and VV [44, 79]. Some members of the *Flaviviridae* such as DV, JEV, and WNV have been shown to activate both RIG-I and MDA5 for type I IFNs production in a variety of skin cell types (Fig. 19.4) [79, 83]. It has been demonstrated that during HSV-1 infection, early in vivo production of type I IFNs is mediated through TLR9 in pDCs, whereas the subsequent IFNs response is derived from several cell types and induced independently of TLR9 [84]. In the latter phase, HSV-1 induces type I IFN expression through a mechanism dependent both on RIG-I and the adaptor MAVS [85]. In addition to the RLRs, the helicases DDX1, DDX21, DHX9, and DHX36 have been linked to recognition of cytoplasmic RNA in DCs [44].

In general, RNA polymerase III (pol III) has the fundamental role in transcribing transfer RNAs and other small noncoding RNA molecules. Recent studies have demonstrated that pol III is also able to generate dsRNA bearing a 5′ ppp moiety with AT-rich DNA as templates and the dsRNA is then recognized by RIG-I [77, 84]. Although POL3 had initially been implicated in the recognition of HSV-1 and adeno virus [84], other studies have found little evidence for the involvement of this pathway during these viral infections [85, 86]. In EBV infection, pol III play a role in the generation of the EBERs (nonpolyadenylated,

noncoding RNA that forms a stem-loop structure by intermolecular base-pairing), giving rise to dsRNA-like molecules, that are subsequently recognized by RIG-I (Fig. 19.4) [84]. However, in this case, pol III acts in its conventional role, that is, in the transcription of functional RNAs, rather than as a sensor *per se* [80].

19.2.6 Other Intracellular DNA Sensors

One area of recent intense research has been the search for cytosolic DNA-sensing pathways that would account for the ability of exogenously added DNA to induce IFN-β. During viral infection, nucleic acids, such as DNA introduced by invading DNA viruses and/or released from damaged host cells, can meet cytosolic sensors and trigger the activation of innate immune responses, especially when DNA from dead cells is not effectively cleared by DNases, including DNase I in the extracellular space, DNase II in lysosomes, and three prime repair exonuclease 1 (TREX1) in the cytoplasm. In addition to the endosomal TLRs-dependent IFN response elicited by intracellular DNA, it has recently been demonstrated that adaptor protein STING (stimulator of IFN genes, also known as MITA, mediator of IRF3 activation) expressed in the endoplasmic reticulum is also essential for IFN-β induction by intracellular dsDNA and/or DNA viruses via activation of the transcription factors IRF3, TANK-binding kinase 1 (TBK1), and NF-κB [77, 78]. Although the identity of the upstream DNA receptors that cause the activation of STING has remained controversial, it has been proposed that the STING-dependent signaling pathway may be activated by multiple DNA sensors; these include DAI (DNA-dependent activator of IFN-regulatory factors), IFI16, the helicase DDX41, DNA-PKcs (DNA-dependentprotein kinase), and cGAS (cyclic GMP-AMP synthase; Fig. 19.3) [77, 78].

A new cytosolic receptor termed DAI, also known as Z-DNA binding protein (ZBP-1), was found to bind multiple types of exogenously added B-form dsDNA, but not Z-form dsDNA, including viral DNA, leading to the activation of IRF3 which participates in the induction of IFN-β expression in a IRF3- and NF-κB–dependent pathway [87–90]. DAI has been shown to play a role in human fibroblasts during HCMV infection, and another DNA receptor, IFI16 (See Capture 20.2.1.4), DDX41, and the catalytic subunit of DNA-dependentprotein kinase (DNA-PKcs), induce IFN-β in response to intracellular DNA in a variety of skin cell types during HSV-1 infection (Fig. 19.3) [44, 77]. It is of note that IFI16 shuttles between the nucleus and the cytoplasm and senses DNA in both compartments [77]. Several groups have demonstrated that DNA damage can result in the production of type I IFNs [91, 92], suggesting that there are links between viral infection, DNA damage, and the innate immune response. Recent study has shown that DNA damage factors, DNA-PKcs and its binding partners Ku70/80, are required for the production of IFN-β and were also involved in the response to HSV-1 [93]. Although another well-characterized DNA damage factor, Mre11 (Meiotic recombination 11), has also been shown to be required for the

DNA-induced IFN response in mouse bone-marrow–derived DCs, Mre11 was not involved in the recognition of pathogens such as HSV-1 [94]. A newly identified enzyme, cGAS, binds intracellular DNA and mediates the production of cyclic GMP-AMP (cGAMP), which functions as a second messenger that binds and activates STING [78]. Thus, cGAS acts as a DNA receptor, and indeed, knockdown of cGAS inhibits IFN-β induction in THP-1 macrophages or the L929 fibroblastic cell line by DNA viruses such as HSV-1 and VV [77, 78].

During HSV-1 and HIV-1 infection, the viral DNA can be recognized by another sensor, high mobility group box 1 (HMGB1), a DNA-binding nuclear protein [91]. HMGB1 may influence HIV infection at three levels at least; HMGB1 can bind DNA and RNA with high affinity, and promote the activation of TLRs, RIG-I, and intracellular DNA receptors in response to their nucleic acid ligands [92]. HMGB1 have recently been identified to be released outside the cell, and act as a potent soluble factor that coordinates cellular events that are crucial for the amplification of the inflammation and establishment of early innate immune response [91, 92]. HMGB1 is therefore considered as the leading member of the DAMPs, named "alarmins". HMGB1 is a nuclear protein and, to function as DAMP in the extracellular milieu, it must be released. This occurs either *via* passive release from necrotic cells, or by active secretion by cells of the innate immune system [92, 93]. Interestingly, extracellular HMGB1 has been shown to inhibit HIV-1 *de novo* infection, but increases HIV-1 production by persistently infected cells [92].

19.2.7 Antimicrobial Peptides (AMPs)

Virtually all human tissues and cells typically exposed to microbes are able to produce small antimicrobial peptides (AMPs). Defensins and cathelicidins are AMPs that are produced by leukocytes and epithelial cells, and that have an important role in antiviral innate immunity. In addition to their antimicrobial activity, the AMPs also play a role in chemoattraction of lymphocytes and participate in cell growth regulation and wound healing (see Chap. 17). In contrast, CCL20, a representing well-known chemokine, was shown also to be a potent AMP [94]. The known cutaneous AMPs can identify more than 20 individual proteins that have shown antimicrobial activity, including dermcidin, which is constitutively secreted by eccrine sweat glands and psoriasin which was first isolated from psoriatic epidermis, are also the important effector molecules of cutaneous innate immunity (see Chap. 17).

Recent advances in understanding the mechanisms of the antiviral actions of AMPs indicate that they have a dual role in antiviral defense, acting directly on the virion and on the host cell [95]. Human α-defensin-1, -2, -3, and -4 are designated as human neutrophil peptides (HNP1, HNP2, HNP3, and HNP4) because they are mainly expressed by neutrophils, whereas human epidermal keratinocytes or genital epithelial cells can produce human α defensin-5 (HD5), HD6, and human β defensin-1 (HBD1), HBD2, HBD3, HBD4, and the sole cathelicidin in humans,

LL-37. Certain defensins (e.g., HBD1) are expressed constitutively, and others (e.g., HBD2 and 3) show increased expression in response to inflammation or infection. For example, HIV-1 and human rhinovirus induce mRNA expression of HBD2 and HBD3 in normal human oral and bronchial epithelial cells, respectively [96, 97]. Since similar upregulation of HBD gene expression is induced by polyinosinic-polycytidylic acid (polyI:C), a ligand for TLR3 [102], TLR3-mediated recognition of dsRNA generated from the viruses might be involved in the upregulation of the HBDs expression. In addition, stimulation of TLR2 and TLR4 has been shown to induce HBD2 expression by vaginal epithelial cells [103].

An emerging body of evidence now indicates the antiviral actions of AMPs. For example, AMPs such as HNP1-4, HBD2, and HBD3 have been reported to inhibit HIV-1 infectivity [95, 98]. Furthermore, α-defensin HNPs (e.g., HNP1, HNP2, HNP3, and HNP4) have inhibitory effects on the infectivity of various skin-associated viruses including HSV-1, HSV-2, and VV. Similarly, cathelicidins and β-defensins, HBD1 and HBD2, also have an inhibitory effect on the infectivity of VV [95]. Cathelicidins are highly expressed by neutrophils and by epidermal or genital epithelial cells, and recent work identified vitamin D3 as a major factor involved in its regulation. Its peptide form, LL-37, was shown to influence TLR signaling in immune cells through interaction with the cellular membrane and epidermal growth factor receptor transactivation and to increase intracellular Ca^{2+} mobilization [99].

The mechanisms of antiviral action by AMPs are multiple and complex and include direct effects on the virion, as well as effects on the target cell and on innate and adaptive immunity [95]. Recently, we found that HSV-2 significantly increased the expression of hBD2, hBD3, hBD4, and LL-37 in normal human keratinocytes, and surprisingly, LL-37 strongly upregulated the expression of HIV receptors in LCs thereby enhancing their HIV susceptibility [30]. These results may explain the in vivo observation that HSV-2 infection facilitates HIV-1 acquisition. Thus, as many PRRs do, AMPs not only act as beneficial to the host, but also contribute viral infectivity and pathogenicity.

19.3 Intrinsic Antiviral Immunity in Skin

The intrinsic antiviral immunity provides an immediate and direct antiviral defense mediated by intrinsic restriction factors that are mostly pre-existent in certain cell types. Unlike TLRs and RLRs, which inhibit viral infection indirectly by activating signaling cascades that result in the transcription of genes encoding antiviral factors such as type I IFNs (Figs. 19.2 and 19.4), intrinsic restriction factors bind viral components and directly inhibit all steps of viral replication (Fig. 19.5) [100]. Note that as most of these factors are encoded by ISGs, they can be further induced by type I IFNs to amplify their antiviral activity [80].

HIV-1 can infect various skin cells that express CD4 and CCR5, including LCs, DCs, macrophages, and CD4+ T cells. However, the magnitude of infection with

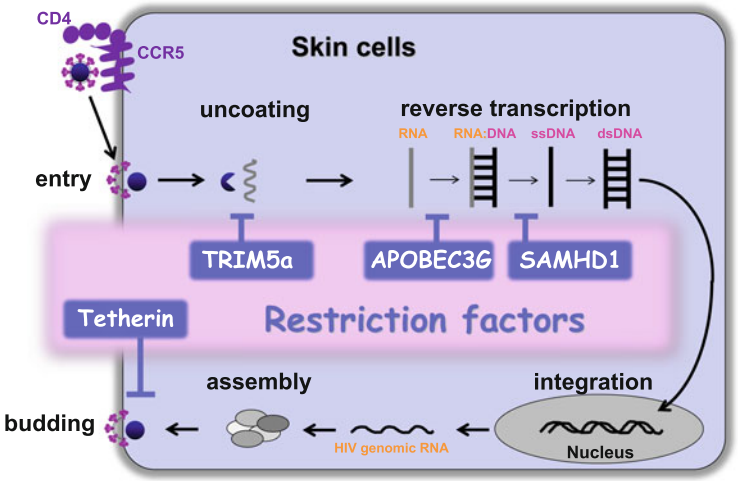

Fig. 19.5 Potential impact of intrinsic antiviral restriction factors on HIV-1 cycle. Certain skin cell types, especially LCs and DCs, express intrinsic antiviral restriction factors, such as TRIM5α, APOBEC3G, SAMHD1, and Tetherin, capable of binding viral components and directly blocking different stages of HIV replication cycle

HIV-1 is cell type specific, and CD4+ T cells are more susceptible to infection with HIV-1 than LCs/DCs and macrophages. Recent studies have revealed that the distinct susceptibility to HIV-1 among those cell types is largely attributed to the host intrinsic restriction factors, such as APOBEC3G (apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like 3G) and SAMHD1 (SAM domain- and HD domain-containing protein 1; Fig. 19.4) [80, 100]. APOBEC3G was one of the first intrinsic antiviral factors identified as acting against HIV-1. In cells productively infected with HIV-1, host APOBEC3G is packaged into HIV-1 virions, and in the infection to other target cells, APOBEC3G edits C to U in HIV DNA, which results in a G-to-A mutation in the HIV genome, leading to diminished replication. The deoxynucleoside triphosphate SAMHD1 degrades deoxynucleoside triphosphates (dNTPs), and inhibits HIV-1 reverse transcription by decreasing the level of cellular dNTPs to below the level required for the synthesis of viral DNA. Intriguingly, in DCs, SAMHD1 has an additional role in limiting the induction of type I IFNs in response to HIV-1 replication [101]. The capsid-binding protein TRIM5α (tripartite motif 5α) also acts as an intrinsic restriction factor and binds viral capsid targeting the virus for proteasomal degradation prior to reverse transcription. Human TRIM5α mediates mild restriction of HIV-2, but does not cause a significant inhibition to HIV-1, whereas rhesus TRIM5α restricts HIV-1. Tetherin has also been identified as the restriction factor responsible for blocking the release of nascent HIV-1 particles from infected cells [80, 100]. At the HIV-1 budding site, tetherin incorporates into the cell and virus membranes and prevents efficient viral release by tethering HIV-1 particles to the cell, and consequently, tethered particles are internalized by endocytosis and are subsequently degraded in the endosomes

(Fig. 19.4). Tetherin has an additional immunological role: the binding of tetherin to ILT7, a membrane receptor selectively expressed in pDCs, leads to the inhibition of TLR-mediated IFN responses [102].

In the initial phase of sexual transmission of HIV-1 *via* mucosa or skin, although DCs, macrophages, and T cells in submucosa or dermis can be potential targets for HIV-1, an emerging body of evidence now indicates that LCs located within the epithelium/epidermis are initial cellular targets and play crucial roles in spreading HIV-1 because keratinocytes are not susceptible to HIV-1 and intercellular desmosomes and tight junctions provide a potent protective barrier to viral invasion [24, 25, 27–29]. During the first week after sexual exposure to HIV-1, HIV-1 infection does not appear to trigger a strong innate immune response in mucosa or skin, probably because HIV-1 may avoid triggering antiviral innate immune responses by not replicating efficiently in LCs. In addition to langerin-mediated degradation of HIV-1 in LCs as mentioned above, the host restriction factors may be associated with the poor replication in LCs. Indeed, we have recently found that LCs do functionally express the intrinsic restriction factors, APOBEC3G and SAMHD1 [30, 103].

Intrinsic immunity mediated by host intracellular restriction factors play important roles in restricting not only HIV-1 but also other viral replication. For instance, tetherin targets many other enveloped viruses, such as Ebola virus and KSHV. Moreover, IFITM (IFN-inducible transmembrane) proteins have been recently identified as a host restriction factor for influenza A virus and they also restrict cytoplasmic entry of some flaviviruses, such as DV and WNV [80, 100]. By contrast, the primate lentiviruses such as HIV-1 have had to evolve multiple mechanisms to antagonize the restriction factors. For example, the Vpx and Vif proteins target SAMHD1 and APOBEC3G, respectively, for ubiquitination and subsequent degradation in virus-producing cells [100]. Furthermore, the lentiviral Vpu protein antagonizes tetherin by altering its normal subcellular localization.

Herpes viruses are subjected to intrinsic immune responses mediated by cellular restriction factors such as promyelocytic leukemia protein (PML), speckled protein of 100 kDa (Sp100), human death domain-associated protein (hDaxx), or alphathalassemia/mental retardation syndrome X-linked (ATRX) [104]. All these factors have been found to be constituents of a cellular, subnuclear structure referred to as nuclear domain 10 (ND10, also called PML-NBs, PML nuclear bodies), and are able to repress the initial transcription of several herpes viruses [105]. The antiviral function, however, is antagonized by viral effector proteins by a variety of strategies, including degradation of PML, relocalization of ND10 proteins, or repressing expression of ATRX [105].

19.4 Acquired Antiviral Immunity in Skin

Cutaneous innate and intrinsic immunity is thought to play a paramount role in the host immune responses against skin-associated viruses, however, the acquired immune mechanisms mediating viral antigen-specific responses are also important for long-term or permanent immunity against the viruses. Earlier studies have elucidated a role of neutralizing antibody in antiviral humoral immunity and an emerging body of evidence now highlights the crucial role of cellular immunity, particularly CD8+ T cell-mediated immunity, in various viral infections. Nevertheless, in most skin-associated viral infections, it has still been obscure or not even addressed which skin cells prime virus-specific CD8+ T cells and how the cytotoxic T cells (CTLs) contribute effective acquired antiviral immunity in the skin. Therefore, to illustrate the cutaneous acquired antiviral immunity, this chapter focuses on the most characterized cutaneous viral infection, HSV.

In cutaneous HSV lesions of humans and in murine models, keratinocytes, DCs, and infiltrating lymphocytes, especially HSV-specific CD4+ and CD8+ T cells, were shown to play a central role in controlling primary and recurrent HSV infections [106–108]. The innate cytokines such as pro-inflammatory cytokines (e.g., IL-6) and type I IFNs (and following activation of ISGs) also play critical roles in decreasing susceptibility to HSV infection and controlling local HSV replication independent of T and B cells [109–115]. HSV infection of keratinocytes leads to the production of the innate cytokines but also β chemokines, which attract HSV-specific CD4+ and CD8+ T cells into lesions [116]. Numerous studies have indicated a significant role for $CD8^+$ T cells in controlling HSV infections, largely through the production of IFN-γ, however, a minor role for CD4+ T cells has been described, with these cells providing some degree of protection in the absence of other immune effectors (reviewed in [107]). More recently, the important role of other innate immune effectors, such as mast cells, NK cells, NKT cells, pDCs, macrophages, and γδ T lymphocytes, has been re-emphasized, either in direct immune control or *via* modulation of adaptive immune responses [107, 114, 117].

Cutaneous HSV infection not only induces the production of innate and adaptive cytokines but also leads to the complex interplay between these cytokines, which further regulates both arms of immunity. For instance, although type II IFN, IFN-γ is a weak inhibitor of HSV-1 replication in vitro; IFN-α and IFN-γ synergize to inhibit HSV-1 replication of keratinocytes with a potency which approaches that achieved by a high dose of acyclovir [118, 119]. Consistent with these findings, it has been demonstrated that mice lacking receptors for both type I and type II IFNs exhibited a dramatic increase in HSV susceptibility, whereas the absence of either receptor alone causes a much lesser increase in susceptibility [120, 121]. Although HSV downregulates the expression of MHC class I in keratinocytes, this is reversed by IFN-γ, mainly produced from early infiltrating CD4 T lymphocytes, thus allowing CD8 T cells to recognize infected keratinocytes [106, 116].

In human skin, three DC subsets have been identified: langerin+ LCs, CD14+, and CD1a+ dermal DCs, and in murine skin, a new subsets of dermal DCs,

CD103+, and langerin+ DCs, have been identified [122–124]. Previous studies in mice have suggested that LCs are likely to be involved in initial HSV antigen uptake [125, 126], however, recent studies demonstrated that migrating CD103+, langerin+ dermal DCs are the major transporters of HSV antigens out of skin and, together with resident CD8+ DCs, are the major antigen-presenting cells of HSV antigens to CD8+ T cells in draining lymph nodes [127, 128]. In reactivation of HSV infection, resident memory CD8+ T cells in the skin can proliferate directly through a tripartite interaction with DCs and CD4+ T cells without migration to the draining lymph nodes [129]. It is of note that the cellular restriction structures, ND10, have been implicated in a number of cellular anti-HSV processes, including the induction of apoptosis and MHC class I antigen presentation [130, 131].

19.5 Concluding Remarks

How innate and intrinsic immune receptors expressed in the skin cells sense viruses has been an area of immunological research that has seen remarkable progress in the past several years. Cutaneous innate immune receptors are essential to initiate antiviral immune responses including production of innate cytokines, such as type I IFNs and IL-1β/IL-18. In addition to the innate cytokines, upon percutaneous viral invasion, the skin cells also provide an early warning system by releasing stored and inducible cytokines as DAMPs or "alarmin". For example, we have recently highlighted the important roles of IL-33 as an alarmin in cutaneous HSV infection [115]. Although viral infection of skin rapidly elicits an innate immune response, the viruses have various mechanisms to escape its host's immune responses and increased their capacity to replicate and to persist. For example, HSV, HCMV, and EBV inhibit IRF3 and/or IRF7 activation and the subsequent induction of type I IFNs expression [132–136], and VZV inhibits NF-κB activation during viral infection and inhibits the capacity of pDCs to express IFN-α [137, 138].

Numerous previous studies have been dedicated to address which innate immune receptors are most critical for antiviral innate immunity, however, few have done so using human skin resident cells. Further studies focusing specifically on primary human skin cells are required. Understanding the mechanisms of cutaneous antiviral innate and intrinsic immunity may have important implications for the design of vaccines and antiviral therapy.

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Chapter 20
Contact Dermatitis

Tetsuya Honda

Abstract Allergic contact dermatitis (ACD) is one of the most common skin diseases, prevailing in 15–20 % of the general population all over the world. It is classified as one type of delayed-type hypersensitivity response, and murine contact hypersensitivity (CHS) is a frequently used and suitable animal model of ACD. During the last decade, new subsets of immune cells, such as regulatory T cells (Tregs) and $CD4^+$ T helper 17 (Th17) cells have been identified both in mouse and human, and the important roles of those cell subsets in ACD have been implicated from the studies of CHS. In addition, the discovery of Langerin-positive dermal dendritic cells (DCs) questioned the relevance of epidermal Langerhans cells as key antigen-presenting cells (APCs) in cutaneous immune responses. We summarize the recent reports of CHS and integrate recent advances into the classic view of CHS, and discuss the updated mechanisms of its development.

Keywords Contact dermatitis • Contact hypersensitivity • Th1 • Tc1 • Langerhans cells • Dendritic cells • Regulatory T cells

20.1 Introduction

Allergic contact dermatitis (ACD), such as metal allergy or plant allergy, is one of the most common skin diseases [1]. ACD is a kind of delayed-type hypersensitivity response, an essential immune response that eliminates the pathogens from the host. The antigens in ACD are usually not pathogens and not harmful to our body. However, once the antigen enters into skin and is recognized as harmful (sensitization phase), our host-defense system tries to eliminate them and provokes inflammation in skin, which is called the elicitation phase. In the sensitization phase, antigen-specific effector T cells are induced in the draining lymph nodes (LNs). In elicitation phase, the effector T cells infiltrate to the skin area where the antigens

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enter, and are activated by their specific antigen, and provoke inflammation in ACD.

The contact hypersensitivity model (CHS) is a classic but easy and appropriate animal model for ACD [2]. By using CHS as a model of ACD, a number of studies have been performed and our knowledge of the immunological mechanisms of ACD has significantly expanded. In the past decade, several kinds of new types of immune cells have been discovered, such as regulatory T cells (Tregs) or langerin positive dermal dendritic cells (DCs). The roles of such new types of cells in ACD have been investigated using CHS, and some of the key dogma in ACD has been changing. In this section, we integrate the recent advancement of immunological mechanisms of both the sensitization and elicitation phases of CHS into the classic view, and discuss updated mechanisms on its development.

20.2 General Protocols of CHS

As this section discusses the mechanisms of ACD mainly based on mouse studies (CHS), we first briefly explain the experimental systems of CHS. For the induction of CHS, dinitrofluorobenzen (DNFB), oxazolone, Trinitrochlorobenzene (TNCB), and fluorescein isothiocyanate (FITC) are generally used as haptens [2]. Although there exist some variations in the protocol of CHS, the basic procedure is as follows. On day 0 (the first day of experiment), mice are painted with hapten (ex. 0.5 % DNFB) on their shaved abdominal skin or ear skin. This is the day of sensitization. Five or 7 days later, the same hapten of less concentration (ex. 0.3 % DNFB) is applied on the ear skin (elicitation), and ear thickness change is evaluated as a parameter of skin inflammation, which peaks at 24–48 h later. Histologically, severe edema and inflammatory cell infiltration are detected in dermis 24–48 h after elicitation, which correspond well with the ear thickness change.

As described above, the experimental system of CHS is quite simple, but well reflects many characteristic features of ACD.

20.3 Mechanisms of Sensitization Phase

20.3.1 Haptens and Keratinocytes

Most of the chemicals that induce ACD are small compounds called haptens, which typically have a molecular mass of less than 500. Haptens need to have chemical interactions with proteins to elicit adaptive immune responses [3]. Upon hapten application, keratinocytes are activated and produce various chemical mediators, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and prostaglandin (PG) E2, which promote the migration and maturation of skin DCs

[4–6]. Keratinocytes are activated by haptens through innate immune systems, such as Toll-like receptors (TLRs) and cytosolic NOD-like receptors (NLRs). Among the NLR family, NACHT-, LRR-, and pyrin (NALP) 3 control the production of pro-inflammatory cytokines through activation of caspase-1. Without NALP3 or its adaptor protein ASC, the production of IL-1β and IL-18 from keratinocytes was inhibited, which resulted in impaired DC migration and T cell priming, and led to impaired CHS [7–10]. Innate immune activation is also important for dendritic cell activations.

20.3.2 Roles of Cutaneous DC Subsets in Sensitization

There are at least three subsets of DCs in mouse skin: Langerhans cells in epidermis, langerin$^+$ dermal DCs, and langerin–dermal DCs in dermis. After haptens enter into skin, they are captured by cutaneous DCs, which become mature and migrate to draining lymph nodes (dLNs), and present the antigen to naïve T cells to induce effector T cells. For the activation of DCs in the sensitization phase, both TLR and NOD pathways are required [11]. For example, among TLRs, TLR2 and TLR4 play critical roles for DCs maturation in CHS induced with trinitrochlorobenzene (TNCB), oxazolone (Ox), and fluorescein isothiocyanate (FITC) [12]. Among cutaneous DCs, LCs have long been considered to be central cells for antigen presentation in the sensitization phase of CHS. However, novel depletion systems of LCs (langerin–diphtheria toxin receptor [DTR] knockin mice) have revealed that langerin$^+$ dermal DCs, but not LCs, may play a crucial role in sensitization, because depletion of langerin$^+$ dermal DCs reduced CHS response whereas depletion of LCs did not [13–16]. Furthermore, it has also been reported that LCs play a regulatory role, rather than a stimulatory role, during sensitization in CHS by producing IL-10 [17, 18] or inducing ICOS$^+$Foxp3$^+$Tregs in dLNs [19]. We and others have, however, recently reported that these two populations work in a compensatory manner to initiate sensitization in CHS [20, 21]. Consistently, Batf3-deficient mice that lack langerin$^+$ dDCs exhibited a normal CHS phenotype, suggesting the compensation of its function by other DCs [22].

Thus, although the function of LCs in sensitization remains controversial, langerin$^+$ dDCs probably exert stimulatory effects during sensitization. As for the function of langerin$^-$ dDCs in sensitization, it has not yet been investigated intensively. However, they also appear to have stimulatory functions in sensitization, because ablation of both LCs and langerin$^+$ dDCs prior to sensitization impairs CHS response but is unable to abrogate it completely [13, 20].

20.3.3 Metal Allergy and Innate Immune Activation

Nickel (Ni^{2+}) is one of the most frequent causes of ACD, although it rarely occurs in mice. Earlier studies have reported that coadministration of adjuvant, such as complete Freund's adjuvant or lipopolysaccharide, efficiently induced Ni^{2+} allergy in mice, suggesting the important role of TLR4 signaling in efficient sensitization with Ni^{2+}. Very recently, Schmidt et al. reported that Ni^{2+} directly activates human TLR4 but not mouse TLR4, and that the transgenic expression of human TLR4 in TLR4-deficient mice resulted in efficient sensitization and elicitation to Ni^{2+} [23]. These reports indicate the crucial roles of TLR4 in Ni^{2+} allergy in both mice and humans.

Together, these hapten-induced irritant effects through innate immune systems are essential to the activation of cutaneous APCs and thereby determine the allergenic potential of a hapten during the sensitization phase.

20.3.4 Role of Mast Cells in Sensitization Phase

As the other authors describe this topic in the chapter, "Mast Cells and Basophils in Cutaneous Immune Response," in this book, we just briefly explain this here. The role of mast cells in CHS has been controversial, because conflicting results have been reported in the CHS model using mice lacking mast cells constitutively [24–27]. However, a novel mast cell ablation system was recently established in two independent groups [28, 29]. In these mice, mast cells can be conditionally depleted through the administration of DT. Both groups reported that mice depleted of mast cells exhibited reduced CHS. It has also been revealed that mast cells stimulated DCs via intercellular adhesion molecule (ICAM)-1 or lymphocyte function-associated antigen (LFA)-1 interaction and by membrane-bound TNF- α on mast cells, and stimulated migration/maturation and subsequent T cell priming in the sensitization phase [28].

Overall, it would be very likely that mast cells play stimulatory roles both in the sensitization and elicitation phases in CHS.

20.4 Mechanisms of Elicitation Phase

20.4.1 Antigen Nonspecific Inflammation: Keratinocytes, Mast Cells, and Neutrophil Activation

The mechanism of inflammatory cell infiltration in the elicitation phase has been explained using two different inflammatory signals: antigen nonspecific and antigen specific [2, 30, 31]. Keratinocytes, neutrophils, and mast cells are the main players

to create the antigen nonspecific inflammation at elicitation. First, haptens stimulate keratinocytes to produce pro-inflammatory cytokines such as IL-1β and TNF-α in an NLR-dependent manner [7, 10]. Those cytokines then activate vascular endothelial cells to express adhesion molecules such as ICAM-1 and P/E-selectins, which guide T cells in the blood to transmigrate to tissues. Haptens also increase vascular permeability through mast cell-derived histamin [29], which help neutrophils infiltrate the skin. In addition, haptens activate mast cells and keratinocytes to produce neutrophil-recruiting chemokines, such as CXCL1 and CXCL2, which further contribute to the neutrophil recruitment [25]. The initial neutrophil recruitment is proposed to be essential for subsequent T cell infiltration, because the depletion of neutrophils reduces the $CD8^+$ T-cell infiltration and leads to impaired CHS [32, 33]. The aforementioned mechanism creates the first round of antigen nonspecific inflammation, which is an important step for subsequent antigen specific inflammation (Fig. 20.1a).

20.4.2 Antigen Specific Inflammation: T-Cell Activation, $CD4^+$ T (Th1/17) Cells, and $CD8^+$ T (Tc1/Tc17) Cells

Following the antigen nonspecific inflammation, T-cell–mediated antigen-specific inflammation is initiated. When T cells infiltrate the skin, they are activated by cutaneous APCs, and produce cytokines such as IFN-γ and IL-17. In fact, stable interaction between skin DCs and T cells was observed in live imaging analysis [34] (Fig. 20.1b), and inhibition of CD86 expression by siRNA resulted in reduced inflammation [35], suggesting that effector T cells are activated locally by hapten-

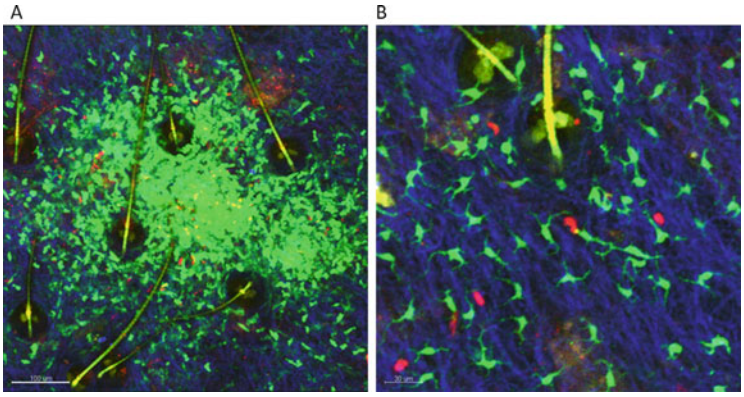

Fig. 20.1 Representative image of neutrophil infiltration and T cells–DCs interaction in skin during elicitation phase. (**a**) Accumulation of neutrophils in dermis 24 h after elicitation (*Green*: neutrophils in Lysm-eGFP mice. *Red*: transferred CD4/CD8 T cells). (**b**) The interaction between T cells and dendritic cells in skin 24 h after elicitation (*Green*: Langerin positive cells in Langerin-GFP mice. *Red*: transferred CD4/CD8 T cells)

carrying APCs. Intriguingly, it has been reported that depletion of skin DCs in hapten-sensitized mice enhanced the effector phase of CHS [36], suggesting the existence of some DC subsets that play a regulatory function in the elicitation phase.

Cytokines produced by activated T cells then stimulate skin resident cells, which lead to further recruitment of T cells and amplify the inflammation. Each T-cell subset (i.e., Th1/Tc1, Th2, Th17/Tc17) activates the skin resident cells differently and forms their specific type of inflammation.

CHS was first considered to be a $CD4^+$ T cell-mediated response as a representative of delayed-type hypersensitivity, but it is now recognized that both $CD4^+$ and $CD8^+$ T cells are important in the elicitation of CHS. $CD8^+$ T cells mainly have pro-inflammatory effector functions, whereas $CD4^+$ T cells have both pro- and anti-inflammatory functions that are dependent on their cytokine production pattern or subset.

$CD4^+$ T helper (Th) cells and $CD8^+$ T cytotoxic (Tc) cells can be subdivided into at least three subsets that are relevant for cutaneous immune responses: Th1/Tc1, Th2/Tc2, and Th17/Tc17 cells, respectively. Th1/Tc1 cells are characterized by the secretion of interferon (IFN)-γ; Th2/Tc2 cells by IL-4, IL-5, and IL-13; and Th17/Tc17 cells by IL-17A and IL-22 production. Although there exists some controversy regarding roles of each cytokine in CHS, the general trend is that IFN-γ from Tc1 is the major effector cytokine that provokes inflammation [37], and IFN-γ from Th1 and IL-17 from Th17/Tc17 also contribute for the full development of CHS [38–40]. It remains unknown whether Th2 cells contribute to the development of CHS, but several reports suggest that Th2 cells may also play important roles for the development of CHS in certain situations, depending on mouse strain or haptens [41].

IFN-γ or IL-17 produced by activated T cells induces various chemokine production from keratinocytes. Keratinocytes are an important source of chemokines in skin, and produce multiple chemokines, such as CXCL1, CXCL2, CXCL9, CXCL10, CCL8, CCL17, and CCL27. CXCL10 is a ligand for CXCR3, which are strongly expressed on Th1 cells and regulates their infiltration into skin [42, 43]. The CCL27-CCR10 and CCL17/22-CCR4 axes are another important mechanism for T-cell recruitment to skin [44, 45]. Neutrophil-recruiting chemokines also play important roles, because a blockade of CXCL1 or a deficiency of its receptor (CXCR2) leads to reduced CHS [32, 46].

20.5 Regulation of Inflammation: Regulatory T Cell (Treg)s

Evidence has accumulated regarding the regulatory mechanisms of Tregs in CHS [47]. Transfer of Tregs before elicitation suppresses the ear-swelling response [48], and depletion of endogenous Tregs before sensitization or elicitation enhances CHS response [49–51], indicating that Tregs play essential roles not only for the resolution of inflammation but also the initiation of T-cell priming. Studies of nickel

Table 20.1 An overview of recently published papers about Tregs and CHS

	Major findings	References
Sensitization	Attenuated sensitization by Treg induced by orally administered antigen in an oral tolerance model	[54]
	Treg attenuate sensitization by modifying DC function through gap junction formation	[55]
	Treg acquire an activated phenotype by means of ATP in draining LNs	[56]
	Enhanced ear swelling response resulting from the depletion of endogenous Treg	[49]
	Enhanced ear swelling response resulting from the depletion of endogenous Treg	[51]
Elicitation	Reduced ear swelling response resulting from the inhibition of the leukocyte influx through IL-10 from Treg	[48]
	Reduced ear swelling response resulting from the inhibition of the leukocyte influx through adenosine from Treg via CD39/CD73 (inhibition of E- and P-selectin expression in endothelial cells)	[53]
	Treg acquire activated phenotype by means of ATP in blood	[56]
	Enhanced and prolonged ear swelling response resulting from depletion of endogenous Treg	[50]
	Prolonged ear swelling response resulting from depletion of endogenous Treg	[51]

allergy illustrated the existence of antigen-specific Tregs in healthy control individuals [52], suggesting the important roles of Tregs for the tolerance to allergens. As for the Tregs suppression mechanisms in CHS, several mechanisms such as IL-10 or CD39/73 dependent pathways are proposed [48, 53]. Table 20.1 summarizes the recent reports of Tregs function in CHS. Moreover, it has been revealed that Tregs display their inhibitory function by recirculating from skin to the draining [50]. As skin is an organ full of Tregs, such inhibitory mechanisms may also work in other skin diseases.

20.6 Conclusion

Taken together, the broad view of CHS development during the sensitization and elicitation phases is summarized in Fig. 20.2 and Table 20.2.

The CHS model has provided us with valuable lessons on the mechanisms of ACD as discussed above. However, there still remains a compelling need to reveal whether such findings in CHS are relevant to human ACD. In addition, recent reports suggest that innate immune cells, such as innate lymphoid cells, macrophages, natural killer T cells, or gamma-delta T cells contribute to the development

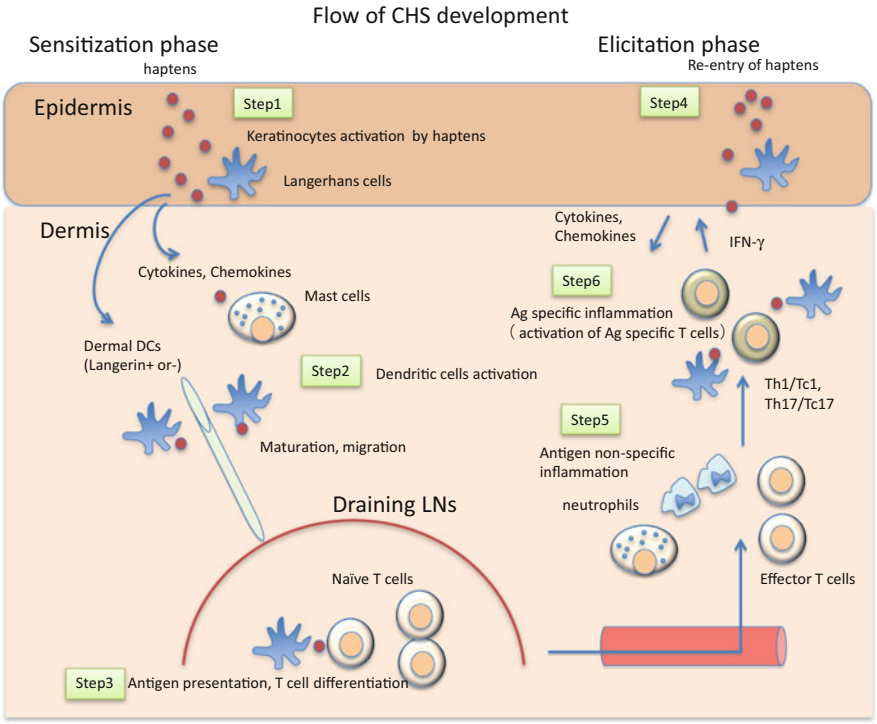

Fig. 20.2 A schematic view of the development of CHS. *Step 1*: Haptens activate keratinocytes (KCs) and mast cells directly or indirectly through innate immune systems. The activated KCs and mast cells produce various chemical mediators, which activate cutaneous DCs. *Step 2*: The activated DCs capture antigens and start maturation and migrate to the dLNs via afferent lymphatics. *Step 3*: Migrated DCs present antigen to naïve T cells in dLNs. Antigen-specific clones differentiate and proliferate into effector T cells. Tregs affect DCs function and play a suppressive role in effector T cell generation. *Step 4*: Upon re-exposure to haptens, KCs and mast cells are activated and produce various chemical mediators, which activate endothelial cells and cause inflammatory cell infiltration, including antigen-specific T cells. *Step 5*: Infiltrated antigen-specific effector T cells are activated and produce pro-inflammatory cytokines and chemokines, which activate KCs and cause further inflammatory cell infiltration. *Step 6*: In addition to effector T cells, Tregs infiltrate inflammatory sites and exert a suppressive function. Some infiltrated Tregs return to dLNs and may contribute to the resolution of inflammation

of many more diseases than previously thought. Investigation of the roles of such cells in CHS would also be important for the understanding of ACD, which may lead to an innovative therapy for allergic skin inflammation.

Table 20.2 Summary of the functions of immune cells in CHS

	Cell subsets	Sensitization phase	Elicitation phase
	Keratinocytes	Initiation of DC migration and maturation via production of TNF-α, IL-1β, IL-18, PGE_2	Recruitment of inflammatory cells through chemokine (CXCX1, CXCL2, CCL17, CCL27, CXCL9, CXCL10) production
	Mast cells	Initiation of DC migration and maturation via TNF-α	Endothelial activation and promotion of inflammatory cell infiltration via TNF-α, histamine, CXCL1
	Neutrophils		Production of pro-inflammatory cytokines (TNF-α, etc), which contributes to Ag-nonspecific inflammation and subsequent Ag-specific inflammation
Cutaneous dendritic cells	Langerhans cells	Ag presentation in dLNs to promote or suppress T cell differentiation and proliferation. Peak of migration: around 72–96 h after haptens application	Possible Ag presentation in dLNs and skin
	$Langerin^+$dDCs (approximately 10 % of dDCs)	Ag presentation in dLNs to promote effector T cell differentiation and proliferation. Peak of migration: around 24–48 h after haptens application	
	$Langerin^-$dDCs (approximately 90 % of dDCs)	Ag presentation in dLNs to promote effector T cell differentiation and proliferation	
T cells	Th1/Tc1		IFN-γ production: stimulate KCs to produce chemokines, and amplify the inflammation
	Th2		IL-4 production: stimulate/regulate the inflammation
	Th17/Tc17	Promotion of effector T cell generation by IL-17	IL-17 production: stimulate KCs to produce chemokines and amplify the inflammation
	Treg	Suppression of T cells differentiation and proliferation by inhibiting the function of DCs	Suppression of endothelial cell activation and inhibition of T cell infiltration via IL-10 and/or adenosine degradation through CD39/73

Ag; Antigen

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Chapter 21
Atopic Dermatitis: Common Extrinsic and Enigmatic Intrinsic Types

Yoshiki Tokura

Abstract AD can be categorized into the IgE-high, extrinsic type and the IgE-normal, intrinsic types. They have also been called several different series of names: mixed AD versus pure AD, allergic AD versus nonallergic AD, and classical AD versus atopiform dermatitis. Although extrinsic AD is the classical type with high prevalence, the incidence of intrinsic AD is approximately 20 % with female predominance. There have been a considerable number of findings regarding the barrier condition and immunological dysregulation in extrinsic AD, whereas the causes and mechanisms of intrinsic AD remain unclear. The skin barrier is perturbed in the extrinsic, and filaggrin gene mutation represents a typical cause of barrier impairment. In extrinsic AD, allergic conditions may be preceded by skin barrier impairment. Protein antigens can penetrate through the disrupted barrier, and epidermal Langerhans cells serve as antigen-presenting cells to Th2 cells. On the other hand, intrinsic AD is immunologically characterized by the higher expression of interferon-γ, and nonprotein antigens, such as metals and haptens, may induce dermatitis in intrinsic AD. In fact, intrinsic AD shows significantly higher percentages of positive reactions to nickel and cobalt than extrinsic AD.

Keywords Atopic dermatitis • Extrinsic • Intrinsic • Filaggrin • Barrier • Stratum corneum • Langerhans cell • Keratinocytes • T cell • Metal

21.1 Introduction

Atopic dermatitis (AD) is a chronic-intermittent, genetically predisposed, eczematous dermatitis that starts at infancy or early childhood and persists for a large part of life. Although several criteria for its definition have been widely approved, there still exist variations in the diagnosis of AD because of its heterogeneous aspects. A large number of clinical, laboratory, and experimental studies have been performed, but the pathophysiology of AD remains to be clarified.

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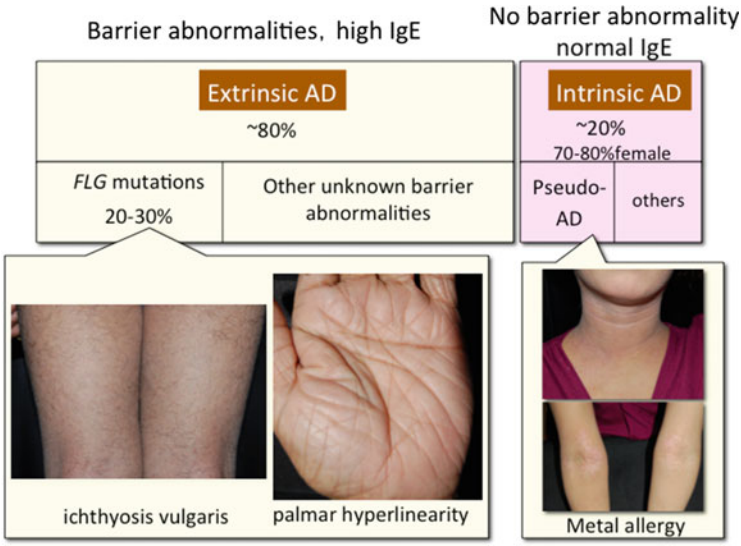


Fig. 21.1 AD spectrum based on extrinsic and intrinsic AD dichotomy. AD can be divided into barrier-disrupted high-IgE extrinsic type and barrier-nondisrupted normal IgE intrinsic type. Some of the patients with extrinsic AD have *FLG* mutations and exhibit ichthyosis vulgaris and palmar hyperlinearity. Intrinsic AD affects mainly women and shows metal allergy in a considerable percentage of patients

The clinical phenotype of AD has been classified into the extrinsic and intrinsic types [36, 66] (Fig. 21.1). Historically, this dichotomy was first used for asthma. The terminology of extrinsic or allergic asthma was first introduced by Rackeman in 1947 and referred to the triggering role of allergens in asthma. By symmetry, he described intrinsic or nonallergic asthma as a disease characterized by later onset in life, female predominance, higher degree of severity, and more frequent association with nasosinusal polyposis. As intrinsic asthmatic patients were not improved by conventional treatments, intrinsic asthma was considered to be caused by a nonallergic unknown phenomenon [52].

In AD, the extrinsic and intrinsic types began to be adopted in the late 1980s [72]. They are also called several different series of names: mixed AD versus pure AD, allergic AD versus nonallergic AD, and classical AD versus atopiform dermatitis. Historically, the intrinsic and extrinsic types appear to correspond to "pure" and "mixed" types, and the latter has concomitant respiratory allergies [9]. Because there is still no sufficient consensus whether the intrinsic type is a distinct entity, some researchers denominate it atopiform dermatitis [2]. However, the classification into the extrinsic and intrinsic AD has been widely used especially since the millennium. Recently, various kinds of clinical studies have been performed under this dichotomy in many countries, including Germany [11, 36, 56], the Netherlands [2], Hungary [47], Italy [22, 28], and other European countries and Asian countries such as Korea [45, 50] and Japan [19, 30].

The original concept of "intrinsic" type represents the nonallergic nature [36], however, the intrinsic type may also show eosinophilia. More fundamentally, both types represent eczematous dermatitis, a manifestation of the delayed-type or late-phase reaction. Therefore, the intrinsic type AD is not a simple nonallergic type, but is induced via some immunological mechanism. This chapter focuses on the differences between intrinsic and extrinsic AD to represent heterogeneous causes and barrier states in AD patients.

21.2 Definition of Extrinsic and Intrinsic AD

Extrinsic AD and intrinsic AD are defined according to IgE-mediated sensitization, namely the presence or absence of specific IgE for environmental allergens and food allergens [30, 45, 71]. Based on this fundamental concept, intrinsic AD has different features from extrinsic AD as described throughout this Chapter (Box 21.1). According to the EAACI nomenclature task force, the term "atopic eczema/dermatitis syndrome" can be used to cover the different subtypes of AD. In this nomenclature, the intrinsic type is termed nonallergic atopic eczema/dermatitis syndrome, which shows normal IgE levels, no specific IgE, no association with respiratory diseases (bronchial asthma or allergic rhinitis), and negative skin-prick tests to common aeroallergens or food allergens [73]. Inasmuch as total serum IgE values are significantly associated with the allergen-specific IgE status [40], total IgE can be regarded as a clinically useful parameter to differentiate between the extrinsic and intrinsic types in both adults [11, 30] and children [40]. The reported mean values of total serum IgE in the intrinsic type are from 22.2 to 134 kU/L, or alternatively, IgE values less than 150 or 200 kU/L have been used for an indication of intrinsic AD [60]. Our study of Japanese patients also showed that the mean value of total serum IgE was 110.5 kU/L [30] or 125 kU/L [19]. Considering the relatively higher serum IgE levels in Japanese than westerners, these mean values are very low.

Among specific IgE antibodies, infantile AD patients are more allergic to food [45], whereas environmental antigens are common in adults. It should be noted that some allergens may not be useful to discriminate the two types. For example, IgE to *Malassezia sympodialis* was found in patients with the intrinsic type as well as the extrinsic type [3]. IgE levels of *Dermatophagoides* (*D*) *pteronyssinus* (DP) and *D. farinae* (DF) can be used for categorization of extrinsic and intrinsic AD as well as total IgE levels [74]. Serum IgE specific to these mites is graded into seven classes (class 0–6). Intrinsic AD can be defined as serum IgE levels $\leq$200 kU/L or 200<IgE$\leq$400 plus class 0 or 1 of IgE specific to DP or DF, and extrinsic AD defined as 400<IgE levels or 200<IgE$\leq$400 plus class 2 or more of the specific IgE [74].

Box 21.1 Characteristics of Intrinsic AD

1. Definition

   Normal total serum IgE. Absence of specific IgE for environmental and food allergens

2. Incidence

   Percentage of intrinsic AD in total AD: 10–45 %
   Female predominance (70–80 %)

3. Clinical features

   Dennie–Morgan fold
   No ichthyosis vulgris or palmar hyperlinearity
   No nonspecific hand or foot eczema
   Lower colonization of *Staphylococcus aureus*
   Relative late onset
   Milder severity

4. Skin barrier

   No markedly disturbed barrier function
   No high incidence of *FLG* mutations

5. Immunological features

   High percentage of IFN-γ–producing T cells

6. Contact allergens

   High prevalence of metal allergy (Ni and Co)

21.3 Prevalence of Extrinsic and Intrinsic AD

21.3.1 Incidence

Because extrinsic AD is the prototype of AD, dermatologists know its prevalence at their daily clinics. On the other hand, the frequency of intrinsic AD has been a matter of investigation. Schmid–Grendelmeier et al. [60] summarized the 12 reports that had been published from 1990 to 2000 and documented the clinical features of extrinsic and intrinsic AD. According to their review paper, the frequency of intrinsic AD was 10–45 %. More recently, the incidence of extrinsic AD and intrinsic AD were reported as follows: 73 % versus 27 % [40] and 63 % versus37 % [41] in German children, 88 % versus 12 % in Hungarian adults [47], 78.2 % versus 21.8 % in Dutch patients from 13 to 37 years of age [2], and approximately 80 % versus 20 % in Koreans [5]. These data are in accordance with the empirical

knowledge that about 20 % of AD patients show normal IgE levels and lack of sensitization towards environmental allergens. Intrinsic AD is seen in various countries, but the prevalence may depend on local areas, as it was reported that intrinsic AD was higher in incidence in East Germany than West Germany, although the exact reason remains unclear [56].

21.3.2 Female Predominance of Intrinsic AD

Although extrinsic AD equally affects both males and females, the female predominance in intrinsic AD is well known and has been observed by a number of studies [2, 19, 28, 36, 37]. Our observation disclosed that 76.5 % of AD patients were female [30]. More extremely, 14 intrinsic AD patients enrolled in a study were all female [37].

21.3.3 Adults and Children

Extrinsic AD starts at infancy or early childhood and persists at adulthood with or without transient remission. The clinical course of intrinsic AD is an issue to be clarified. Several reports may provide an implication that the intrinsic type is seen at higher frequencies in children than adults [19]. A Korean group of AD investigators showed that the intrinsic type is more prevalent in infancy, and even the third group of the indeterminate type between the intrinsic and extrinsic ones can be identified in this younger generation [45]. A prospective birth cohort study followed for 5 years by a German group demonstrated that one third of child AD was the intrinsic one, and more common in females [24]. Another German group indicated the low prevalence of the intrinsic AD among adult patients [11]. They showed 6.9 % patients fulfilled the criteria of intrinsic AD, and after follow-up, the incidence was declined to 5.4 % because some patients developed respiratory allergies or IgE-mediated sensitizations. These observations may suggest that the intrinsic type is more prevalent in children than adults.

However, it should be noted that a considerable number of the above infantile or juvenile intrinsic AD patients possibly develop the extrinsic type as they grow and show high levels of serum IgE. Furthermore, the later onset was reported to be a feature of intrinsic AD [2]. It is tempting to speculate that the juvenile IgE-normal AD group contains two types, the genuine intrinsic AD and the IgE level-normal stage of extrinsic AD. In addition, it appears that a part of adult intrinsic AD may occur or deteriorate after high school age in Japan.

21.4 Clinical Features of Extrinsic and Intrinsic AD

The skin manifestations of the two types of AD are indistinguishable. As described below, however, a part of extrinsic AD patients have filaggrin (FLG) gene mutations; they may exhibit ichthyosis vulgaris (or severe dry skin) and palmar hyperlinearity (Fig. 21.1). Keratosis pilaris, pityriasis alba, and nonspecific hand or foot eczema are the features of extrinsic AD.

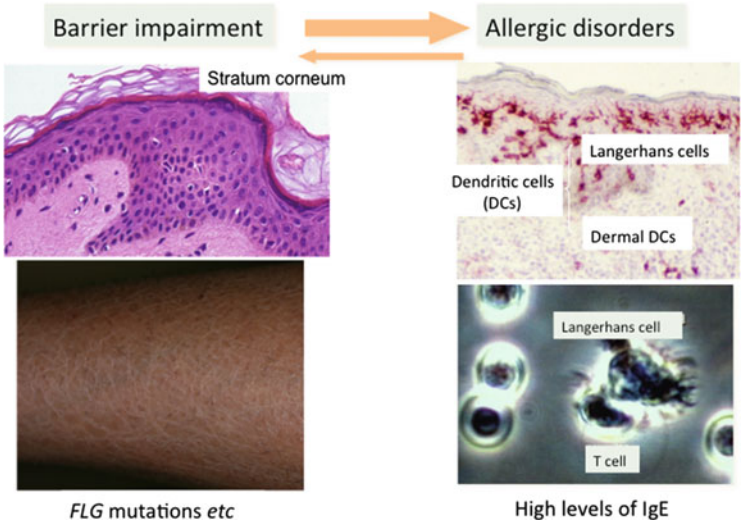
Intrinsic AD shares the vast majority of features with extrinsic AD. However, Brenninkmeijer et al. extensively studied the clinical features of intrinsic AD [2] and found that the Dennie–Morgan fold is significantly more often present in the intrinsic type. The later onset of disease and milder disease severity are also characteristics of intrinsic AD. The features that are negatively associated with intrinsic AD include personal or family history of atopy, recurrent conjunctivitis, palmar hyperlinearity, keratosis pilaris, pityriasis alba, nonspecific hand or foot eczema, and influence of emotional or environmental factors [6]. As mentioned below, some of these nonassociated features are considered to stem from the lack of barrier disruption and/or filaggrin gene mutations in intrinsic AD.

21.5 Skin Barrier Function in Extrinsic and Intrinsic AD

21.5.1 Barrier Function of Stratum Corneum *and Pruritus Perception*

The barrier function is usually assessed by transepidermal water loss (TEWL) and skin surface hydration (capacitance). Extrinsic AD patients have increased TEWL and lower skin surface hydration, whereas intrinsic AD patients have comparable levels of TEWL and skin surface hydration to those of healthy subjects [5]. On the antecubital fossae, both types of AD patients have higher TEWL and decreased capacitance. We examined the skin surface hydration and TEWL at the nonlesional forearm and lower leg of patients and normal volunteers in a comparison between the extrinsic and intrinsic types [30]. The level of skin surface hydration was significantly lower in extrinsic AD than in normal control subjects. On the other hand, there was no significant difference in the hydration level between intrinsic AD and healthy control. The extrinsic type tended to be lower than the intrinsic type at both sites. Thus, the skin barrier function was impaired in extrinsic AD and relatively preserved in intrinsic AD. The barrier impairment induces allergic responses to external antigen in extrinsic AD (Fig. 21.2).

The skin perception threshold of electric current stimuli is one of the indices of itch. The electric current perception threshold significantly correlates with the skin surface hydration and inversely with TEWL in intrinsic AD patients as well as healthy individuals. In contrast, extrinsic AD patients do not exhibit such a



Fig, 21.2 Basic concept of extrinsic AD. In extrinsic AD, barrier impairment, which is typically associated with *FLG* mutations, induces allergic responses to external antigens, especially protein allergens. Langerhans cells (LCs) serve as antigen-presenting cells to protein antigens, and serum IgE is elevated as a result of Th2 responses

correlation. Therefore, intrinsic AD patients retain the normal barrier function and sensory reactivity to external pruritic stimuli [73].

21.5.2 High and Low Frequencies of FLG *Mutations in Extrinsic and Intrinsic AD, Respectively*

The recent identification of loss-of-function mutations in *FLG* as a widely replicated major risk factor for eczema sheds new light on the mechanisms of AD [43]. These mutations also represent a strong genetic predisposing factor for atopic eczema, asthma, and allergies in various countries [35]. Profilaggrin is the major component of the keratohyalin granules within epidermal granular cells. During epidermal terminal differentiation, the profilaggrin polyprotein is dephosphorylated and rapidly cleaved by serine proteases, such as kallikrein-5 [54], to form monomeric FLG, which is further degradated into natural moisturizing factor (Fig. 21.3). Perturbation of the skin barrier function as a result of reduction or complete loss of FLG expression leads to enhanced percutaneous transfer of allergens (Fig. 21.2). The association of the *FLG* mutations in particular with the extrinsic type of AD was observed [19, 69].

Furthermore, *FLG* mutations are significantly associated with palmar hyperlinearity in patients with AD (Fig. 21.1), which represents a shared feature of AD and ichthyosis vulgaris (Fig. 21.1). This is in accordance with the finding that

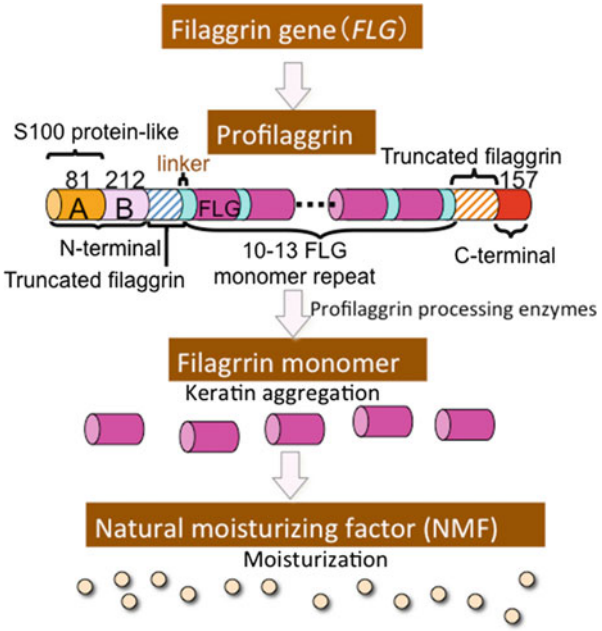


Fig. 21.3 Profilaggrin processing. *FLG* gene product profilaggrin is cleaved by processing enzymes at the linker site [28] and then degraded into FLG monomer, which is further converted to NMF

palmar hyperlinearity is negatively associated in the intrinsic type [2, 19]. We investigated *FLG* mutations in IgE-high and IgE-normal Japanese AD patients. Although 5–9 % of IgE-normal AD cases had *FLG* mutations, about 33–44 % IgE-high patients possessed *FLG* mutations [19, 74], suggesting that *FLG* mutations are less prevalent in the IgE-normal group (normal controls, 3.7 %). In the IgE-high patients, there was no statistical difference in SCORAD or IgE level between the *FLG* mutation-bearing and *FLG* mutation-lacking patients. It has also been reported that *FLG* mutations predispose to early-onset and extrinsic AD [70].

21.6 Characteristics of T Cells, Cytokines/Chemokines, and Dendritic Cells (DCs) in Extrinsic and Intrinsic AD

21.6.1 Th1 and Th2 Cells

AD is well known as a Th2-polarized disease. However, some differences in systemic cytokine polarization between the two types of AD have been reported. As expected with elevated total serum IgE, extrinsic AD patients show high levels of Th2 cytokines, such as IL-4, IL-5, and IL-13, and intrinsic AD is linked with much lower levels of IL-4 and IL-13 [28]. Along with the elevation of IL-5 [33, 48],

eosinophil counts [45] and eosinophil cationic protein levels [41] are increased in extrinsic AD. On the other hand, there was a report demonstrating that both extrinsic and intrinsic patients showed increased production of IL-5 and IL-13 [61]. In that study, however, when peripheral blood mononuclear cells were stimulated with anti-CD3 antibody, extrinsic AD patients had a decreased capacity to produce interferon-γ (IFN-γ) and GM-CSF as compared to the intrinsic AD [61]. The apoptosis of circulating memory/effector Th1 cells is confined to extrinsic AD patients, whereas intrinsic AD patients show no evidence for enhanced T-cell apoptosis in vivo [1].

Circulating IFN-γ^+ T cell frequency was higher in intrinsic than extrinsic AD in our study [19]. In addition, although not statistically significant, there was a tendency that the frequencies of circulating IL-4^+ or IL-5^+ Th2 cells were higher in extrinsic AD than in intrinsic AD. Intrinsic AD may have a less Th2-skewing state and rather shows a high expression level of IFN-γ (Fig. 21.4). The overproduction of IFN-γ may further downregulate IgE production in intrinsic AD, as suggested by our in vitro study [19].

In the skin lesions, eosinophils infiltrate the dermis more markedly in the extrinsic than the intrinsic type, and the extrinsic type exhibits more prominent deposition of eosinophil granular protein and higher staining for eotaxin [23, 50]. Although the levels of mRNA expression for IL-5, IL-13, and IL-1β are higher in both types of AD patients than nonatopic subjects, extrinsic AD shows even higher levels than intrinsic AD [23]. The expression of IFN-γ, IL-12, and GM-CSF, IL-4, and IL-10 are elevated without differences between extrinsic and intrinsic AD [1]. However, a recent study using lesional skin showed that higher activation of all inflammatory axes, including Th2, was seen in patients with

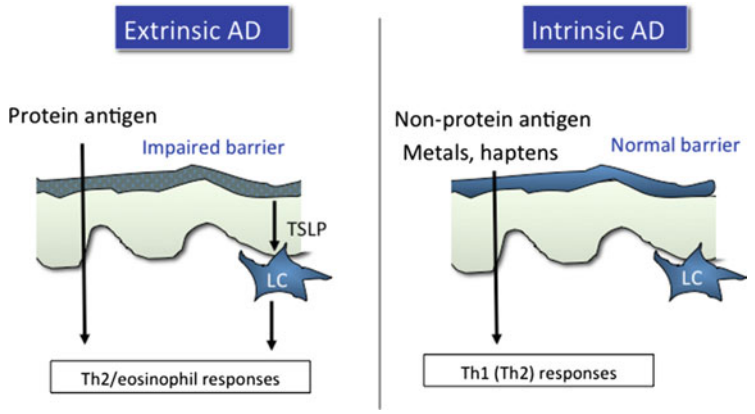


Fig. 21.4 Differences between extrinsic and intrinsic AD in the barrier status and immune responses. In extrinsic AD, the impaired *stratum corneum* barrier allows protein antigen to penetrate through the skin. The external stimuli via the impaired barrier also stimulate keratinocytes to produce TSLP, which subsequently renders LCs to serve as antigen-presenting cells to Th2 cells. In intrinsic AD, nonprotein antigens, such as metals and haptens, can penetrate and function as antigen through the unimpaired barrier

intrinsic AD [62], suggesting an important role of Th2 cells in the development of intrinsic AD lesions as well as extrinsic AD lesions.

21.6.2 Th17 Cells

We found that Th17 cells, producing IL-17A and IL-22, were increased in the peripheral blood of AD, and Th17 cells infiltrated the acute skin lesions more markedly than in the chronic lesions [20]. There was a tendency that the frequency of circulating Th17 cells was higher in intrinsic AD than in extrinsic AD [19]. In the lesional skin, another group of investigators reported that positive correlations between Th17-related molecules and SCORAD scores were only found in patients with intrinsic AD, whereas only patients with extrinsic AD showed positive correlations between SCORAD scores and Th2 cytokine (IL-4 and IL-5) levels [62]. In AD, the acute skin lesion corresponds to the late phase reaction evoked by Th2 cells and eosinophils, whereas the chronic skin lesion corresponds to the delayed-type hypersensitivity induced by Th1/Tc1 cells [42]. Because Th17 cells already exist in the Th2-associated acute lesions, they seem to disappear gradually in the progression to the chronic lesion where Th1/Tc1 cells infiltrate.

21.6.3 Chemokines and Others

With regard to chemokines, patients of both types showed high serum amounts of CCL17/TARC and CCL22/MDC and high peripheral blood mononuclear cell expression of CCL17 and CCL22 at comparable levels [44]. We investigated serum CCL17/TARC levels in extrinsic and intrinsic patients. Both groups had higher levels of serum CCL17 than healthy controls. However, its value was significantly higher in extrinsic than intrinsic AD [19, 42]. The blood levels of soluble receptors derived from lymphocytes correlate to the activity in various diseases. There is no significant difference in the elevated amounts of sCD23, sCD25, and sCD30 between the two types [68].

21.6.4 Dendritic Cells (DCs) and Langerhans Cells (LCs)

DCs as well as $CD4^+$ and $CD8^+$ T cells are comparably increased in both extrinsic and intrinsic AD. The extrinsic type is characterized by a significantly high level of the expression of IgE high-affinity receptor (FCεR) on the $CD1a^+$ DCs compared to the intrinsic type [36, 39]. When the high-affinity/low-affinity expression ratio was used as a disease marker for AD, the values for intrinsic AD fell below the diagnostic cut-off level, suggesting that intrinsic AD can be distinguished by

phenotyping of epidermal LCs [36, 39]. In accordance with these data from the lesional skin, the surface expression of the high- and low-affinity receptor for IgE and the IL-4Rα chain are significantly elevated in circulating monocytes from extrinsic AD patients [52].

As described below, it is possible that epidermal LCs in the barrier-disrupted skin produce high amounts of Th2 and eosinophil chemokines. Recent accumulating evidence indicates that upon external stimulation, epidermal keratinocytes produce thymic stromal lymphopoietin (TSLP), which stimulates LCs possessing TSLP receptors (Fig. 21.4) [18]. Protein antigen is more essential than hapten as the cause of extrinsic AD. Upon the epicutaneous application of ovalalbumin (OVA), conditional LC depletion attenuated the development of clinical manifestations as well as serum OVA-specific IgE increase, OVA-specific T-cell proliferation, and IL-4 mRNA expression in the draining lymph nodes [32]. Consistently, even in the steady state, permanent LC depletion resulted in decreased serum IgE levels, suggesting that LCs mediate the Th2 local environment. In addition, mice deficient in TSLP receptors on LCs abrogated the induction of OVA-specific IgE levels upon epicutaneous OVA sensitization [32]. Thus, LCs initiate epicutaneous sensitization with protein antigens and induce Th2-type immune responses via TSLP signaling, further suggesting that LCs play a mandatory role in extrinsic AD.

21.7 Relationship Between Barrier Status and Skin Immune Responses in Extrinsic AD

21.7.1 Epidermal Cytokine Production in Barrier-Disrupted Skin

The skin immune status is closely associated with the disordered condition of the skin barrier (Fig. 21.4). Studies using a mouse model of contact hypersensitivity (CHS) have shown that CHS responses to hapten are increased when a hapten is applied to the barrier-damaged skin [34]. Barrier disruption of the skin is experimentally performed by extraction of epidermal lipids with acetone or removal of corneocytes by tape stripping. Both procedures can induce elevated CHS responses. Not only increased permeability of hapten through the epidermis but also altered immune functions of epidermal cells potentiate T-cell activation in acute barrier disruption [34]. Such augmentation of immune reactivity may be critical to elimination of environmental noxious agents that penetrate easily into the barrier-disrupted epidermis, and it is also closely related to the mechanism underlying extrinsic AD.

21.7.2 Epidermal Chemokine Production in Barrier-Disrupted Skin

Regarding epidermal chemokines of the barrier-disrupted skin, the mRNA expression levels of Th1 chemokines (CXCL10, CXCL9, and CXCL11), Th2 chemokines (CCL17 and CCL22), and eosinophil chemoattractant (CCL5) are high in the epidermal cells from Th2 response-prone mice. In particular, we found that CCL17, CCL22, and CCL5 were remarkably elevated in BALB/c mice [38]. Tape stripping induced dermal infiltration of eosinophils in BALB/c mice, and the late-phase reaction was increased with infiltration of Th2 cells as well as eosinophils, when challenged via the tape-stripped skin. Notably, Th1 chemokines (CXCL9 and CXCL10) and Th2 chemokines (CCL17 and CCL22) are derived mainly from keratinocytes and LC, respectively [31]. Therefore, it is likely that LCs serve not only as protein antigen-presenting cells [32] but also as a Th2-attracting chemokine source [31].

21.7.3 Implications for the Difference Between Extrinsic and Intrinsic AD

The above findings suggest that Th2 and eosinophil responses and the resultant late-phase reaction are prone to take place in the skin with a damaged barrier by the modulated function of LCs. This may provide the mechanism of Th2-polarized immunophenotype of the extrinsic AD. On the contrary, LCs may not be stimulated to produce Th2 chemokines in intrinsic AD because of the presence of normal *stratum corneum*. Protein antigens penetrating the damaged barrier further induce the Th2-shifted response in extrinsic AD, whereas nonprotein antigens exert the Th1 response in intrinsic AD (Fig. 21.4).

It has been reported that Th2 cytokine IL-4 suppresses the enhancement of ceramide synthesis and cutaneous permeability barrier functions, which further aggravates the barrier [14]. This “outside-to-inside, back to outside” paradigm [10] is applicable for the pathogenesis of extrinsic AD. Neutralization of the normally acidic *stratum corneum* has deleterious consequences for permeability barrier homeostasis and *stratum corneum* integrity/cohesion attributable to serine protease activation leading to deactivation/degradation of lipid-processing enzymes and corneodesmosomes [13]. Hyperacidification improves permeability barrier homeostasis, attributable to increased activities of two key membrane-localized, ceramide-generating hydrolytic enzymes, which correlate with accelerated extracellular maturation of *stratum corneum* lamellar membranes. Thus, the surface pH may be another important factor to differentiate between the extrinsic and intrinsic types of AD. These several different lines of evidence suggest the relationship between the skin barrier condition and helper T-cell polarization in AD.

21.8 Patch Tests in Extrinsic and Intrinsic AD and Metal Allergy in Intrinsic AD

21.8.1 Patch Tests for Mite Antigens

An Italian group performed a patch test with house dust mites at a concentration of 20 % in petrolatum in the extrinsic and intrinsic types of adult male AD patients [22]. The patch test was positive in 47.4 % of extrinsic AD and in 66.6 % of intrinsic AD, and in 12.2 % of healthy subjects [22]. Inasmuch as extrinsic AD patients usually have high levels of IgE specific for mites, the authors wondered why the patch test was highly positive in the intrinsic AD. However, patch tests can reflect mostly the T-cell–mediated contact sensitivity, and the IgE-high extrinsic nature does not promote the patch test reactions. Rather, given that IFN-γ is produced at a higher level in the intrinsic than the extrinsic type, the higher frequency of positive reaction in the intrinsic type seems to be reasonable.

21.8.2 Patch Tests for Metals

It is known in patients with AD that the most frequent contact allergens are metals [12]. In 137 atopic children, 19.3 % patients were positive to metals [12]. In 1965, Shanon reported that patients with metal allergy occasionally exhibit a skin manifestation indistinguishable from AD under the name of "pseudo-atopic dermatitis" [8, 57], and chrome is the causative in their report [57]. Some patients with AD were improved by intake of a metal-free diet and elimination of metals [8].

The percentage of IFN-γ-producing Th1 cells is significantly higher in the peripheral blood of intrinsic AD than extrinsic AD [19]. Protein antigen is known to induce Th2 responses [18], and therefore, the impaired barrier of extrinsic AD may allow protein allergens to penetrate the barrier and to evoke Th2 responses. In this scenario, Th1-inducing nonprotein antigens, such as metals, might be causative for intrinsic AD [28]. In fact, high frequencies of positive patch test reactions to metals have been reported in AD patients [16, 53]. Nickel (Ni), cobalt (Co), and chrome (Cr) are the three major metals, and a high frequency of positive patch test to at least one of them is higher in AD patients than in non-AD patients [26]. However, the intrinsic and extrinsic types were not separately analyzed in those studies. Metals are administered orally as food and may be excreted from sweat at high concentrations as well as urine [16]. This notion raises the possibility that the concentrations of metals are high in the sweat of intrinsic AD patients.

In our study, intrinsic AD showed significantly higher percentages of positive reactions than extrinsic AD to Ni and Co (Fig. 21.1) [74]. The positivity to Co also tended to be higher in intrinsic than extrinsic AD. The prevalence of metal allergy to one or more of the three metals was more than twice higher in intrinsic AD (61.3 %) than extrinsic AD (25.5 %). In the IgE≤100 group, the incidence of

positive reactions to one or more of Ni, Co, and Cr was 63.6 %, whereas the 400<IgE group exhibited 25.0 % positivity. FLG deficiency may represent a risk factor for contact sensitization to allergens such as Ni [73]. Therefore, the frequencies of a positive metal patch test were analyzed in relation to *FLG* mutation. In the total patients, there was no significant difference in the patch testing results of three metals between the *FLG* mutation-bearing and nonbearing groups [74]. The concentration of Ni was significantly higher in the sweat of intrinsic AD than extrinsic AD patients [74]. When the sweat Ni concentration was analyzed as the function of serum IgE values, there was an inverse correlation between them. Metals such as Ni, Co, and Cr are known to cross-react with each other [25]. Cross-reactivity may occur in patch tests depending on the concentration of metals and the moieties of vehicles. Metal allergy is one of the potential causes of intrinsic AD. Interestingly, Co allergy is more prevalent in females than males [19], which is in accordance with the female preponderance of intrinsic AD.

21.8.3 Th1-Skewing Responses of Ni and Co Allergy

Haptens and metals are representatives of nonprotein, small antigenic molecules. The mechanisms underlying the Th1-polarizing action of metals remain unclear. Recently, Ni has been shown to activate Toll-like receptor 4 (TLR4) signaling in antigen-presenting cells (APCs) such as DCs [59]. The same TLR4 simulation occurs with Co, and the necessity of histidines H456 and H458 of human TLR4 is evident for activation of APCs by Co [49]. Thus, metals can interact with not only major histocompatibility/self peptide complex [55] but also with TLR4. TLR4 stimulation induces NF-κB activation and conversion of proIL-1β to IL-1β [49, 59], which has no skewing ability to Th1 or Th2 cells. Accordingly, Ni, Co, and Cr show a mixed Th1- and Th2-type cytokine response in peripheral T cells from sensitized patients [29], which is different from Th2-stimulatory protein antigens [18].

21.8.4 Significance of Metal Allergy in Intrinsic AD

Individuals highly ingesting or exposed to metals in daily life possibly develop AD via metal allergy. It has been shown that environmental Ni exposure is more important than genetic disposition, such as *FLG* mutation, in the development of Ni allergy [64]. Metals are administered with foods and applied to the skin with jewelry [7, 16]. Personal habits may increase the risk of development of contact dermatitis to metals. For example, women show a higher sensitization rate to Ni than men perhaps by wearing Ni-containing jewelry [27], which might result in the female preponderance of Ni allergy in intrinsic AD. It was reported that only

piercing women in the AD group had a higher incidence of sensitization to Ni but AD patients without piercing had no increased risk of Ni allergy [65].

Ni- or Co-rich food items include peanuts, hazelnuts, almond, chocolate, cocoa, sunflower seeds, beans, dried beans, porridge oats, licorice, lucerne seeds, oatmeal, and wheat bran [6, 58, 67]. Excess intake of these foods allows metal ions to be extraordinarily administered. Serum Ni levels correlate with Ni-rich food items. When Ni-allergic patients avoided Ni intake, their serum Ni levels were lowered compared to controls [27]. It is thought that metals are excreted through sweat, and therefore, sweating possibly may elicit dermatitis by serving as contactants. We found that the concentration of Ni was higher in the sweat of intrinsic than extrinsic AD patients [74], suggesting that metal allergy may be more significant in intrinsic AD. Eczematous lesions preferentially occur on the flexor aspects of the limbs and around the neck of AD patients. The degree of sweat secretion has been variously reported in AD. The possibility remains that Ni concentration is different depending on the skin sites, which might explain the predilection areas of intrinsic AD. A metal-free diet and elimination of metals improve skin eruptions in some AD patients [8, 57]. When Ni-allergic patients avoided Ni intake, their serum Ni levels were lowered compared to controls [27].

21.9 Skin Infections in Extrinsic and Intrinsic AD

Both extrinsic and intrinsic AD patients suffer from recurrent bacterial and viral infections [15]. A higher colonization of *Staphylococcus aureus* was observed in the extrinsic (71 %) versus the intrinsic children (49 %) [51]. The expression of human β-defensin-3, an antimicrobial peptide, is decreased in both types of AD as compared to normal skin and psoriatic skin [15]. Therefore, skin infection with microorganisms, in particular *Staphylococcus aureus*, may be more severe in the extrinsic type because of barrier perturbation, but it remains unclear whether the defense responses are different between the two types.

21.10 Neurotrophins and Neuropeptides in Extrinsic and Intrinsic AD

Given the original idea that external protein allergens are not causative in intrinsic AD, neurogenic inflammation induced by neuropeptides might be more important in this type [46]. Neurotrophins, nerve growth factor (NGF), and brain-derived neurotrophic factor (BDNF), are increased in both extrinsic and intrinsic AD, suggesting a similar pathophysiological background implicating a neuroimmune network [48]. However, there is a significant correlation between BDNF and SCORAD only in intrinsic AD [48]. Maternal NGF levels were significantly higher

in patients with both extrinsic and intrinsic AD than controls [68]. We measured plasma levels of SP in the two groups. The levels of SP in the IgE-high and IgE-low groups were comparable [19]. In both groups, SP levels and VAS for pruritus significantly correlated with each other, indicating no dominancy of neuropeptides for intrinsic AD.

21.11 Animal Models for Intrinsic AD

Most of the mouse models of AD target extrinsic AD [17], inasmuch as barrier abnormalities and/or IgE-related pruritus-prone consequences are thought to be a requirement for the AD model. On the other hand, a non-IgE–associated AD model is regarded as a mode of human intrinsic AD [4]. In the mouse model of AD, IL-18 contributes the spontaneous development of AD-like skin lesions independently of IgE [21]. When the skin barrier was destroyed in the mice and protein A from *Staphylococcus aureus* was topically applied to the skin, the mice developed AD lesions with dermal infiltration of eosinophils and mast cells and showed an increase in serum levels of IL-18, but not IgE [63]. In this model, IL-18 might be important for the development of infection-associated AD by induction of IL-3 from IFN-γ- and IL-13-producing "super" Th1 cells. Because the intrinsic AD shows high levels of IFN-γ-producing cells [19, 61] and normal levels of IgE, this mouse model resembles intrinsic AD and suggests that some intrinsic AD patients may be related to infection. However, we could not find a significant elevation of serum IL-18 in our intrinsic AD patients compared to extrinsic AD patients. Thus, the IL-18 mediation remains unclear in human intrinsic AD.

21.12 Conclusions

There have been a considerable number of findings regarding the barrier condition and immunological dysregulation in extrinsic AD. On the other hand, the causes and mechanisms of intrinsic AD remain unclear. In contrast to extrinsic AD, intrinsic AD can be characterized by normal barrier function [30] and IFN-γ-producing potency [19]. These findings suggest that the intrinsic AD patients are not sensitized with protein allergens, which induce Th2 responses, but with other antigens. Metals might be one of the candidates as antigens [74].

The extrinsic nature may be upmodulated as the patients grow. Therefore, the extrinsic and intrinsic types should be re-evaluated at each stage of life, that is, infancy, childhood, teenage, and adult for the allergological management of patients, including allergen avoidance, second allergy prevention, and immunotherapy. However, the risk of an "atopy march" is significantly lower in children with the intrinsic type [73]. Again, it appears that the intrinsic type is not related to the pure Th2 dominant immunological state. Future studies on the intrinsic type of

AD may clarify the pathophysiology of not only intrinsic AD, but also dermatitis of unknown cause that has been called atopiform dermatitis [2] or pseudo-atopic dermatitis [57].

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Chapter 22
Psoriasis

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Abstract Psoriasis vulgaris is a chronic, debilitating skin disease that affects millions of people worldwide. Although many components, including keratinocytes, dendritic cells (DCs), T cells, neutrophils, and endothelial cells are involved in psoriasis lesional skins, it is an inflammatory skin disease mainly mediated by T cells and DCs. Psoriatic skin contains diversified T cell and dendritic cell populations, such as interleukin (IL)-17–producing T (T17) cells, T helper (Th) 1 cells, Th22 cells, Langerhans cells, plasmacytoid DCs, and myeloid DCs. These cells interact with each other then complete psoriasis lesions. Inflammatory myeloid DCs release IL-23 and IL-12 to activate T17 cells, Th1 cells, and Th22 cells, and to produce the cytokines IL-17, interferon-γ, tumor necrosis factor (TNF), and IL-22. These cytokines mediate effects on keratinocytes to amplify psoriatic inflammation. Therapeutic studies with anticytokine antibodies have shown the importance of the key cytokines IL-23, TNF, and IL-17 in this process. Genetic studies also point to the importance of IL-23 in psoriasis pathogenesis. These findings indicate the central role of the IL-23/T17 axis in psoriasis. Our current model for disease pathogenesis emphasizes the central role of IL-23 in controlling activation of lymphocytes that produce IL-17 and a number of emerging therapies for psoriasis are targeted to the IL-23/T17 response axis.

Keywords Interleukin (IL)-17 • IL-23 • Tumor necrosis factor (TNF)α • Inflammatory myeloid dendritic cells • IL-17–producing T (T17) cells • T helper (Th)1 cells • Th22 cells • Dendritic cell lysosomal-associated membrane protein (DC-LAMP) • TNF and inducible nitric oxide synthase-producing dendritic cells (TIP-DCs) • Therapeutics

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22.1 Introduction

Psoriasis is an immune-mediated inflammatory skin disease that affects millions of people across the world. Psoriasis vulgaris, the most common form of psoriasis, is characterized by recurrent episodes of red and scaly plaques that are sharply demarcated from adjacent normal skin. The lesions often appear in areas susceptible to epidermal trauma such as elbows and knees. It may remain localized or become generalized over time. Although a great deal of complexity exists in gene circuits activated in psoriasis [61-63], this chapter focuses on immunological characteristics of psoriasis and treatment of psoriasis with immune targeted therapeutics.

22.2 Genetic Links

Genetic studies suggest immune activation is important in disease pathogenesis. Traditional linkage studies have mapped a number of psoriasis susceptibility loci (PSORS). There are often numerous candidate genes at each PSORS locus that may be contributing to the disease. The psoriasis-associated susceptibility locus 1 (PSORS1) at chromosomal position 6p21.3, also identified by single nucleotide polymorphism (SNP) rs 1265181, provides the strongest linkage with psoriasis [4, 37]. PSORS1 lies within the class I region of the MHC, and there is consensus that HLA-C is the most likely PSORS1 gene [11]. Given our understanding that HLA class I molecules are important for antigen presentation to $CD8^+$ T cells, this locus clearly links the genetics of psoriasis with T lymphocyte activation.

Central roles for interleukin (IL)-23 and IL-17–producing T (T17) cells are implied by disease association with a number of significant SNPs, including IL-12B (IL-12/23p40), IL-23A (p19), and the IL-23R (encoding a subunit of the IL-23 receptor) [44, 45]. The IL-23R risk allele has been shown to promote IL-23–induced T helper (Th)17 cell effector function compared to the protective allele [10]. Many other identified genes, such as tumor necrosis factor (TNF)α-induced protein 3 and TNFα-induced protein 3 interacting protein 1, seem to relate to TNF signal pathways that are likely to be enrolled in psoriasis [44]. Recently, a defect in IL36RN has been identified as a cause for pustular psoriasis [41, 49]. IL36RN serves as an antagonist of signals by IL-36α, β, and γ cytokines, suggesting that this axis has the potential to induce massive neutrophil influx if dysregulated. Thus genetics has identified key cytokine pathways that are discussed later in this chapter.

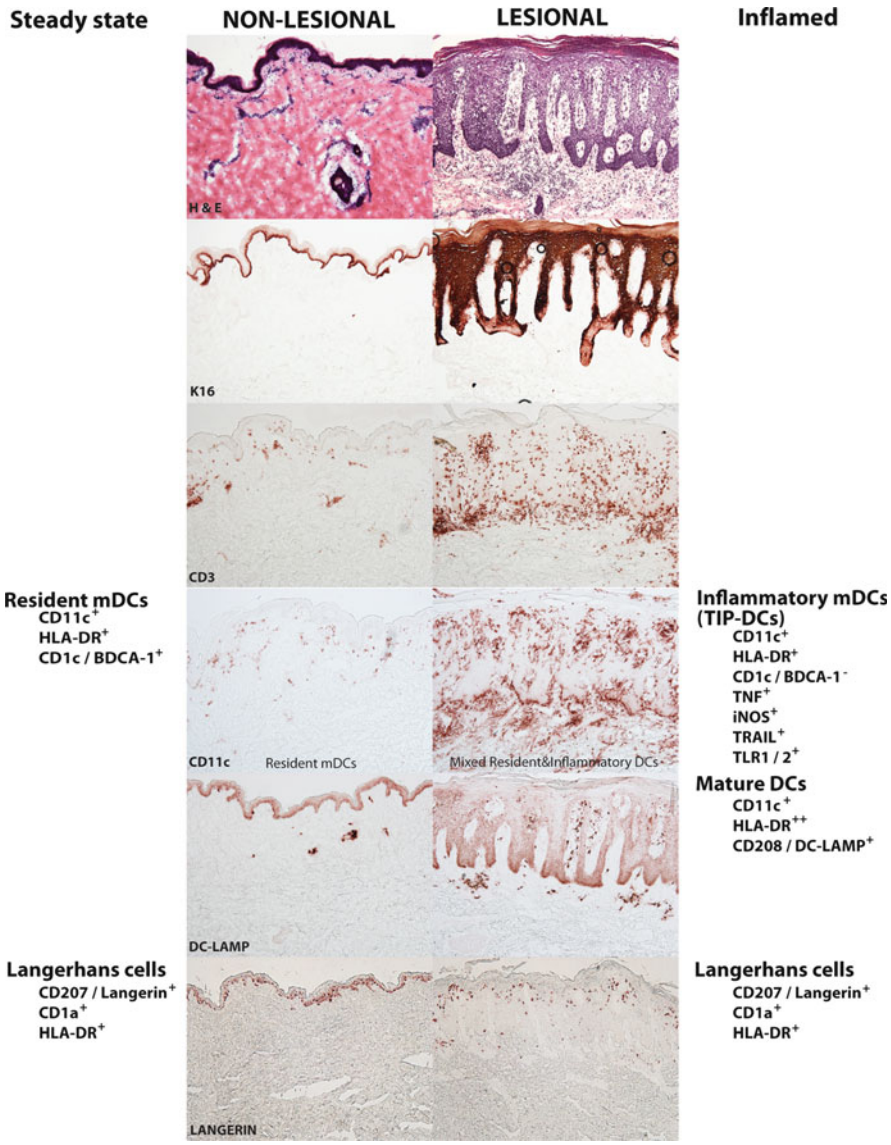

Fig. 22.1 Cellular components of healthy and inflamed skin. Representative immunohistochemistry of normal appearing, nonlesional skin of psoriasis patients, and lesional psoriasis skin, and summary of immune cellular components and their surface receptors. With hematoxylin and eosin stain (H&E), lesional psoriasis skin shows a greatly thickened epidermis (acanthosis) with elongations into the dermis (rete ridges). Retention of nuclei (parakeratosis) can be seen in the thickened *stratum corneum*. There is a dramatic increase in the number of cells in the dermis, composed predominantly of dendritic cells (DCs) and T cells. There are increased $CD3^+$ T cells in lesional psoriasis skin, often forming lymphoid-like clusters with DCs. Keratin 16 (K16) stains basal epidermis in nonlesional skin, but full thickness epidermis in psoriasis. In the steady state, $CD11c^+CD1c$/ blood dendritic cell antigen (BDCA)-1^+ resident myeloid DCs (mDCs) are found in the upper dermis in nonlesional skin.

22.3 Cellular Infiltrates in Psoriasis

Histologically, psoriasis has a defining appearance (Fig. 22.1). The epidermis is greatly thickened (acanthosis), due to increased proliferation of keratinocytes. As the normal process of differentiation cannot occur, there is loss of the normal granular layer, thickened *stratum corneum* (hyperkeratosis) and retention of nuclei in the upper layers and *stratum corneum* (parakeratosis). There is increased keratin 16 staining throughout the epidermis. There are collections of neutrophils in the epidermis and *stratum corneum* (Munro's microabscess).

In the dermis, there are abundant mononuclear cells, predominantly $CD3^+$ T cells and $CD11c^+$ dendritic cells (DCs). Psoriasis lesions contain prominent aggregates of intermixed T cells and CD208/DC-lysosomal–associated membrane protein $(DC\text{-}LAMP)^+$ mature DCs in the dermis. Langerhans cells exist mainly in the upper part of the epidermis, showing redistribution. In lesional skin, a greater number of dilated dermal blood vessels are seen. Endothelial cells are activated in psoriatic lesions, as is indicated by staining for intracellular adhesion molecule-1 (ICAM-1, also known as CD54), vascular cell adhesion molecule-1 (VCAM-1, or CD106), and E-selectin (CD62E). Leukocytes can gain entry to skin parenchyma by transmigration through reactive vessels, but resident skin leukocytes might also expand to create the dense infiltrates seen in psoriatic lesions.

For many years, there was a debate whether the primary process in psoriasis involved hyperplastic keratinocytes with secondary immune activation or vice versa. In part, this debate was fueled by lack of knowledge regarding therapeutic mechanisms of commonly used agents. Corticosteroids and some immunosuppressants could be used to treat psoriasis, but on the other hand, systemic agents such as methotrexate were viewed as keratinocyte-directed agents. Although cyclosporine, a calcineurin antagonist, had a dramatic effect for disease activity, this agent has direct effects on keratinocytes and immune cells, so an immune pathogenesis could not be proven solely with this agent [12, 25]. The first specific indication that the immune system could be playing a more integral role came with the clinical trial targeting T cells with the DAB_{389}IL-2 agent, a fusion protein also called denileukin diftitox, that causes apoptosis in activated T cells expressing functional IL-2

Fig. 22.1 (continued) During psoriatic inflammation, there is an increase in $CD11c^+$ inflammatory mDCs in the epidermis and the dermis that are CD1c/BDCA-1^-. These cells express HLA-DR, TRAIL, and Toll-like receptor (TLR)1/2. Among increasing $CD11c^+$ DC populations, tumor necrosis factor (TNF) and inducible nitric oxide synthase (iNOs)-producing DCs (TIP-DCs), which express high levels of TNF and iNOS, are also found. In addition, CD208/DC-lysosomal–associated membrane protein $(DC\text{-}LAMP)^+$ mature DCs exist in the dermis, forming aggregates with $CD3^+$ T cells. Resident mDCs are stable in number between nonlesional and lesional skin, and are both $CD11c^+$ and BDCA-1^+. CD207/langerin$^+$ Langerhans cells are found scattered in the lower epidermis in nonlesional skin and are found higher up in the thicker epidermis in lesional skin. Langerhans cells are also identified by CD1a and HLA-DR. All images 10× magnification

Table 22.1 Systemic therapeutics for psoriasis

Target	Agent	Drug
General T-cell immunosuppressive agents tested or used in psoriasis treatments with known molecular target		
Calcineurin	Cyclosporin	Neoral
IL-2R	DAB_{389}IL-2	Denileukin diftitox
CTLA4	CTLA4-Ig	Abatacept
LFA-3	LFA-3-Ig	Alefacept
CD11a	Anti-CD11a	Efalizumab
Protein kinase C (PKC)θ	PKC inhibitor	AEB071
		(Sotrastaurin)
Phosphodiesterase type 4 (PDE4)	PDE4 inhibitor	CC-10004
		(Apremilast)
Cytokine targeted agents or therapies for psoriasis		
TNF	Anti-TNF	Infliximab
		Adalimumab
TNF	TNFR-Ig	Etanercept
IL-12/23	Anti-IL-12/23p40	Ustekinumab
		Briakinumab
IL-23	Anti-IL-23p19	LY2525623
		SCH 900222
		CNTO 1959
		AMG 139
IL-17A	Anti-IL-17A	Secukinumab
		Ixekizumab
IL-17RA	Anti-IL-17RA	Broadalumab

receptors [23]. This study showed that specific depletion of activated T cells in psoriasis lesions could cause clinical and histological disease resolution.

Hence, the DAB_{389}IL-2 study set up the general hypothesis that psoriasis is a disease mediated by activated T cells that are present in focal skin regions. This view has been solidified and refined by the availability of a series of immune-targeted drugs that have been tested in psoriasis patients (Table 22.1). CTLA4-Ig (abatacept) was used to block B7-mediated costimulation to T cells [1]. At high doses, consistent improvements in psoriasis were detected that correlated with depletion of DC and T cell subsets from diseased skin regions. Therefore, this study was the first to show that disease activity could potentially be restrained by a specific T-cell antagonist that did not deplete T cells as its primary mechanism of action. Subsequently, two biologics targeted to T-cell activation pathways became FDA approved therapeutics for psoriasis. One of these agents was an LFA-3-Ig fusion protein (alefacept) that blocks CD2-mediated T-cell activation. With this agent, strong clearing of psoriasis lesions was seen in a subset of patients where the drug induced large decreases in T cells and DC populations in the skin [7]. Memory T cells were often depleted in the peripheral circulation of patients treated with

alefacept [6], providing additional evidence for pathogenic actions of activated T cells that infiltrate skin lesions of psoriasis.

Another T-cell targeted biologic approved for use in psoriasis was a monoclonal antibody to the integrin CD11a (anti-CD11a, efalizumab). T cells selectively use the integrin CD11a/CD18 (LFA-1) for migration to peripheral tissues and as a part of T-cell costimulation. Efalizumab blocks T-cell migration and activation responses in psoriasis patients, again without inducing T-cell cytotoxicity as a primary mechanism. Strong improvements in psoriasis lesions were seen in patients that had accompanying reductions in T-cell and DC subsets that infiltrate skin lesions [38]. Subsequently, the role of different T-cell subsets in psoriasis, including Th1, Th17, and Th22, has been dissected through testing of a range of cytokine antagonists [47], listed in Table 22.1.

22.4 Effector Immune Pathways in Psoriasis

In the early 2000s, the Th1 pathway was regarded as the dominant pathogenic model for psoriasis [35]. At that time, it was appreciated that there was a strong interferon (IFN)-γ signature in psoriasis lesions: IFN-γ–producing Th1 cells were abundant in psoriasis lesions and blood, and these Th1 cells were reduced with successful therapy. Recently, clinical studies have been conducted with individual cytokine antagonists that suggest a central role for IL-23 and IL-17, along with TNFα, in driving disease pathology. Therefore, the current pathogenic model of psoriasis emphasizes the IL-23/T17 axis, but also contains other T-cell subsets. For this chapter, IL-17–producing T cells are denoted as T17.

In the steady state, there are three DC populations residing in the skin: Langerhans cells, resident dermal myeloid DCs (mDCs), and plasmacytoid DCs (pDCs). During psoriatic inflammation, both mDCs and pDCs increase in number, but the greater increase comes from the mDC population [46, 71]. Blood dendritic cell antigen (BDCA)-$2^{+}CD123^{+}$ pDCs have been proposed to have an important role in the triggering of lesions, although mDCs are appreciated to be key proximal cells in the pathogenic psoriatic pathway. Psoriasis can be triggered by many factors, including injury and trauma (termed the Koebner effect), infection, medications, and the topical biological response modifier Imiquimod (Fig. 22.2a). Injury to the skin may cause cell death and increase production of the antimicrobial peptide LL37 by keratinocytes. DNA/LL37 complexes bind to intracellular Toll-like receptor (TLR)9 in pDCs, which causes activation and production of type I interferons, IFNα/β. LL37/RNA complexes can activate pDCs through TLR7, and mDCs can be activated by the LL37/RNA complex as well as by type I interferons, driving T-cell activation and the production of cytokines found in psoriasis [17, 19, 31]. In addition, murine studies have shown that topical Imiquimod (a TLR7 agonist) may induce psoriasiform skin inflammation, mediated by the IL-23/IL-17 axis and activated DCs [64]. mDCs existing in the skin are generally characterized using the classic DC definition, $CD11c^{+}HLA\text{-}DR^{+}$ cells. The major resident

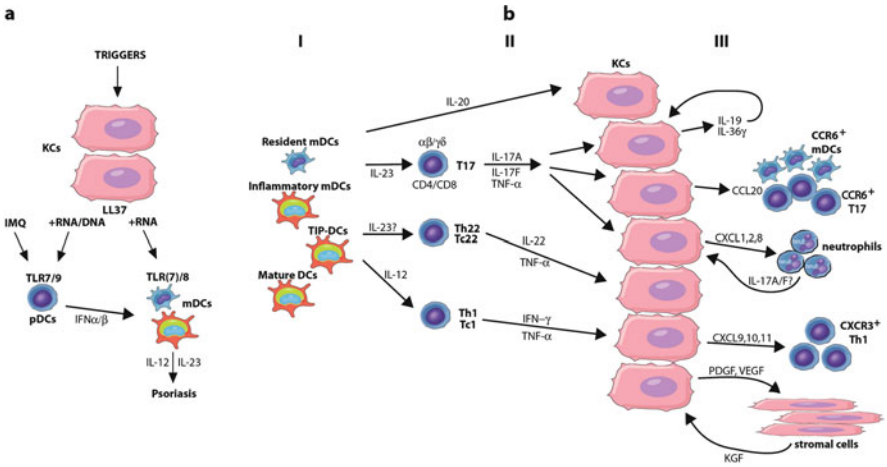

Fig. 22.2 Pathways for initiation and maintenance of psoriasis. (**a**) Initiation phase. Imiquimod (IMQ), a Toll-like receptor (TLR)7 agonist, can activate plasmacytoid dendritic cells (pDCs) to produce interferons (IFNs). LL37, a peptide derived from cathelicidin, may have an important role in the initiation of psoriasis lesions via the pathway. LL37 released from keratinocytes (KCs) can bind to nucleic acids to activate pDCs to release IFNα/β. LL37/RNA complexes can also activate myeloid DCs (mDCs) to produce IL-12 and IL-23, key psoriatic cytokines. (**b**) Maintenance phase (chronic disease). Major pathogenic pathway in psoriasis with (*I*) resident and infiltrating DCs producing cytokines such as interleukin (IL)-23 and IL-12. (*II*) These cytokines activate IL-17–producing T (T17), T helper (Th)22, and Th1 cells, to contribute to the cytokine milieu and further act on KCs. (*III*) Upon activation, KCs can produce chemokines to attract DCs, T cells, and neutrophils to the skin. Cytokines produced by KCs act as keratinocyte autocrine and/or paracrine growth factors. *TNF* tumor necrosis factor, *TIP-DCs* TNF and inducible nitric oxide synthase-producing dendritic cells, *PDGF* platelet-derived growth factor, *VEGF* vascular endothelial growth factor, *KGF* keratinocyte growth factor

population of mDCs in normal dermis is identified phenotypically with a single monoclonal antibody, CD1c, which is also known as BDCA-1. In the steady state, CD1c/BDCA-1$^+$ mDCs are relatively immature with modest T-cell stimulatory ability [70]. However, many CD11c$^+$ DCs in psoriasis lesions express markers of DC maturity, such as CD208/DC-LAMP and CD83 [38], thus they could function as conventional DCs in terms of presenting antigens to T cells for triggering of acquired immune responses. During psoriasis inflammation, there appears to be an additional mDC population that did not costain BDCA-1, and were subsequently termed inflammatory mDCs or TNF- and inducible nitric oxide synthase (iNOS)-producing DCs (TIP-DCs) [68]. They are likely derived from circulating precursors migrating into the skin due to inflammatory and chemotactic signals. Inflammatory mDCs express inflammatory molecules that include TRAIL, TLR1, TLR2, and S100A12 [69]. TIP-DCs express high levels of TNF and iNOS, thus referred to as TIP-DCs [38]. Pathogenicity of TIP-DCs in psoriasis is suggested by the rapid downmodulation of TIP-DC products TNF, iNOS, IL-20, and IL-23 during treatment with effective therapies [26, 67]. TIP-DCs can also stimulate the differentiation and activation of Th17 T cells [68]. Activated DCs in psoriasis produce IL-23

and IL-12 [68], which stimulate the three populations of resident T cells, T17, Th22, and Th1 cells. IL-23 activates T17 cells to produce IL-17A and IL-17F, which drive keratinocyte responses. mDCs also produce IL-20 in psoriasis lesions [65], and this could be a driver of epidermal hyperplasia. Interestingly, both resident (BDCA-1$^+$) and inflammatory (BDCA-1$^-$) mDCs have been shown to induce the proliferation of T cells and induce allogeneic T cells to produce IFN-γ and IL-17 [68], which are abundant in lesions [39].

Whereas keratinocytes might be viewed only as bystander cells in terms of immune activation, it is more likely that they are active participants in the recruitment and activation of leukocytes in psoriasis lesions (Fig. 22.2). IFN-γ induces keratinocytes to produce an array of pro-inflammatory chemokines, including CXCL9, 10, and 11, to recruit more CXCR3$^+$Th1 cells into the lesion [48]. By contrast, IL-17 induces the expression of chemokines (CXCL1, 2, and 8) that are involved in neutrophil recruitment into the site of inflammation. Aside from neutrophil-attraction chemokines, IL-17 induces the elaboration of CCL20, which recruits more CCR6$^+$CD11c$^+$ mDCs and CCR6$^+$ T17 T cells from peripheral circulation thus potentially creating a self-amplifying loop of inflammation [48]. LL37, which is also induced by IL-17 [54], may establish another self-amplifying inflammatory loop by forming complexes with self-RNA, and inducing mDCs to mature and express DC-LAMP [17]. Furthermore, activated T17 cells also elaborate TNF-α [39], which has synergistic effects with IL-17 as they share common transcriptional regulatory elements (NF-κB and CCAAT/enhancer-binding protein) [57]. IL-17 induces IL-19 and IL-36γ in psoriasis lesions, which may then lead to proliferative responses in keratinocytes. IL-19, IL-20, and IL-22 have similar trophic effects on the epidermis [55], and transgenic models have shown psoriasis-related pathologies in mouse skin for IL-20, IL-22, and IL-36 cytokines, reviewed in [40]. In humans, Th22 T cells are the main producers of IL-22. IL-17 and IL-22 cooperatively enhance the expression of epithelial antimicrobial peptides [36]. In addition, keratinocyte-derived cytokines such as platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) influence the growth of supporting stromal cells. Activated stromal cells overproduce factors such as keratinocyte growth factor (KGF) that can induce proliferation of keratinocytes [14].

After DC activation, activation and differentiation of T-cell subsets are supported by IL-12 and IL-23, which are produced mainly from mDCs in the skin. Psoriasis lesions contain T cells that discretely produce IFN-γ, IL-17, and IL-22, with initial labeling of these cells as Th1, Th17, and Th22, respectively. There are also CD8$^+$ T cell populations that make the same range of cytokines, thus these have been termed Tc1, Tc17, and Tc22, respectively. More recently, γδT cells have been found to be IL-17–producing cells in psoriasis, so we have adopted the more general term T17 to encompass IL-17–producing lymphocyte subsets in the skin. Figure 22.2b shows a current pathogenic model for the involvement of T cells and DC subsets in sustaining disease activity in psoriasis.

22.5 Therapeutic Agents Target Activated Immunity in Psoriasis

Current therapeutic options for psoriasis include various topical agents for limited disease (corticosteroids and vitamin D analogues), oral systemic immunosuppressive agents (such as methotrexate, retinoids, and cyclosporine), and phototherapy. More recently, biologics such as anti-TNF and anti-IL-12/23 antibodies have been FDA approved, and there are evolving agents such as anti-IL-23p19 and anti-IL-17 currently in clinical trials (Table 22.1). Therapeutic effectiveness of various agents may be related to how well this cycle of inflammation is broken.

22.5.1 New T-Cell Targeted Agents

Anti-inflammatory agents, including protein kinase C (PKC) inhibitor and phosphodiesterase (PDE)4 inhibitor are also in clinical trials. PKC isoforms have been shown to play key roles in cellular signaling, proliferation, differentiation, migration, survival, and death. PKCα and PKCθ as well as PKCβ and PKCδ are functionally important for T and B cell signaling, respectively [24, 42, 59]. PKCθ plays an essential role in T-cell activation because it is the only isoform that is selectively translocated to the T-cell/antigen-presenting cell contract site immediately after cell–cell interaction [43]. Furthermore, PKCθ is crucial for IL-2 production, a prerequisite for the proliferation of T cells [2]. AEB071 (sotrastaurin) is a novel PKC inhibitor that has strong and specific activity on PKCθ, PKCα, and PKCβ and lesser activity on PKCδ, PKCε, and PKCη. Actually, AEB071 both abolishes the production of several cytokines by activated human T cells, keratinocytes, and macrophages in vitro and inhibits an acute allergic contact dermatitis response in rats [58]. Clinical severity of psoriasis patients who were administered AEB071 was reduced up to 69 % compared with baseline after 2 weeks of treatment [58]. The improvement in psoriasis patients was accompanied by the histological improvement of skin lesions and may be partially explained by a substantial reduction of $p40^+$ dermal cells [58], which are known to mediate psoriasis.

PDEs comprise a family of enzymes that uniquely hydrolyze and degrade cyclic adenosine monophosphate (cAMP). One of 11 subtypes, PDE4 is widely expressed in numerous cell types, including hematopoietic cells, keratinocytes, endothelial cells, and nerve cells [9, 27]. Among its various cellular functions, PDE4 regulates immune and inflammatory processes through control of intracellular cAMP levels and downstream protein kinase A pathways [50]. With PDE4 inhibition, and the resulting increases in cAMP levels in immune and nonimmune cell types, expression of a network of pro-inflammatory and anti-inflammatory mediators can be modulated. In T cells, elevated levels of cAMP decrease the intracellular signaling triggered by the CD3-TCR complex on the cell surface. CC-10004 (apremilast) is

an orally available PDE4 inhibitor that modulates a wide array of inflammatory mediators involved in psoriasis, including decreases in the expression of iNOS, TNF-α, IFN-γ, IL-12, IL-23, CXCL9, and CXCL10 and the increase in IL-10 [3, 5, 56]. A Phase II open-label study in recalcitrant plaque psoriasis showed that mean percent decreases (improvements) from baseline in psoriasis patients administered apremilast were −59 % for PASI score and −53 % for body surface area at week 12. Skin samples taken from patients after treatment showed significant reductions of CD11c, CD3, and CD56 positive cells [22]. It indicates that CC-10004 reduced mDC, T-cell, and NK-cell or NK-T cell infiltration into the epidermis and the dermis. Reduced inflammatory leukocytes, with a pattern of broad partial inhibition, suggested reduced IL-23/T17 and Th22 response pathways.

22.5.2 Specific Cytokine Antagonists as Therapeutics

TNFα blockade was the first widely used anticytokine therapy for psoriasis. In psoriasis, TNFα is produced by keratinocytes, DCs (particularly TIP-DCs), Th1 cells, Th17 cells, and Th22 cells [13, 38, 39]. Blockade of TNFα is very efficacious, with >50 % of subjects achieving a psoriasis area and severity index (PASI)75 by 3 months. There are three FDA-approved anti-TNFα agents approved to treat psoriasis: infliximab is a chimeric monoclonal antibody that binds soluble and membrane TNF; etanercept is a soluble TNFα receptor-IgG fusion protein; and adalimumab is a fully human anti-TNFα IgG1 monoclonal antibody. Insights to the mechanism for the effective therapy with TNF modulators come from genomic studies that follow psoriasis lesions with etanercept treatment. These studies found that disease improvement correlated with the rapid downmodulation of IL-23 and Th17 cell products, and final disease resolution correlated with the late downmodulation of Th1-associated genes [67, 72]. Furthermore, comparison of responders and nonresponders to etanercept treatment revealed that successful response to treatment was dependent on the inactivation of mDC products and subsequently the Th17 pathway [72]. Thus, the TNFα blockade may be linked to suppression of the IL-23/Th17 axis.

To date, direct blockade of IL-17 appears to be the antipsoriatic therapeutic strategy with the most rapid efficacy. The studies presented below suggested that IL-17 ligands are the key pathogenic psoriatic cytokines, and that inhibiting this axis is essential for disease resolution. IL-17 and its downstream genes are turned off by many other psoriasis treatments we have studied, including cyclosporine [26], and narrow band ultraviolet B radiation [29]. Our group showed that TNFα blockade with etanercept reduced T cells, T17 cell products, and IL-17 induced genes, and that efficacy was associated with reduction in the IL-17 gene expression signature [67, 72]. Hence, for disease resolution, IL-17 must be switched off, implying that IL-17 signaling is required for disease presence. Three of the six known IL-17 ligands are overexpressed in psoriasis: IL-17A, IL-17F, and IL-17C. IL-17A and IL-17F bind to a heteromeric IL-17 receptor (IL-17R) composed of

IL-17RA and IL-17RC, whereas IL-17C binds to an IL17RA/IL17RE complex [16]. There have been three anti-IL-17 agents tested in psoriasis, with slightly different targets. Secukinumab and ixekizumab selectively bind IL-17A [18, 28, 30, 33], whereas brodalumab inhibits the IL-17RA subunit [52, 53]. Hueber et al. first presented clinical improvement in psoriasis with secukinumab [28]. Studies with brodalumab and ixekizumab, in subjects with moderate-to-severe psoriasis, have shown rapid cellular and molecular responses to IL-17 blockade [30, 33, 52, 53]. Both brodalumab and ixekizumab appear to work quickly because there were significant improvements by 1–2 weeks. Furthermore, a large proportion of patients achieved PASI90 and PASI100 responses (75 % and 62 %, respectively, with brodalumab), indicating IL-17 pathway blockade eliminates an essential component of psoriasis. The extent to which IL-17 inhibitors reverse cellular, molecular, and clinical phenotypes of psoriasis was surprising, because multiple T-cell subtypes are coactivated in psoriasis. There are approximately 160 genes that are synergistically regulated by IL-17 and TNFα [8]. However, anti-IL-17A treatment of psoriasis reduced sixfold greater number of genes than anti-TNFα treatment at 2 weeks [30]. Also, genes synergistically regulated by IL-17 and TNFα were blocked to a higher magnitude by anti-IL-17A than anti-TNFα treatment. Synergism between IL-17 and IL-22 has also been shown [66], but may also exist with other IL-20 family cytokines (IL-19, IL-20, and IL-24) that bind a common receptor. Overall, this supports the crucial role of IL-17 in driving psoriasis pathogenesis and the complex synergistic effects of these pro-inflammatory cytokines.

IL-23 is the critical driver behind T17 activation, as well as production of IL-17. IL-23 is a heterodimer of a unique IL-23p19 and shared IL-12/23p40 chains. IL-23 is produced by both resident and inflammatory mDCs, as well as macrophages in psoriasis [15, 68, 69]. IL-12, a heterodimer of IL-12/23p40 and IL-12p35, is produced by the same cells and is more important in activating Th1 cells. Both IL-12/23p40 and IL-23p19 are elevated in psoriasis, whereas IL-12p35 is not [32], implying that the effects of IL-23 might be dominant in psoriasis. Ustekinumab, an FDA-approved monoclonal antibody to IL-12/23p40, has been very successful for psoriasis patients; >60 % achieve a PASI75 response [34, 51]. Briakinumab, a second IL-12/23p40 inhibitor, shows similar efficacy [20, 21, 60]. Studies are underway to block the unique p19 chain of IL-23, which targets IL-23 specifically and spares IL-12. Head-to-head comparison of IL-23–specific inhibition and IL-12/23p40 inhibition will help determine how these agents differ in the clinical setting, and the specific role, either positive or negative, of IL-12 in psoriasis. Overall, disease improvements mediated by TNFα, p40, and IL-17 antagonists support the concept that inflammatory loops in psoriasis can be targeted at different points in the pathogenic cascade.

22.6 Concluding Remarks

In this chapter, we have highlighted the central role of the IL-17 cytokines in psoriasis, and their position in a positive feedback loop that maintains the inflammatory psoriatic state. Other important signaling proteins in this loop include IL-23, immediately upstream of IL-17A and IL-17F, and TNF, which can synergize with IL-17. It has also become clear that the inflammatory state in psoriasis may be better thought of as a complex network of cytokines interacting in positive and negative feedback loops rather than as a linear pathway or signaling cascade. Therapies targeted against IL-17 signaling, TNFα, or the IL-23 subunits have all shown varying levels of efficacy. Future goals should include developing strategies of treatment that do not require continuous, long-term immune suppression, that is, strategies to restore immune regulation or tolerance in this disease, and to derive a better understanding of the specific antigenic triggers that may induce and sustain T-cell activity in focal skin lesions. It is hoped that this approach taken to psoriasis pathogenic dissection and treatment will be a model for the approach to other inflammatory diseases of the skin and to inflammatory diseases in other tissues that are less accessible to direct analysis by tissue biopsy.

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Chapter 23
Urticaria

Marcus Maurer, Markus Magerl, Torsten Zuberbier, and Karsten Weller

Abstract Urticaria is a heterogeneous group of diseases and highly frequent. Wheals and angioedema are the signature signs and itch is the key symptom. Most cases of urticaria resolve within days. Those that do not tend to be of long duration and associated with severely impaired quality of life. Some patients with chronic urticaria develop wheals and angioedema exclusively in response to specific triggers (inducible urticaria), but most do not (spontaneous urticaria). The aim of chronic urticaria management is complete relief of signs and symptoms, which is often achieved by symptomatic rather than curative treatment. Modern, second generation H1-antihistamines are the first-line therapy.

Keywords Urticaria • Hives • Wheals • Angioedema • Itch • Mast cells • Histamine

23.1 Definition

Urticaria is a heterogeneous group of mast cell-mediated diseases characterized by itchy wheals, angioedema, or both [42]. Urticaria wheals are short-lived superficial itchy skin swellings. As they develop, these wheals are initially whitish in color (Fig. 23.1). They then develop a surrounding flare (erythema) before they resolve completely over the course of minutes to hours without showing subsequent skin changes. Urticaria wheals are usually itchy but may also come with a burning or stinging sensation. Angioedema is defined as a rapid swelling (edema) of the dermis and subcutaneous tissue or of the mucosa and submucosal tissue (Fig. 23.2). In contrast to wheals, angioedema is not usually itchy but sometimes painful, and it lasts longer. In urticaria, angioedema most commonly occurs in the face (lips, around the eyes) but may also affect the extremities and other skin sites.

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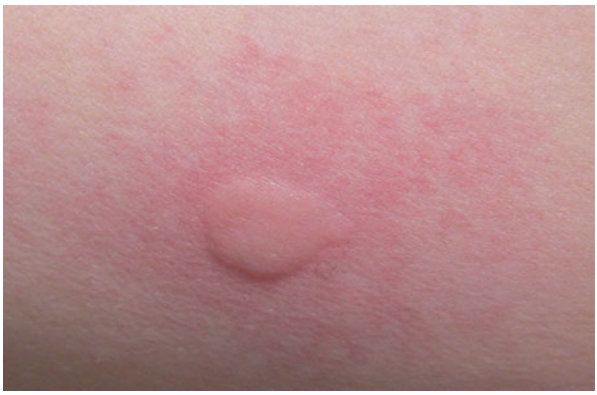

Fig. 23.1 Newly developing wheal

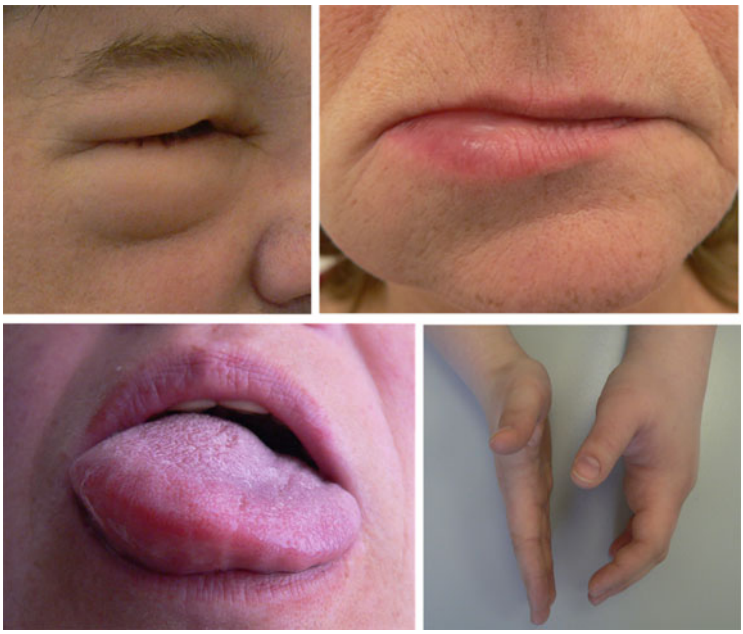

Fig. 23.2 Angioedema

23.2 Classification

Urticaria is classified according to (1) its duration and (2) its triggers of exacerbation (Table 23.1). In acute urticaria, the signs and symptoms occur for less than 6 weeks, whereas chronic urticaria is of more than 6 weeks duration [42]. The development of wheals and angioedema in urticaria patients can either be

Table 23.1 Classification of urticaria

Acute urticaria	*Acute spontaneous urticaria*	Spontaneous appearance of wheals, angioedema, or both <6 weeks due to known or unknown causes
	Chronic spontaneous urticaria	Spontaneous appearance of wheals, angioedema, or both ≥6 weeks due to known or unknown causes
Chronic urticaria	*Chronic inducible urticaria*	*Physical urticarias*
		Symptomatic dermographism[a]
		Cold (contact) urticaria
		(Delayed) pressure urticaria
		Solar urticaria
		Heat (contact) urticaria
		Vibratory angioedema
		Cholinergic urticaria
		Contact urticaria
		Aquagenic urticaria

From Zuberbier et al. [42]
[a]Also called *urticaria factitia*, dermographic urticaria

unpredictable and unprompted, in spontaneous urticaria, or it can occur only in response to specific triggers and eliciting situations, in inducible urticarias. In the inducible urticarias, specific triggers, which can be exogenous and acting on the skin directly (physical urticarias, contact urticaria, aquagenic urticaria) or not (cholinergic urticaria), are responsible and required for the induction of signs and symptoms. Triggers of physical urticaria are skin contact with cold and heat (cold urticaria, heat urticaria), mechanical triggers such as friction, pressure and vibration (symptomatic dermographism, pressure urticaria, vibratory urticaria, respectively), or ultraviolet or visible light (solar urticaria) [42]. Triggers of symptoms in contact urticaria are skin contact with urticariogenic substances, and in the case of aquagenic urticaria exposure to water. In cholinergic urticaria, symptoms are brought about by exercise or passive warming (sauna, hot showers, spicy food). Frequently, patients show more than one urticaria type, for example chronic spontaneous urticaria and symptomatic dermographism.

23.3 Epidemiology

Urticaria is a very common disease and usually of short duration (acute urticaria). Virtually everyone, at one point during his or her life, develops acute urticaria. Most commonly, this is acute contact urticaria, caused by skin contact with urticariogenic substances derived, for example, from stinging nettles or jellyfish (Fig. 23.3). Acute spontaneous urticaria is also very common, and it is estimated that its lifetime prevalence is up to 20 % [25]. Acute spontaneous urticaria rarely progresses to chronic spontaneous urticaria. Nonetheless, chronic spontaneous urticaria, which is thought to be twice as frequent as chronic inducible urticaria, is estimated to have a

Fig. 23.3 Contact urticaria induced by stinging nettle

point prevalence of 0.5–1 % [25]. Chronic spontaneous urticaria can affect all age groups with a peak incidence between the twentieth and the fortieth year of life; that is, patients are primarily affected during important years of their working age [25]. Women are affected about twice as often as men [25].

23.4 Pathogenesis

The signs and symptoms of urticaria, both spontaneous and inducible, are due to the activation and degranulation of skin mast cells and the effects of pro-inflammatory mediators released in the process. Mast cells are long-lived resident cutaneous cells that predominantly localize around blood vessels and sensory nerves [26] as well as in the upper papillary dermis. They contain cytoplasmic granules in which preformed mediators are stored that are released into the cell's vicinity by degranulation in response to activation [36]. These preformed mast cell mediators include histamine, heparin, and proteases (e.g., tryptase, chymase) [7] as well as several cytokines. Upon activation and degranulation, skin mast cells also rapidly produce and secrete prostaglandins, leukotrienes, and platelet activating factor [36]. Mast cells are thought to function as sentinel cells of the skin and a first line of defense against bacteria and other pathogens [27]. The inflammatory effects of their mediators include the activation of sensory skin nerves (itch, burning sensation, pain), the dilatation of skin blood vessels (erythema, hyperthermia) and the induction of plasma extravasation (wheals and angioedema). Histamine plays a critical role in skin mast cell-mediated vasodilation and extravasation, by acting on H1 receptors [8]. The degranulation of skin mast cells in urticaria patients also results in the

recruitment of basophils, neutrophils, eosinophils, and other immune cells to the site of activation [41]. The mechanisms of mast cell activation in urticaria are largely unclear. In chronic urticarias, mast cells are typically not activated by the binding of environmental allergens to specific IgE bound to cell surface IgE receptors [42], as is the case in allergies such as allergic rhinitis and anaphylaxis. Reported candidates for relevant signals involved in mast cell activation in chronic spontaneous urticaria include autoantibodies to IgE [11] or the IgE receptor [15], IgE autoantibodies directed against autoantigens (autoallergens) such as thyreoperoxidase [2], complement components such as C5a [10], as well as neuropeptides [4], for example, substance P.

23.5 Clinical Picture

23.5.1 Acute Spontaneous Urticaria

Acute spontaneous urticaria usually resolves within a few days to weeks. Viral infections of the upper airways as well as nonsteroidal antiphlogistics (e.g., ibuprofen, diclofenac, acetylsalicylic acid) and other drugs are common causes. But in many acute spontaneous urticaria patients no relevant cause can be identified. The spectrum of signs and symptoms ranges from a few short-lived wheals to severe angioedema attacks with persistently reoccurring multiple and confluent wheals that affect large body areas.

23.5.2 Chronic Spontaneous Urticaria

The symptoms of chronic spontaneous urticaria are generally the same as in acute spontaneous urticaria, but in contrast, chronic spontaneous urticaria is characterized by a long duration with up to 50 % of patients affected for more than 10 years [35]. The mean length of chronic spontaneous urticaria seems to be around 4–7 years. In up to half of all patients both wheals and angioedema occur, and in about one in ten patients only angioedema develops [25]. The remaining patients solely show wheals. Most patients with moderate or severe disease activity have symptoms every day or almost every day [37]. Disease activity may change markedly over time in the same patient, but the natural course also varies considerably between different subjects.

23.5.3 Chronic Inducible Urticaria

In chronic inducible urticaria, the development of wheals and angioedema is always provoked by the exposure to specific triggers. These conditions are, therefore, more predictable than spontaneous urticaria and disease activity depends on the frequency (and avoidance) of exposure to the relevant trigger at above threshold strength. The sensitivity to symptom-inducing triggers tends to be stable in individual patients over time. Triggers of inducible urticaria include the exposure to low or high temperatures, UV or visible light, as well as pressure and other mechanical forces for the physical urticarias and exposure to urticariogenic substances (contact urticaria), water (aquagenic urticaria), or situations associated with an increase in body temperature (cholinergic urticaria). Skin sites exposed to inducible urticaria triggers such as the hands are therefore more commonly affected. The only exception to this rule is cholinergic urticaria, where triggers (exercise, hot bath, spicy food) do not act directly on the skin but by increasing the body temperature [19]. Similar to chronic spontaneous urticaria, skin lesions in chronic inducible urticaria patients can be accompanied by systemic problems (e.g., hypotension in cold urticaria, malaise in delayed pressure urticaria). These extracutaneous symptoms are thought to be due to the effects of histamine and other pro-inflammatory mediators released at skin sites of trigger exposure and wheal and angioedema development. Chronic inducible urticarias show spontaneous remission in the vast majority of patients, but there are currently no biomarkers or other indicators that allow us to predict, for individual patients, when this will occur. Chronic inducible urticaria usually persists for several years before resolving spontaneously [25].

23.6 Diagnostics

23.6.1 Acute Urticaria

Acute urticaria usually does not require a diagnostic workup, because it is self-limited. The one exception to this rule is suspicion of acute urticaria due to an allergy (i.e., exposure to an allergen in a sensitized patient) or the existence of other eliciting factors such as nonsteroidal antiphlogistics. In this case, allergy tests as well as educating the patients may be useful to allow patients to avoid re-exposure to relevant causing factors.

23.6.2 Chronic Spontaneous Urticaria

The diagnostic workup in patients with chronic spontaneous urticaria should (1) exclude the presence of severe inflammatory conditions, (2) identify the causes in patients with severe and/or longstanding disease, (3) assess disease activity and impact, and (4) exclude differential diagnoses if indicated.

In all patients with chronic spontaneous urticaria, the correct diagnosis should be confirmed by a thorough history, and severe inflammatory conditions should be ruled out by assessing erythrocyte sedimentation rates/C-reactive protein levels and a differential blood count. A physical examination should be performed and all nonsteroidal antiphlogistics should be discontinued and avoided in the future [42].

In patients with long-standing disease and/or high disease activity, underlying causes should be looked for. The search for underlying causes in chronic spontaneous urticaria should be based on clues from the history. Common causes are autoreactivity, autoallergy, chronic infections, and intolerance to food components.

Autoreactivity, that is, a harmful response of the body to itself, is thought to be the relevant cause in one third to half of chronic spontaneous urticaria patients [16]. High disease activity, the development of angioedema, the lack of benefit from antihistamine therapy and autoimmune comorbidities should all prompt the search for autoreactivity.

In addition, most patients with chronic spontaneous urticaria have been found to exhibit IgE antibodies to autoantigen (autoallergens) such as thyreoperoxidase [2] or double-stranded DNA [13], and anti-IgE therapy effectively controls disease activity in these patients [23].

Chronic spontaneous urticaria can also be caused by bacterial infections [42], for example, of the gastrointestinal tract by *Helicobacter pylori* or chronic ear, nose, or throat infections, especially of the teeth, as well as by parasitic infections and, rarely, viral infections. The spectrum of relevant infections varies across geographical regions [42]. Underlying infections may be asymptomatic or associated with mild symptoms. In many patients no systemic signs of inflammation and infections can be detected. It is, therefore, recommended to investigate patients thoroughly when checking for common chronic spontaneous urticaria causing infections.

Many patients suspect that their chronic spontaneous urticaria is due to what they eat and drink. Whereas food allergies are rarely found to be the cause of chronic spontaneous urticaria [43], many patients exhibit food intolerance [6, 21, 44], for example, to taste intensifiers, preservatives, or to naturally occurring aromatic compounds, biogenic amines, and salicylic acid. Chronic spontaneous urticaria due to food intolerance is confirmed by a documented decrease in disease activity after a 4-week diet that is virtually devoid of potentially relevant food components (sometimes called pseudoallergens) and by an increase in disease activity after oral provocation with these food components. Independent studies found beneficial effects of a pseudoallergen-low diet in one third to three quarters of chronic spontaneous urticaria patients [6, 21, 44].

Less frequent causes of chronic spontaneous urticaria include other chronic inflammatory conditions such as gastritis or inflammation of the bile duct, systemic *Lupus erythematosus*, and other autoimmune disorders as well as sensitizations to type I allergens (in less than 1 %).

All patients with chronic spontaneous urticaria should be investigated and monitored for their disease activity, disease impact on quality of life, and disease control. The gold standard for measuring disease activity in spontaneous urticaria is the urticaria activity score (UAS) [29]. To obtain UAS values, patients are usually asked to document every day for seven consecutive days (UAS7) the numbers of wheals and the intensity of pruritus they experienced over the last 24 h using a 0–3 point scale for wheals (0 for none, 1 for <20, 2 for 20–50, and 3 for >50) and pruritus (0 for none, 1 for mild: present but not annoying or troublesome, 2 for moderate: troublesome, but does not interfere with normal daily life activity or sleep, and 3 for intense: interferes with normal daily life or sleep). The UAS7 (minimum = 0, maximum = 42) is then calculated as the sum of the daily totals of the wheal and the itch scores. In chronic spontaneous urticaria patients who develop angioedema, but not wheals, the angioedema activity score (AAS) should be used [40] which is also a validated, prospective diary-type instrument assessing the frequency and severity of angioedema symptoms. Patients with wheals and angioedema should be assessed with both scores, the UAS7 and the AAS.

Disease activity and quality of life impairment are poorly correlated in many patients with chronic spontaneous urticaria. Thus, the UAS7 and/or the AAS should be used together with the disease-specific quality of life questionnaires, that is, the CU-Q_2oL [3] (for patients with wheals), the AE-QoL [38] (for patients with angioedema), or both (for patients with wheals and angioedema).

The urticaria control test (UCT) is a novel and validated tool for assessing disease control in all patients with chronic urticaria (spontaneous or inducible) [39]. The UCT has only four items and a clearly defined cut-off for patients with "well-controlled" versus "poorly controlled" disease, and it is thus ideally suited for the management of patients in routine clinical practice. An overview on available instruments for patients with chronic urticaria is depicted in Table 23.2.

Table 23.2 Tools for assessing disease activity, disease control, and disease impact on quality of life in patients with chronic urticaria

	Chronic spontaneous urticaria			
	Patients with wheals	Patients with wheals and angioedema	Patients with angioedema	Inducible urticaria
Disease activity	UAS	UAS and AAS	AAS	Determination of trigger threshold with specific provocation test
Disease control	UCT	UCT	UCT	UCT
Quality of life	CU-Q_2oL	CU-Q_2oL and AE-QoL	AE-QoL	No instrument available yet

UAS Urticaria Activity Score, *AAS* Angioedema Activity Score, *UCT* Urticaria Control Test, *CU-Q_2oL* Chronic Urticaria Quality of Life Questionnaire, *AE-QoL* Angioedema Quality of Life Questionnaire

Finally, the diagnostic workup in chronic spontaneous urticaria patients should include the consideration of differential diagnoses, especially in patients resistant to standard treatment. Patients with recurrent wheals who do not develop angioedema may have urticaria vasculitis or an autoinflammatory condition such as Schnitzler syndrome or cryopyrin-associated periodic syndromes [17]. Patients with recurrent angioedema who do not develop wheals may have hereditary angioedema or another form of bradykinin-mediated angioedema. A thorough history that includes the right questions and, if indicated, a limited set of investigations is sufficient to confirm or exclude the most common differential diagnoses of chronic spontaneous (and inducible) urticaria (Fig. 23.4) [24].

23.6.3 Inducible Urticarias

The underlying causes of chronic inducible urticarias with the exception of contact urticaria are unknown and routine investigations for underlying causes are therefore not recommended [42]. An exception may occur if there are compelling clues from the history. The diagnostic workup in inducible urticaria patients is aimed at the identification of the relevant elicitation triggers and at measuring trigger thresholds [19].

In symptomatic dermographism, also called urticaria factitia or dermographic urticaria, wheals are induced by scratching. Provocation testing should be done by stroking the skin of the volar forearm or the upper back with a smooth and blunt object such as a closed ballpoint pen or a dermographometer [1], for example, the FricTest (Moxie GmbH, Berlin, Germany, Fig. 23.5), which allows for simultaneous testing of four different trigger strengths, or a pen-shaped dermographic tester with a spring-loaded tip that can be adjusted to exert different strengths of shear force (HTZ Limited, Vulcan Way, New Addington, Croydon, Surrey, UK). Provocation tests with dermographometers are done by placing them vertically on the skin and then moving them across the skin with a defined pressure. The test is positive when a wheal occurs at the provocation site within 10 min. When the test is positive, threshold tests should be performed (Fig. 23.6).

Delayed pressure urticaria patients develop erythematous angioedema-like swellings at skin sites exposed to pressure. These swellings are induced by vertical pressure, for example, by shoulder straps of bags, tight shoes, or prolonged sitting (e.g., bicycle ride). Swellings occur with a delay of four to eight hours and typically persist for several hours, in some patients for several days. Weighted rods or dermographometers are used to test for delayed pressure urticaria. The result is positive when a red palpable swelling is present 6 h after testing.

In patients with vibratory angioedema cutaneous swellings occur within minutes after exposure to vibration at skin contact sites. This can be tested with a laboratory vortex mixer.

In cold urticaria, itchy wheal and flare-type skin reactions or angioedema are induced by exposure to cold, typically within minutes after cold contact (cold air,

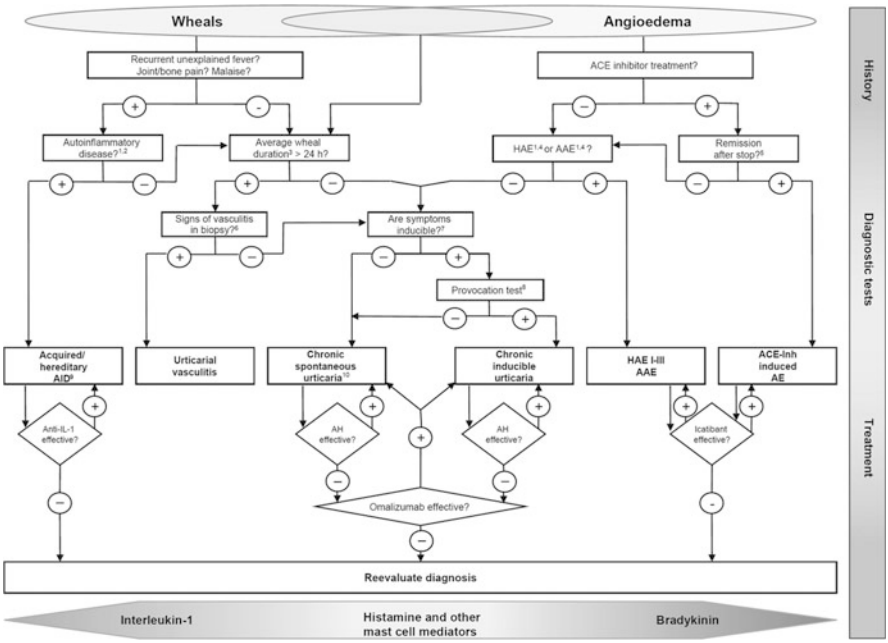

Fig. 23.4 Algorithm for diagnostic workup in patients with recurrent wheals, angioedema, or both (from Maurer et al. [24]). *AAE* Acquired angioedema due to C1-inhibitor deficiency, *ACE-Inh* angiotensin converting enzyme inhibitor, *AE* angioedema, *AH* H1-Antihistamine, *AID* Autoinflammatory disease, *HAE* Hereditary angioedema, *IL-1* Interleukin-1. (*1*) Patients should be asked for a detailed family history and age of disease onset. (*2*) Test for elevated inflammation markers (C-reactive protein, erythrocyte sedimentation rate), test for paraproteinemia in adults, look for signs of neutrophil-rich infiltrates in skin biopsy; perform gene mutation analysis of hereditary periodic fever syndromes (e.g., Cryopyrin-associated periodic syndrome), if strongly suspected. (*3*) Patients should be asked: "How long do your wheals last?" (*4*) Test for Complement C4, C1-INH levels, and function; in addition test for C1q and C1-INH antibodies if AAE is suspected; do gene mutation analysis, if former tests are unremarkable but patient's history suggests hereditary angioedema. (*5*) Wait for up to 6 months for remission; additional diagnostics to test for C1-inhibitor deficiency should only be performed if the family history suggests hereditary angioedema. (*6*) Does the biopsy of lesional skin show damage of the small vessels in the papillary and reticular dermis and/or fibrinoid deposits in perivascular and interstitial locations suggestive of urticaria vasculitis? If yes, direct immunofluorescence should be performed to look for immune complexes (immunoglobulins or complement) in vessel walls. Also, if suggested by the history, systemic vasculitic diseases which may present with urticaria vasculitis (e.g., lupus erythematosus or Sjögren's syndrome) should be ruled out and patients should be screened for antinuclear and extranuclear antibodies where indicated. (*7*) Patients should be asked: "Can you make your wheals appear?" (*8*) In patients with a history suggestive of inducible urticaria standardized provocation testing according to international consensus recommendations should be performed. (*9*) Acquired AIDs include Schnitzler's syndrome as well as systemic-onset juvenile idiopathic arthritis (sJIA) and adult-onset Still's disease (AOSD);, hereditary AIDs include cryopyrin-associated periodic syndromes (CAPS) such as familial cold autoinflammatory syndromes (FCAS), Muckle–Wells syndrome (MWS) and neonatal onset multisystem inflammatory disease (NOMID), more rarely hyper-IgD syndrome (HIDS), and tumor necrosis factor receptor alpha-associated periodic syndrome (TRAPS). (*10*) In some rare cases recurrent angioedema is neither mast cell mediator-mediated nor bradykinin-mediated, and the underlying pathomechanisms remain unknown. These rare cases are referred to as "idiopathic angioedema" by some authors

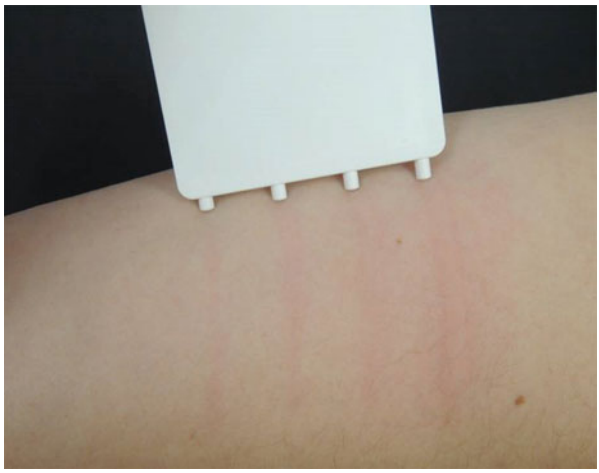

Fig. 23.5 FricTest, a dermographometer for diagnosing symptomatic dermographism and measuring trigger thresholds

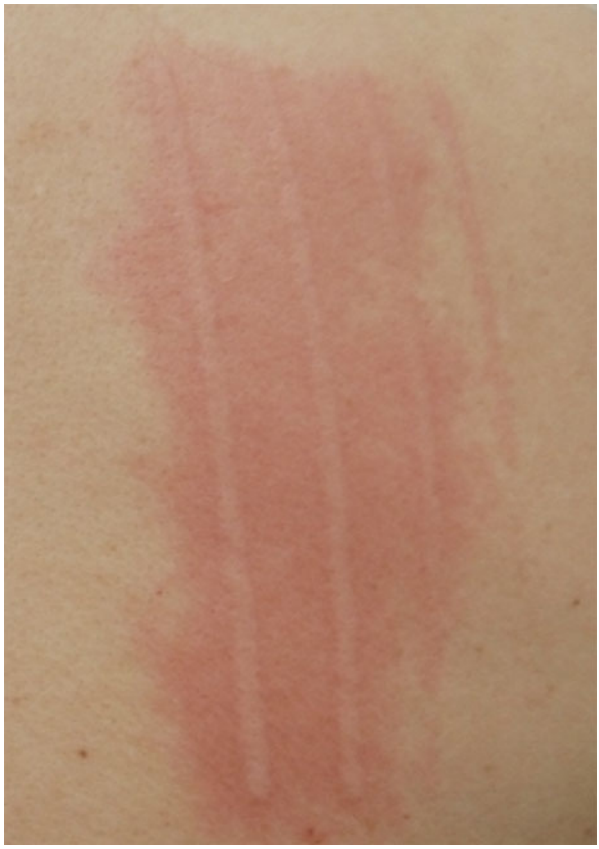

Fig. 23.6 Threshold test performed with the help of FricTest in a patient with symptomatic dermographism

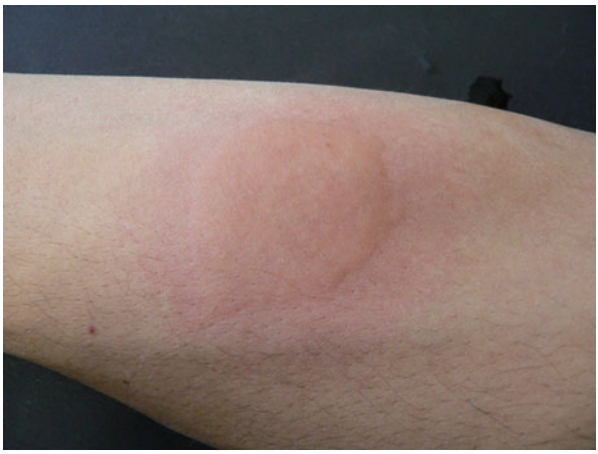

Fig. 23.7 Positive ice cube cold provocation test in cold urticaria patient

cold liquids, or objects). To perform a cold provocation test, a melting ice cube in a thin plastic bag (to avoid cold damage of the skin) is placed on the volar forearm for 5 min and the test response is assessed 10 min later. If the test site shows a palpable and visible wheal, the result is positive (Fig. 23.7). Cold urticaria patients should be evaluated for their individual temperature and/or stimulation time thresholds [1, 28] (Fig. 23.8), for example, by using a TempTest instrument [33]. The latest TempTest instrument (TempTest 4.0, Courage + Khazaka electronic GmbH, Köln, Germany, Fig. 23.9) simultaneously tests for skin responses to all temperatures from 4 to 44 °C (Fig. 23.10). Threshold measurements allow patients and physicians to monitor disease activity and responses to therapy.

In heat urticaria, wheals are usually well-defined and limited to the area of heat exposure. They develop within minutes after heat contact and usually resolve within 3 h. To test for heat urticaria, temperatures of up to 44 °C should be applied to the skin for 5 min (TempTest, metal/glass cylinders, filled with water, hot water bath), and responses should be assessed 10 min thereafter. Heat urticaria patients should also be tested for their temperature thresholds to determine disease status and treatment response.

Solar urticaria is characterized by itchy wheals that occur within minutes after skin exposure to UV and/or visible light. Solar urticaria is diagnosed by provocation tests done with solar simulators (with UV-A and UV-B filters) or monochromators (UV-A and UV-B, visible light). UV-A is tested at 6 J/cm^2 and UV-B at 60 mJ/cm^2 (buttocks). A palpable and clearly visible wheal at 10 min after testing confirms solar urticaria, in which case patients should be threshold tested for their minimal urticaria-triggering dose of radiation.

In cholinergic urticaria, itching and whealing occur in situations associated with a rise in body temperature. Wheals appear within minutes and typically last less than 1 h. To diagnose cholinergic urticaria, patients are first subjected to moderate physical exercise (treadmill or stationary bicycle) that makes them sweat. Patients with a positive result (wheals after 10 min) are then subjected, after a break of at

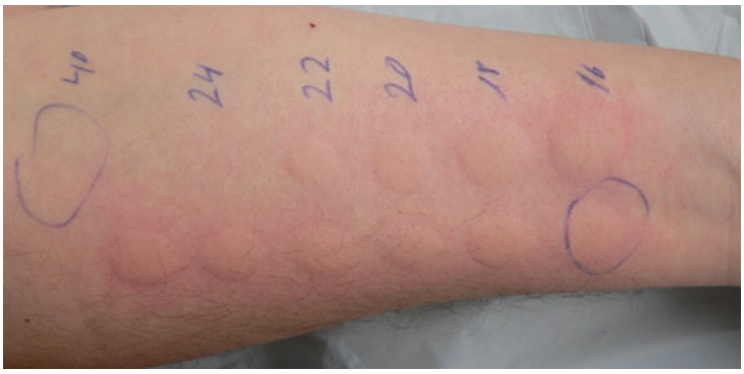

Fig. 23.8 Result of threshold testing performed with the help of TempTest 3.0 in patient with cold urticaria

Fig. 23.9 TempTest 4.0

least 24 h, to a warm bath (42 °C) for up to 15 min, which also usually leads to whealing in cholinergic urticaria patients.

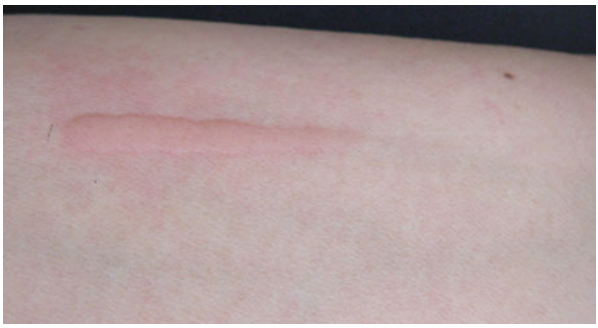

Fig. 23.10 Result of temperature threshold testing with TempTest 4.0

23.7 Therapy

23.7.1 Acute Spontaneous Urticaria

Patients with acute spontaneous urticaria should be advised to avoid eliciting factors, if they are known and avoidable. The therapeutic goal is to control and prevent the development of urticarial lesions until the condition resolves by itself. Mild cases may not require treatment or will respond to oral second generation H1-antihistamine treatment. In more severe cases, doses of nonsedating H1-antihistamines may have to be increased up to fourfold the licensed dose and oral steroids may also be necessary. Oral steroid intake should be limited to short-term treatment and should not be used as long-term treatment [42].

23.7.2 Chronic Spontaneous Urticaria

The aim of treatment in patients with chronic spontaneous urticaria is to stop the reoccurrence of urticarial skin reactions. This can either be achieved by treating patients for underlying causes and triggers or by the prophylactic use of drugs that block the activation of mast cells or the effects of mast cell mediators. Some of the underlying causes of chronic spontaneous urticaria such as relevant infections can be treated and eradicated. In patients where no underlying causes are identified or where underlying causes are identified but cannot be treated, symptomatic treatment is required.

Second generation, nonsedating H1-antihistamines are the first-line symptomatic treatment for chronic spontaneous urticaria [42]. These should be taken as preventive therapy, on a daily basis. In patients who do not respond adequately to standard doses, nonsedating H1-antihistamines should be updosed (up to four times the standard dosage) after 2 weeks. Higher than standard doses have been shown to be safe and to be superior to standard dosages in chronic spontaneous urticaria [9, 34]. Patients who fail to respond adequately even to higher doses of second

generation H1-antihistamines should be treated with add-on omalizumab (anti-IgE), cyclosporin, or montelukast, a leukotriene antagonist (Fig. 23.11) [42]. With the exception of standard-dosed H1-antihistamines, all of these therapies are off-label. For any treatment that results in the complete control of symptoms, it is advisable to check patients for spontaneous remission every 6–12 months.

23.7.3 Chronic Inducible Urticaria

The treatment of inducible urticarias relies on the avoidance of eliciting stimuli and the prevention of symptoms by treatment with inhibitors of mast cell mediators. To completely avoid relevant triggers is often not possible for patients or associated with severe quality of life impairment. For symptomatic preventive therapy, the same treatment algorithm applies as for chronic spontaneous urticaria (Fig. 23.11) and second-generation nonsedating H1-antihistamines are recommended as the first-line treatment [42]. In many patients, higher than standard doses are more efficacious as compared to standard dose treatment and required to sufficiently control symptoms [18, 22, 32]. Patients who remain symptomatic on high-dose H1-antihistamine treatment are recommended to receive additional treatments such as omalizumab. Various treatment options appear to be especially effective in some inducible urticarias, but it is unclear why and controlled studies are missing. For example, UVB light therapy has been reported to be effective in patients with symptomatic dermographism [5], dapsone [12] and anti-TNF [20] in delayed pressure urticaria, antibiotic treatment with doxycycline or penicillin for several weeks in cold urticaria [30], afamelanotide in solar urticaria [14], and injections of botulinum toxin in cholinergic urticaria [31].

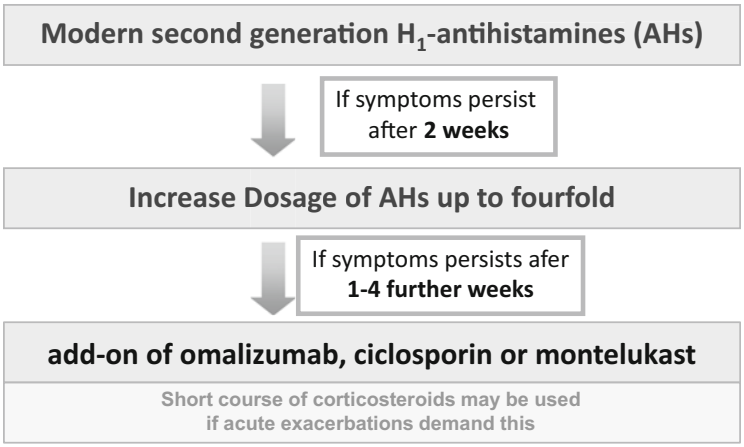

Fig. 23.11 Therapeutic algorithm for treating chronic spontaneous urticaria. (From Zuberbier et al. [42])

In principle, desensitization to eliciting triggers is possible in some types of inducible urticaria such as solar urticaria, cold urticaria, and cholinergic urticaria. However, this desensitization requires an ongoing self-provocation of the patients with their specific triggers to deplete urticaria-eliciting mediators, such as daily cold showers in cold urticaria or ongoing UV-treatment in solar urticaria, which is, for most patients, impossible to maintain over longer time periods.

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Chapter 24
Cutaneous Adverse Drug Reactions: Stevens–Johnson Syndrome and Toxic Epidermal Necrolysis

Riichiro Abe

Abstract Stevens–Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) are life-threatening cutaneous reactions caused by drugs or infections and exhibiting widespread epidermal necrosis. SJS/TEN might be a specific immune reaction initiated by cytotoxic T lymphocytes via an HLA-restricted pathway. Recent advances in pharmacogenomic studies have provided evidence for genetic predispositions to SJS/TEN. Toward explaining how drug-specific (immunological) reactions induce adverse drug reactions, several concepts have been proposed: in addition to the hapten concept and the p-i concept, there is the idea that drugs alter the antigen by binding to the HLA pocket. With regard to keratinocyte death, several cell death mediators, such as FasL and granulysin, have been proposed as playing a role in SJS/TEN pathogenesis. If SJS/TEN is simply the most extreme reaction on a spectrum of drug reactions, then regulating the immunological reaction could influence the severity of the adverse drug reaction. A subset of T lymphocytes, including regulatory T cells, also might play a role in SJS/TEN.

Keywords Hapten • Human leukocyte antigens (HLA) • p-i concept • Stevens–Johnson syndrome • T cell receptor • Toxic epidermal necrolysis

24.1 Introduction

Stevens–Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) are rare, life-threatening mucocutaneous reactions characterized by extensive detachment of the epidermis [37]. They are considered to lie on the same spectrum of diseases, but with different severities. SJS patients have skin detachment on less than 10 % of the body surface area, whereas TEN patients have more extensive lesions [5]. The overall incidences of SJS and TEN have been estimated at 1–6 cases per million person-years and 0.4–1.2 cases per million person-years, respectively. The

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mortality associated with TEN is 25 %. The eruptions initially distribute on the face, trunk, and extremities, but they can spread to the whole body in just a few hours. Mucous membrane involvement is observed in approximately 90 % of cases. Approximately 85 % of patients have conjunctival lesions. Ocular complications include chronic conjunctivitis, conjunctival scarring, corneal vascularization, and corneal damage, which can lead to blindness. Ocular morbidity and visual loss can be caused by acute corneal complications, and progressive conjunctival scarring is also significantly associated with subsequent loss of vision [11]. Several treatments have been attempted, including high-dose corticosteroids, intravenous immunoglobulin (IVIG), and plasmapheresis.

This chapter focuses on immunological phenomena, including antigen (causative drug) presentation, cytotoxic signaling, and immune molecule and T lymphocyte subtypes.

24.2 Interaction of HLAs, Drug Antigens, and T-Cell Receptors

Recent advances in pharmacogenomic studies have provided evidence for genetic predispositions to SJS/TEN. In particular, the strong genetic association between human leukocyte antigens (HLAs) and specific-drug–induced SJS/TEN [9] makes screening tests prior to drug intake practicable in preventing SJS/TEN [6]. However, we still do not fully understand how drug-specific (immunological) reactions induce SJS/TEN.

24.2.1 The Hapten/Pro-Hapten Concept

Because common skin-reaction–inducing drugs are small and, thus, not antigenic on their own, it is thought that their immunogenicity may result from binding of the drugs to other proteins [34]. Stable covalent binding by a chemically reactive drug to a protein is then able to form a neoantigen that can be recognized by T cells after antigen processing of the hapten–carrier complex (Fig. 24.1). The chemical properties of hapten-like drugs are crucial for the generation of antigenic epitopes and the activation of the innate immune system. Some haptens, such as beta-lactam antibiotics, tend to bind to particular amino acids; this has been well characterized for penicillin, which has a propensity to bind to lysine residues [47]. Drug hypersensitivity to a hapten–peptide complex is less likely to be HLA-restricted, as multiple binding sites in a protein imply that after processing, a number of potential drug-bound peptides are available for loading onto different types of HLA alleles. Indeed, there are no proven examples of a hapten-restricted immune response that is strictly associated with an HLA allele. The pro-hapten concept proposes that a

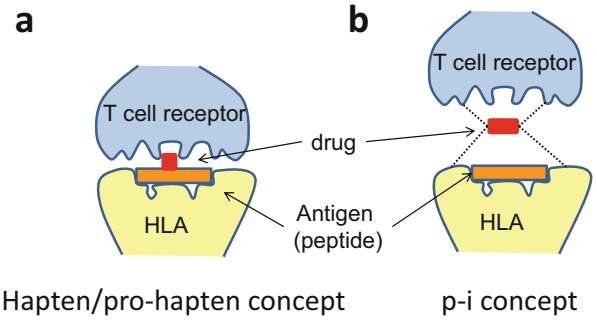

Fig. 24.1 (**a**) The hapten-modified peptide is recognized and stimulates T cells. The hapten may also have the ability to activate the innate immune system. (**b**) The drug binds to the TCR and provides some initial signal. The signal is strengthened by additional interaction with HLA molecules. Drug binding and HLA interactions stimulate T cells as does a normal peptide/HLA complex

chemically inert drug may become reactive after undergoing metabolism, when it is then able to form a hapten and stimulate an immune response [33]. A classic example of this is sulfamethoxazole, which forms a hapten after being metabolized to a reactive compound. It is important to note that neither hapten nor pro-hapten necessarily needs to undergo processing to become antigenic. This was illustrated by Horton et al. [16], who showed that amoxicillin, which forms a hapten by means of a covalent bond, is able to stimulate T cells in a processing-independent manner by binding directly to peptide–MHC complexes.

24.2.2 The p-i Concept

As an alternative to the hapten and pro-hapten concepts, the p-i concept proposes that a drug is able to stimulate T cells directly without forming a hapten, in an HLA-dependent manner [32]. This may occur if a drug that cannot form a covalent bond with a larger carrier interacts directly with T-cell receptors (TCR) or MHC molecules with sufficient affinity.

According to the hapten and pro-hapten concepts, drugs and other substances that are not chemically active and that are therefore incapable of coupling to a protein would not be antigens and could not induce hypersensitivity reactions. However, this hypothesis has been challenged by clinical and immunological evidence that cannot be explained by hapten or pro-hapten models [34].

Consistent with the p-i concept, chemically inert drugs, unable to bind covalently to peptides or proteins, can nevertheless activate certain T cells, if they fit with a sufficient affinity into some of the various T-cell receptors or MHC molecules (Fig. 24.1). Evidence for the p-i mechanism lies in observations in which fixed APC, which are unable to process antigens, are still able to activate specific T-cell

clones. Instead of a drug (hapten) specific immune response, the p-i–stimulated T cells arise from previously primed effector and memory T cells. In vivo, p-i–activated T cells expand and subsequently infiltrate the skin and other organs. This threshold of T-cell activation might be further lowered by the massive immune stimulation that concomitantly occurs during generalized herpes, human immunodeficiency virus (HIV) infections, autoimmune diseases, or malignant tumors. Such immune processes are associated with high cytokine levels and increased expression of MHC and costimulatory molecules. Consequently, T cells more readily react to a minor signal, such as the binding of a drug to its TCR. This would explain the high occurrence of drug hypersensitivities in these diseases. On the other hand, in some cases, metabolites simultaneously react as well as the parent compound. This suggests that the hapten characteristic of a drug is required for p-i stimulation to occur.

24.2.3 Affects of HLA Polymorphism on Drug Antigen Peptide Presentation

In general, peptides associate with HLA molecules by inserting parts of their amino-acid residues into a set of six binding pockets in the HLA [23]. The structure of these pockets is highly allele-specific, thereby dictating peptide-binding preferences for each HLA molecule.

A recent paper showed that a drug can bind directly to some pocket of a specific HLA, but that the drug does not bind to a closely related HLA molecule [17]. These data suggest that HLA and the drug form a complex before the HLA molecules are loaded with peptides inside the cell, thereby altering the pool of self peptides that are bound to the HLA and displayed on the cell surface for T-cell recognition. This shift in the specific HLA-associated cell-surface–peptide display leads to the activation of different T cells. Indeed, activation of a wide range of $CD8^+$ T cells occurs as the cellular basis of abacavir hypersensitivity reactions [7].

Part of the drug protrudes into the HLA molecule's pocket, reducing the pocket's size, which accounts for its preferential binding of smaller amino acids following drug exposure. The structures also revealed that the drug binds to the amino-acid residues that are unique to the HLA molecule, thereby explaining the drug's allele specificity. This shift in the bound-peptide repertoire is a plausible explanation for drug-induced hypersensitivity [36], T cells that lack self-reactivity but have the potential to recognize foreign antigens.

Some self peptides are never encountered during T-cell development, but exposure can occur under pathological conditions [7]. When this occurs, a situation of "mistaken identity" can arise, in which self peptides are perceived as foreign by the immune system.

In complexes that consist of immunogenic HLA, the drug and peptide may be generated either via incorporation of the drug with peptides of the constitutive

repertoire, or within the ER, such that the peptides are presented with an altered conformation, or the stabilization of "novel self" ligands may be absent from the constitutive repertoire but favored in the presence of the drug.

24.3 Cytotoxic Signals and Immune Molecules in SJS/TEN

The phenomenon of antigen (causative drug) presentation seems to be shared by SJS/TEN and nonsevere adverse drug reactions. Indeed, several HLA haplotypes that have been reported to be associated with adverse drug reactions do correlate with severity [46]. With regard to histological findings, an observation of keratinocyte death differentiates SJS/TEN from nonsevere adverse drug reactions. Therefore keratinocyte death may be implicated in the SJS/TEN pathomechanism.

24.3.1 Fas–FasL Interaction

In 1998, French and colleagues reported that the activation of the Fas apoptosis receptor through the Fas ligand (FasL) is an initial important step in keratinocyte apoptosis in TEN [45]. They assumed that both Fas and FasL derived from keratinocytes, and that the FasL expressed by keratinocytes leads to the apoptosis of keratinocytes in TEN [45]. The present author previously showed that the levels of soluble FasL (sFasL) in SJS/TEN patient serum are elevated, that sFasL is secreted by causal-drug–stimulated peripheral blood mononuclear cells, and that TEN patient serum with high levels of sFasL induces apoptosis in cultured keratinocytes [1].

Before disease onset (Day -4~Day -2), seven samples were available, and we detected the high concentrations of sFasL in five out of seven cases (71.4 %). The elevated sFasL level declined rapidly within 5 days after disease onset. In all 32 patients with ODSR and in the 33 normal controls, no elevation of sFasL was detected. Other soluble factor concentrations showed no significant difference for SJS/TEN before disease onset versus for nonsevere adverse drug reactions [29]. Lan also reported a diagnostic role for sFasL secretion by peripheral blood mononuclear cells from patients who had recovered from SJS/TEN [20]. On the other hand, other groups reported that elevated levels of FasL were detected not only in TEN patients but also in the sera and lesional skin of patients with the maculopapular type of adverse drug reactions [41]. Drug-induced hypersensitivity syndrome (DIHS), another severe adverse drug reaction, also shows high serum levels of sFasL [44].

24.3.2 Perforin and Granzyme B

Perforin/granzyme B have been reported to play a key role in keratinocyte death in SJS/TEN [30]. Nassif et al. showed that the cytotoxic effect of TEN blister lymphocytes on keratinocytes could be attenuated by the inhibition of perforin/granzyme B expression, but not by the anti-Fas monoclonal antibody [30]. The activated CTLs and NK cells produce perforin, which can bind and punch a channel to the target cell membrane and promote the entrance of granzyme B into keratinocytes. Once the granzyme B enters into the target cells, it activates the caspase cascade and the succeeding apoptosis [22]. Levels of perforin, granzyme B, TNF-alpha, and FasL have been observed to relate to disease severity of drug hypersensitivity, from mild maculopapular rashes to severe TEN [35].

24.3.3 Granulysin

Granulysin is a cytotoxic molecule that is produced against virus-infected cells, tumor cells, transplant cells, bacteria, fungi, and parasites [18]. It plays an important role in the host defense against pathogens. The 15-kDa granulysin, a cationic cytolytic protein, is secreted extracellularly by CTLs and NK cells via a nongranule exocytotic pathway [19]. The expression level of granulysin rises upon T and NK cell activation. Granulysin has been reported as a serum marker for cell-mediated immunity. Chung and his colleagues reported that granulysin is strongly expressed by blister cells in skin lesions and plays a crucial role in the widespread keratinocyte apoptosis of SJS/TEN [10]. Granulysin has a direct cytotoxic effect on keratinocytes at concentrations detected in the blister fluids. The cytotoxic effect of SJS/TEN blister fluids on keratinocytes can be reduced by granulysin depletion. In addition, injections of granulysin into mouse skin were found to result in blistering and epidermal necrosis mimicking SJS/TEN [10]. They concluded that high levels of secretory granulysin in blistering skin lesions could explain the histopathology observed in SJS/TEN, in which infiltration of sparse dermal mononuclear cells results in extensive epidermal necrosis. In addition, the serum levels of granulysin were found to increase during the early stage of SJS/TEN, but not in patients with drug-induced MPE [2], suggesting granulysin as an early diagnostic marker of SJS/TEN. Indeed the immunochromatographic test for granulysin (with a procedure time of less than 15 min) showed positive results for 4 out of 5 patients with SJS/TEN but only 1 patient out of 24 with nonsevere adverse drug reactions [12]. The results correlate closely with those of ELISA. On the other hand, DIHS also shows high serum levels of granulysin [38].

24.3.4 Other Cytokines/Chemokines

To date, several reports have shown cytokines/chemokines as being involved in the immune reactions of SJS/TEN. These cytokines/chemokines were found to have elevated expression in skin lesions, blister fluids, blister cells, peripheral mononuclear cells, or plasma in SJS/TEN. These cytokines/chemokines include IFN-g, TNF-a, IL-2, IL-5, IL-6, IL-10, IL-12, IL-13, IL-15, IL-18, CCR3, CXCR3, CXCR4, and CCR10 [8]. These cytokines/chemokines may be responsible for the trafficking, proliferation, regulation, or activation of T cells and other leukocytes involved in SJS/TEN. In addition, the α-defensin gene was recently found to be expressed in PBMCs from patients with cutaneous adverse drug reactions [26]. -α-Defensin expression was confirmed by intracellular flow cytometry in mononuclear cells from the patients, including monocytes, NK cells, and T cells from peripheral blood and blister fluid. α-Defensin levels were estimated by ELISA to be higher in blister fluid when compared to simultaneously drawn plasma samples.

24.4 Subtypes of T Lymphocytes

$CD8^+$ lymphocytes are necessary to SJS/TEN pathogenesis. Reports have shown that $CD4^+$ T cells are the predominant population that infiltrates into "maculopapular rash" skin lesions and that most drug-specific T cells are $CD4^+$ T cells [15]. However, in severe cutaneous adverse drug reactions, $CD8^+$ T cells were found to be the predominant population that infiltrated into the epidermis of skin lesions of SJS/TEN patients [10], and HLA-B*1502 was found to be associated with carbamazepine-induced SJS in all cases [9]. In addition, drug-specific $CD8^+$ T cells were found to proliferate predominantly during the acute stages of SJS/TEN [14]. Although drug-specific $CD4^+$ T cells are essential in drug-mediated immune reaction, $CD8^+$ T cells are critical to the development of SJS/TEN.

If SJS/TEN is the ultimate adverse drug reaction along the spectrum of nonsevere adverse drug reaction, then regulation of immunological reaction could influence the severity of the reactions. For example, immunocompromised individuals, such as patents who have AIDS or malignancy, or who were treated with immunosuppresive therapy, tend to be prone to SJS/TEN [24, 40].

24.4.1 Regulatory T Cells

The regulatory T cell (Treg) maintains self-tolerance and suppresses immune responses. Treg has been reported to be involved in the pathogenesis of SJS/TEN [4, 42]. Treg function is profoundly impaired in TEN, even though the cells are present in normal frequency [42]. These functional defects in TEN are restored

upon recovery. These findings indicate that transitory impairment in their function during the acute stage of TEN may relate to severe epidermal damage, whereas a gradual loss of their function after resolution of DIHS may increase the subsequent risk of autoimmune disease occurrence [42]. Indeed, in an animal model of TEN, Treg cells were found to prevent experimentally induced epidermal injury mimicking TEN [3, 4].

Recent reports have shown that $CD4^+Foxp3^+$ Treg exists in heterogeneous subpopulations divided by the level of CD45RA expression [25]. Human $CD4^+Foxp3^+$ Treg is divisible into three functionally and phenotypically distinct subsets: $CD4^+CD45RA^+$ Foxp3low resting Treg (rTreg), $CD4^+CD45RA^-$Foxp3high activated Treg (aTreg), and cytokine-secreting $CD4^+CD45RA^-$Foxp3low nonsuppressive T cells (non-Treg). rTreg and aTreg have potent immunosuppressive activity, whereas non-Treg lacks such activity and has the potential to secrete pro-inflammatory cytokines such as IFN-γ and IL-17 [25]. Furthermore, the relative frequencies of the three $CD4^+Foxp3^+$ Treg subpopulations differ with various disease conditions [31, 39, 48]: in active systemic lupus erythematosus, for example, the number of aTreg cells is lower and that of non-Treg cells is higher than those of healthy controls, enabling an autoimmune reaction [25]. SJS/TEN might also be considered susceptible to development under the imbalance of $CD4^+Foxp3^+$ Treg subpopulations. The relative frequencies of the three Treg subpopulations and cytokine-secreting activity are found to differ between SJS/TEN patients and healthy controls. These results indicate that the imbalance of Treg subpopulations is involved in the pathogenesis of SJS/TEN.

24.4.2 Th17 Cell

Th17 cells are a recently described effector $CD4^+$ T-cell subset that produces IL-17 and IL-22 and that has been implicated in the pathogenesis of various autoimmune and allergic diseases [21]. The proportion of circulating IL-17–producing $CD4^+$ T cells, but not $CD8^+$ T cells, is significantly higher in patients with SJS/TEN than in patients with erythema multiforme, as well as in healthy subjects [43]. IL-17–producing $CD4^+$ T cells in a CLA^+CCR4^+ subset with skin-homing properties are found in significantly higher proportion in this subset of patients with SJS/TEN [43]. The proportion of circulating Th17 cells decreases significantly after disease improvement. Collectively, these results suggest that skin-homing Th17 cells are involved in the pathogenesis of SJS/TEN. Th17 cells might be involved in inflammation and tissue damage in patients with SJS/TEN through regulation of the recruitment of neutrophils and other inflammatory leukocytes.

The percentages of Th17 tended to be high in SJS/TEN (2–6 days after onset) as compared to normal subjects and MPE patients [13].

24.4.3 CD94/NKG2C$^+$ CTLs

Cytotoxic T lymphocytes (CTLs) with NK-like activity (NK-CTLs) have been shown to express T-cell receptors restricted by the HLA-Ib molecule HLA-E [28]. Alternatively, the HLA-E-specific activating receptor CD94/NKG2C can trigger T-cell receptor-independent cytotoxicity in CTLs. In SJS/TEN lesions, keratinocytes from affected skin express HLA-E and that HLA-E sensitizes keratinocytes to killing by CD94/NKG2C$^+$ CTLs [27]. The frequencies of CD94/NKG2C$^+$ peripheral blood T and NK cells were found to be increased in patients with SJS/TEN during the acute phase. The Morel report indicates that CD94/NKG2C might be involved in triggering cytotoxic lymphocytes in patients with SJS/TEN.

24.5 Conclusion

SJS/TEN have been considered part of a spectrum of adverse cutaneous drug reactions showing severe and extensive skin detachment. However, a specific HLA haplotype, such as HLA-B*15:02, was reported as phenotype-specific, suggesting that other factors might be essential for exacerbation, and that fact clearly distinguishes SJS/TEN from nonsevere adverse drug reactions. SJS/TEN are "sudden disasters." As medical professionals, we have a responsibility to reveal their mechanisms and to establish diagnostic and therapeutic methods. Saving patients requires a perspective from both bench and bedside.

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Chapter 25
Pemphigus

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Abstract Pemphigus is a group of autoimmune bullous diseases of the skin and mucous membranes, which are characterized by IgG autoantibodies against desmogelin 3 (Dsg3) and/or Dsg1. Recent molecular biology techniques such as the phage display system allowed us to isolate monoclonal antibodies from peripheral blood of patients with pemphigus and to characterize their pathogenic activities and their epitopes on desmogleins. Through these studies, hot epitopes as well as a characteristic consensus sequence of CDR3 regions of pathogenic antibodies were identified. Concurrently, an active disease mouse model of pemphigus was developed by adoptive transfer of peripheral lymphocytes from Dsg3$^{-/-}$ mice. Dsg3-specific CD4^{+} T cells were isolated from pemphigus model mice and, subsequently, Dsg3-specific T cell receptor (TCR) transgenic mice were generated. These studies have revealed that Dsg3-specific CD4^{+} T cells were able to induce, not only antibody production with B cells and acantholysis, but also interface dermatitis. These studies have provided better understanding of pathophysiological mechanisms for pemphigus as well as deep insights for T-cell–mediated inflammatory skin diseases.

Keywords Autoimmunity • Autoantibodies • Autoreactive T cells • Tolerance • Pemphigus • Desmoglein • Mouse model

25.1 Introduction

Autoimmune bullous diseases are caused by autoantibodies leading to blister formation on the skin and in mucous membranes. These diseases fall into two basic categories; pemphigus, characterized by intraepidermal blisters, and pemphigoid, which causes subepidermal blisters. The main target antigen of autoantibodies in pemphigus is desmoglein (Dsg) 1 and 3, constituting components of desmosomes that play a crucial role in cell–cell adhesion in the epidermis. Autoantibodies in the pemphigoid subtype generally target proteins that compose

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essential parts of hemidesmosomes involved in dermal–epidermal cohesion (e.g., BP180, Type VII collagen).

Features of pemphigus diseases are intraepidermal blisters caused by acantholysis (i.e., loss of cell–cell adhesion of keratinocytes) and in vivo bound immunoglobulin (Ig) directed against the cell surface of keratinocytes. Pemphigus can be divided into three major subtypes: pemphigus vulgaris (PV), pemphigus foliaceus (PF), and paraneoplastic pemphigus (PNP). PV and PF are known as the classic forms of pemphigus. All PV patients have mucosal erosions, and more than half also have cutaneous blisters and erosions. PV blisters occur in the deeper part of the epidermis, just above the basal layer. On the other hand, PF patients have only cutaneous involvement without mucosal lesions, and the blister is in the superficial layer of the epidermis. PNP is generally linked to an underlying neoplasm, usually lymphoproliferative disorder.

Because many immunologically important findings have been provided from basic research on pemphigus, we chose to focus on major progress in recent pemphigus studies in this chapter.

25.2 Pathogenic Monoclonal Antibodies That Are Able to Induce Pemphigus Blisters

Currently, treatments for pemphigus include suppressing systemic immunity through the use of corticosteroid and immunosuppressive agents. However, autoantibodies against Dsg that cause blisters account for a small portion of the whole repertoire in patients with pemphigus, and innovative therapeutic strategies targeting only pathogenic autoantibodies are desirable. To achieve this goal, characterization of autoantibodies in pemphigus is essential to find a novel target for therapy. However, analyses of antibodies binding to Dsg in patients have been technically difficult due to various polyclonal antibodies responsible for the disease. Recent technology used to isolate monoclonal antibodies (mAbs) has facilitated investigation of antigen–antibody reaction features in pemphigus and contributed tremendously to elucidation of the pathophysiology of pemphigus.

Herein, we introduce new insights, focusing on research using mAbs in pemphigus in the first part of this chapter.

25.2.1 Epitopes on Desmogleins for Pathogenic Monoclonal Antibodies in Pemphigus

Upon detection of circulating autoantibodies against Dsg, autoreactive B cells recognizing Dsg are believed to contribute to the pathomechanism in patients with pemphigus. These cells differentiate into plasma cells that produce

autoantibodies against Dsg following activation. Several approaches have been introduced to isolate mAbs from patients with pemphigus, allowing for the analysis of autoantibodies derived from patients with active disease.

The phage display system is a useful technique to build an antibody library from pemphigus patients and isolate mAbs against Dsg (Fig. 25.1a). In the first step, mAbs with the single chain fragment variables (scFvs) form, composed of cDNA from the variable heavy chain and light chain regions of Ig, are amplified by reverse transcription polymerase chain reaction (RT-PCR) from mRNA extracted from B cells expressing Ig on the cell surface as a transmembrane receptor in the peripheral blood. These scFvs are randomly combined with heavy and light chains, and the entire repertoire is cloned into phagemid vectors. At this point, some scFvs are believed to reflect a combination of the variable heavy and light chain regions of autoreactive B cells recognizing Dsg in the patient among the innumerable repertoire of the scFv library. In the next step, scFvs binding to recombinant Dsg are selected from the library. Selected phages can be amplified by infection to *Escherichia coli*. Next, scFvs with a higher affinity to Dsg can be efficiently isolated with several steps of selection, or "panning". The pathogenicity of scFvs isolated from patients with PV and PF has already been shown by injection into organ-cultured human skin and neonatal mice [1, 2]. ScFvs are a monovalent form of mAbs comprising a single antigen-binding domain without FC effector function. Findings that scFvs can induce blisters as well as bivalent antibodies are important while considering the pathophysiology of pemphigus. As shown in previous studies using serum-derived Fab′ fragments, results are consistent with direct disruption of Dsg interactions by pemphigus autoantibodies, which is caused by "steric hindrance" [3].

From an adoptive transfer mouse model of pemphigus (details are in the latter part of this chapter), a pathogenic mAb against Dsg3 has been isolated using a cell fusion method [4]. Epitope analysis using Dsg1/Dsg3 domain-swapped molecules and point-mutated Dsg3 molecules revealed that the pathogenic mAb recognized the amino terminal extracellular domain of Dsg3 that is predicted to form the intercellular transadhesive interface, and nonpathogenic mAbs recognized the membrane proximal extracellular domain of Dsg3 (Fig. 25.1b). Another study isolated anti-Dsg3 mAbs using B cells immortalized by Epstein–Barr viral infection derived from the peripheral blood of a PV patient [5]. One of the pathogenic mAbs also bound to the amino terminal extracellular domain of Dsg3. Epitope mapping analysis using peptide libraries revealed that this pathogenic mAb bound to the part associated with *cis*-binding between neighboring Dsg3 molecules, predicted by positioning of homologous peptides in the C-cadherin crystal structure [6]. Results from a competition enzyme-linked immunosorbent assay (ELISA) using PV sera and this mAb suggested that the epitope can be shared with most sera of PV patients in the study (Fig. 25.1b).

Unlike many other autoantibody-mediated diseases, such as pemphigoid, in which the constant regions of antibodies are required for blister formation to activate complement, or bind FC receptors on inflammatory cells, the variable regions of antibodies are sufficient to cause blisters in pemphigus. Based on

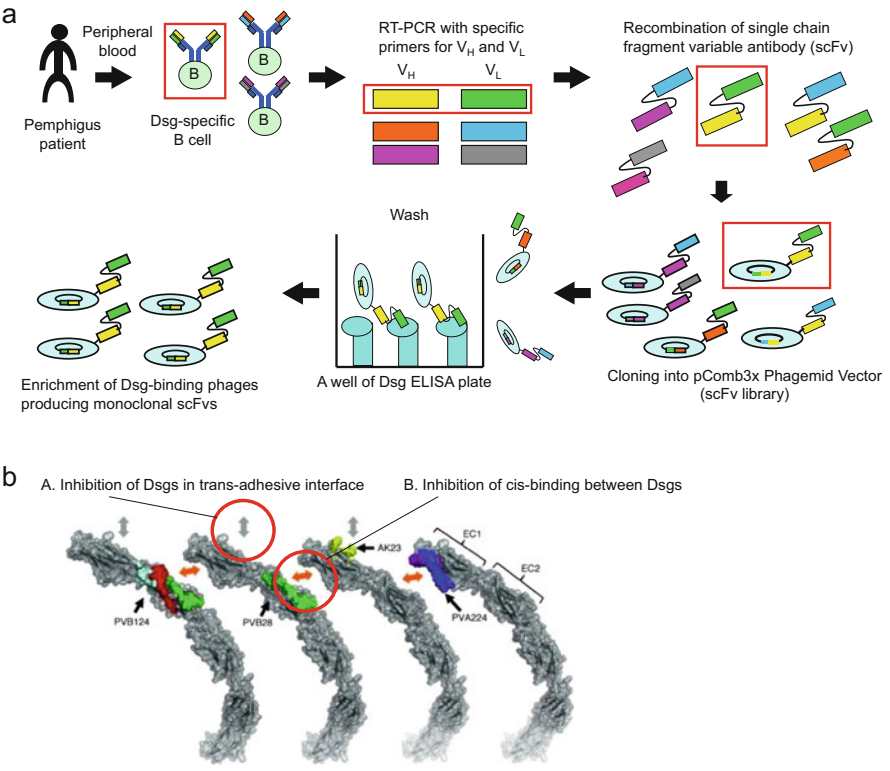

Fig. 25.1 (**a**) Schematic of construction of the scFv antibody library and isolation of monoclonal antibodies (mAbs) with specific binding to desmoglein (Dsg) using phage display. (**b**) Direct disruption of Dsg interactions by pemphigus autoantibodies (steric hindrance). AK23, a pathogenic mAb, binds to the transadhesive interface between Dsg molecules (*A*) and other mAbs, such as PVA224, PVB28, and PVB124, and reacts with *cis*-elements between neighboring Dsg molecules (*B*) [5]

cadherin ultrastructure (steric hindrance) and epitope mapping studies using mAbs, several reports have suggested that pathogenic PV and PF autoantibodies bind to calcium-sensitive, conformational epitopes in the amino terminal extracellular domains that form the key molecular interactions for Dsg intercellular adhesion [4, 7].

25.2.2 Common Sequences of CDR3 Region of Pathogenic Monoclonal Antibodies in Pemphigus and Their Binding Target on Hot Spot of Desmogleins

Several studies have been able to isolate mAbs against Dsgs. Inasmuch as mAbs are divided into two groups, pathogenic mAbs and nonpathogenic mAbs, there should be diversity in pathogenicity among autoantibodies in an in vivo situation [8]. It is sometimes observed that an ELISA titer is not necessarily associated with disease severities while following patients with pemphigus. As mentioned here previously, one of the factors determining pathogenicity is the epitope, or the part of the antigen where antibodies are binding. Further characterization of the structure of pathogenic mAbs will probably aid in the discovery of a promising target for a novel therapy in the future.

A series of mAbs against Dsg1 and Dsg3 in the form of scFv have been cloned from patients with PV and PF. Epitopes defined by pathogenic mAbs were shared by sera from various PV and PF patients, suggesting that pathogenic antibodies bind similar sites on Dsg and, therefore, might share common idiotypes. To investigate whether these shared idiotypes might be associated with homologous amino acid sequences in various pathogenic mAbs, variable regions of the mAbs were sequenced. Immunoglobulin has three complementarity determining regions (CDR1, CDR2, and CDR3) in each variable heavy chain and light chain region. Specifically, CDR3 in the heavy chain (H-CDR3), the junction of VH, D, and JH genes, varies the most in structure and plays a crucial role in determining the antigen specificity of an antibody (Fig. 25.2a, b), which is why H-CDR3 was focused on to determine a common motif among pathogenic mAbs. In six out of nine pathogenic clones, a consensus amino acid motif (D/E-X-X-X-W) was found in which an acidic amino acid (aspartic acid [D] or glutamic acid [E]) was arranged for amino acids upstream from a tryptophan (W) [9]. On the other hand, all 21 nonpathogenic mAbs previously cloned did not contain this H-CDR3 consensus motif.

To determine whether the H-CDR3 sequence must be unique for the binding activity and/or pathogenicity of each mAb, the H-CDR3 sequence of a pathogenic anti-Dsg1 mAb containing the consensus motif was randomized. Nine clones of Dsg1-binding mAbs derived from the mAb with H-CDR3 randomization were obtained. Based on ELISA results, all nine mAbs showed specific binding to Dsg1 and cell surface staining in the epidermis on indirect immunofluorescence using normal human skin. Injection into normal human skin organ culture showed that seven of the nine clones were pathogenic, indicating that a given H-CDR3 imparting pathogenicity to an anti-Dsg mAb was not necessarily unique for each mAb. These results also revealed that antibody binding to Dsgs can be uncoupled from pathogenicity. Although two pathogenic randomized clones shared the consensus motif D/E-X-X-X-W, others did not, suggesting that this consensus motif is not necessary for pathogenicity. However, all randomized clones contained at least one tryptophan (W) in the H-CDR3, suggesting the importance of W for mAbs that

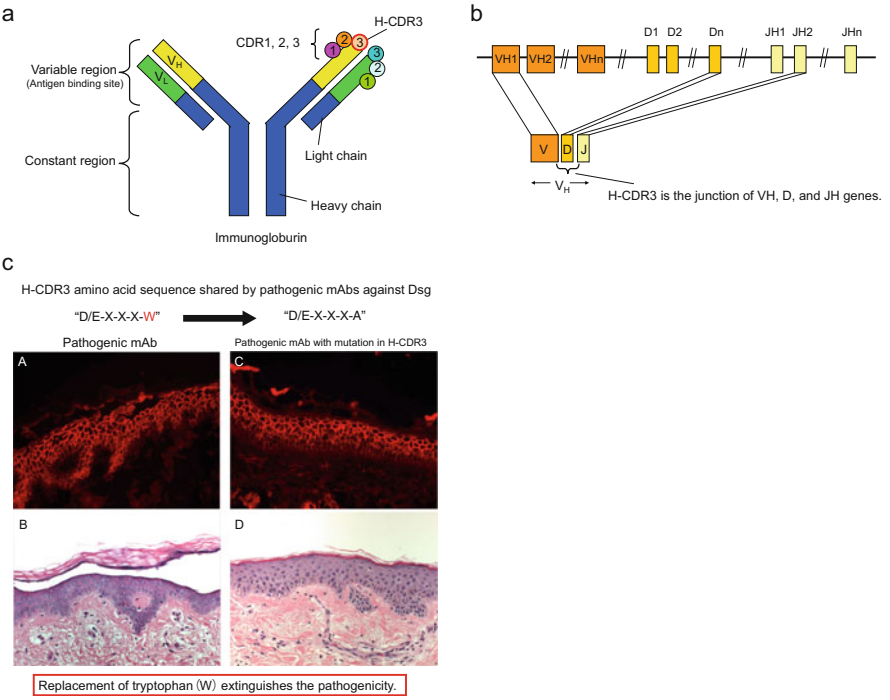

Fig. 25.2 (**a**, **b**) Complementarity-determining regions (CDR1, CDR2, and CDR3) in variable regions of the immunoglobulin chain. Specifically, CDR3 in the heavy chain (H-CDR3), the junction of VH, D, and JH genes, plays a crucial role in determining the antigen specificity of an antibody. (**c**) Pathogenicity impaired by a single amino acid mutation in H-CDR3. A pathogenic mAb had the consensus motif D/E-X-X-X-W in H-CDR3, and the tryptophan (*W*) was replaced with an alanine (*A*). The original pathogenic mAb without mutation caused blisters upon injection into human skin organ culture (*A*, *B*), whereas the mAb with the W replacement still bound to the epidermis, but lost pathogenicity (*C*, *D*) [9]

bind to Dsg1 and those that cause pathogenicity. To confirm the importance of W in the H-CDR3 of pathogenic mAbs, the W was mutated to an alanine (A) by site-directed mutagenesis. These changes inhibited the pathogenicity of mAbs but not their binding activities, confirming the importance of W for pathogenicity. These findings suggest that W in the H-CDR3 is critical for pathogenicity but not binding, which is in agreement with the randomization studies discussed above (Fig. 25.2c). It is believed that, in desmosomal cadherin, such as Dsg, W is critical in the trans-interaction and, thus, antibodies that interfere with binding may make use of a W through inhibition [6].

These observations suggest that autoantibodies have a "hot spot" that determines pathogenicity and, therefore, could be a novel therapeutic target.

25.3 Dsg3-Specific Autoreactive T Cells as a Commander in Chief in Pemphigus

Although the characteristics of pathogenic anti-Dsg3 autoantibodies were discussed above, it is also believed that autoreactive T cells are required to induce autoantibody production. The following evidence supports the commitment of autoreactive $CD4^+$ T cells in the pathogenesis of PV. Strong association of PV with certain HLA class II alleles including DRB1*0402 in Jewish and DQB1*0503 in non-Jewish individuals indicates HLA class II-restricted recognition of Dsg3 by $CD4^+$ T cells [10–14]. Mutations in CDR3 of anti-Dsg3 antibodies suggest $CD4^+$ T cell-dependent somatic hypermutation [1]. However, analysis of human Dsg3-specific T cells is technically difficult because there is no experimental system to elucidate whether analyzed T cells have the capacity to induce anti-Dsg3 antibody production and the PV phenotype in vivo through controlling Dsg3-specific B cells. Over 10 years ago, we reported the experimental mouse model of PV that paved the way for analyzing the pathogenicity of T cells in vivo and gave us new insights into the pathogenesis of pemphigus as well as inflammatory skin diseases [15].

25.3.1 Development of an Active Disease Mouse Model for Pemphigus

It was difficult to make a pemphigus mouse model by repetitive immunization of a wild-type mouse with recombinant Dsg3 protein; this was probably due to strictly regulated immune tolerance against Dsg3. Then, an autoantigen-deficient mouse was utilized to elicit an immune response against the autoantigen, as the deficient mouse does not establish immunological tolerance against the autoantigen. To generate a humoral autoimmune response against Dsg3 that causes pemphigus vulgaris, a $Dsg3^{-/-}$ mouse was immunized with recombinant Dsg3, thus producing an anti-Dsg3 antibody (Fig. 25.3a). Because the $Dsg3^{-/-}$ mouse does not express Dsg3 in the stratified squamous epithelium, the antibody produced does not bind the skin and oral mucosa or induce the PV phenotype. Splenocytes primed with rDsg3 are then transferred to a $Rag2^{-/-}$ mouse, physiologically expressing Dsg3. Transferred splenocytes begin to produce an anti-Dsg3 antibody, which binds to Dsg3 molecules in the skin and oral mucosa. Therefore, the recipient $Rag2^{-/-}$ mouse starts to show the PV phenotype, including weight loss and erosions on the face, where the mouse tends to scratch frequently, followed by gradual spreading to the whole body. Weight loss is believed to be caused by difficulty in eating; specifically, a suprabasilar acantholytic blister, a characteristic feature of PV, is observed in the pathology of the hard palate and esophagus in the recipient (Fig. 25.4a). In addition, IgG deposition on keratinocyte cell surfaces was detected by immunofluorescent staining and anti-Dsg3 IgG titer peaks at 2–3 weeks after transfer, which was confirmed by ELISA (Fig. 25.4a). Unlike PV patients, the PV mouse model

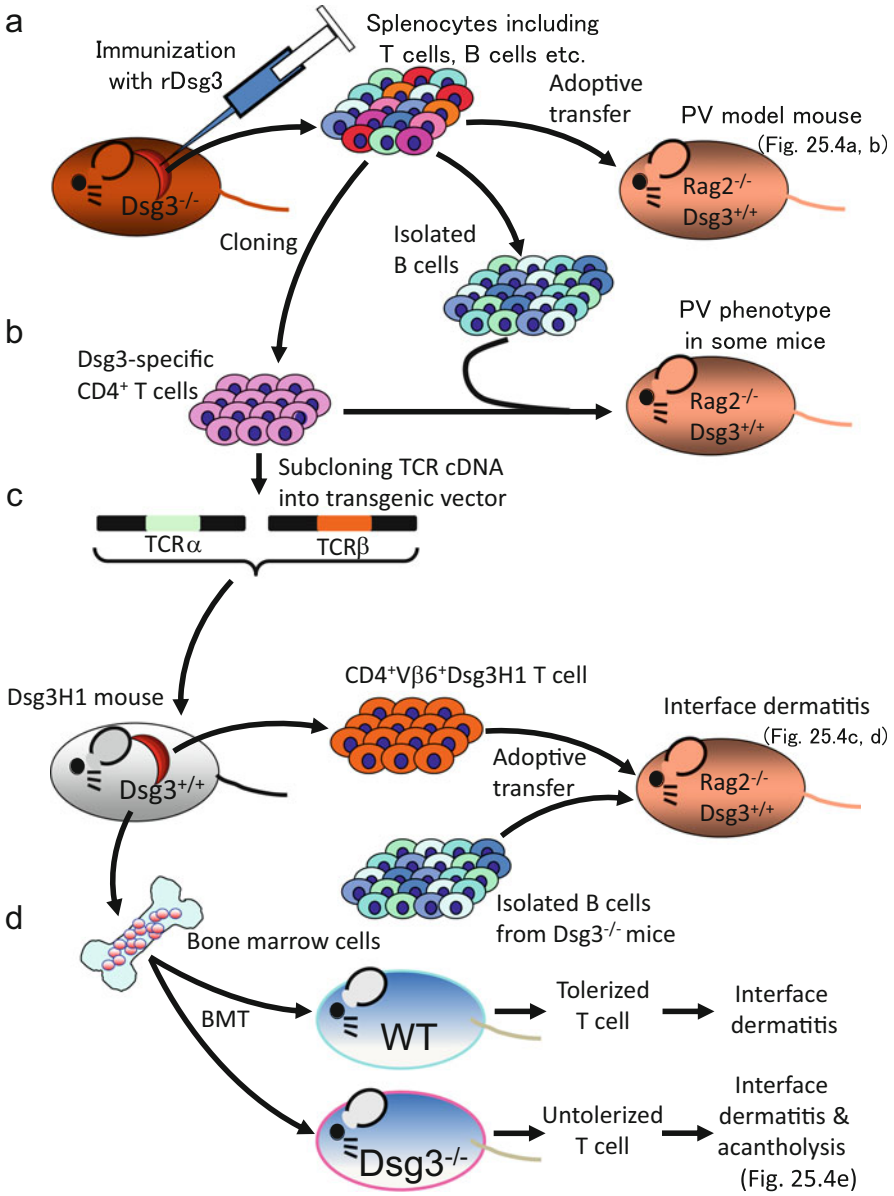

Fig. 25.3 Experimental outline. (**a**) Approach to make the PV mouse model. (**b**) Pathogenicity evaluation of Dsg3-specific T-cell clones. (**c**) Generation of Dsg3H1 mice and pathogenicity evaluation of transgenic T cells. (**d**) Bone marrow transfer (BMT) experiment and comparison between tolerized and untolerized transgenic T cells

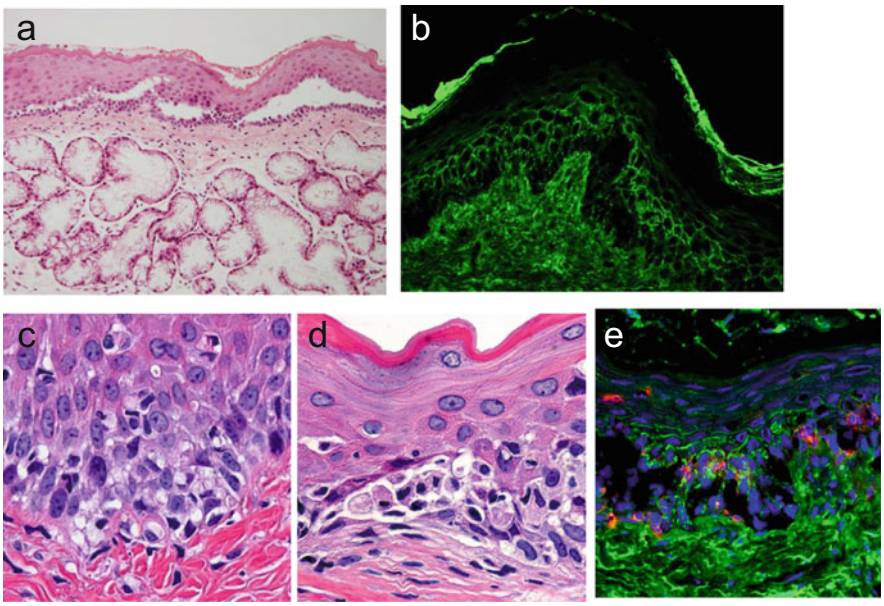

Fig. 25.4 (**a**) Histological study of the palate in the PV mouse model. H&E staining. (**b**) Direct immunofluorescence of the palate in the model. IgG is visualized in *green*. (**c**, **d**) Histological study of the palate in the recipient of Dsg3-specific transgenic T cells isolated from Dsg3H1 mice. Liquefaction degeneration and lymphocyte infiltration at the dermal–epidermal junction (**c**) and degenerated keratinocytes (**d**) are shown. (**e**) Direct immunofluorescence of the palate in the recipient of untolerized Dsg3-specific transgenic T cells and Dsg3$^{-/-}$ B cells. T cells are shown in *red*, IgG is shown in *green*, and the nucleus is shown in *blue*.

showed telogen hair loss caused by Dsg3 dysfunction that is strongly expressed in the follicular epithelium and contributes to cell adhesion during the telogen hair cycle.

25.3.2 Pathogenic Heterogeneity Among Dsg3-Reactive T-Cell Clones

Further investigation revealed that both T cells and B cells derived from Dsg3$^{-/-}$ mice are required to obtain the PV phenotype. When either T cells or B cells are derived from a wild-type mouse, the recipient mouse does not show the PV phenotype, revealing that transferred T cells and B cells must contain the pathogenic population with the ability to induce anti-Dsg3 antibodies through T–B interaction for PV phenotype induction [16]. Utilizing this result, we sought to evaluate the pathogenicity of Dsg3-reactive T cell clones established from rDsg3-immunized Dsg3$^{-/-}$ mice (Fig. 25.3b) [17]. Seven of 20 T-cell clones induced the PV phenotype after adoptive transfer with Dsg3$^{-/-}$ B cells, and the residual clones

did not, demonstrating "pathogenic heterogeneity;" there are pathogenic and nonpathogenic T cells for inducing the PV phenotype, although all are reactive to Dsg3. By comparing cytokine expression patterns between pathogenic and nonpathogenic clones, IL-4 and IL-10 were identified as significantly expressed cytokines in pathogenic clones. Furthermore, a cytokine blockade study demonstrated that IL-4 neutralization suppressed the severity of the PV phenotype induced by transfer of a pathogenic T-cell clone and $Dsg3^{-/-}$ B cells, and IL-4 plays critical roles in PV phenotype induction in mice. In a study on PV patients, Dsg3-reactive T cells from peripheral blood mononucleocytes (PBMCs) in the active phase of PV significantly expressed IL-4, but not during remission or in Dsg3-reactive T cells from normal healthy controls, consistent with mouse studies [18].

25.3.3 Dsg3-Specific T Cells Induce Not Only Pemphigus but Also Interface Dermatitis, a Skin Inflammatory Disease with Unknown Etiology

cDNA coding Dsg3-specific TCRα8 and β6 chains derived from a Dsg3-reactive T-cell clone were utilized to generate a Dsg3-specific TCR transgenic mouse (Dsg3H1 mouse, Fig. 25.3c) [19]. In the Dsg3H1 mouse, approximately 30% of $CD4^+$ T cells in the spleen and lymph nodes (LNs) express the TCRβ6 chain. The transgenic TCR reconstituted in T cell hybridoma cell was able to recognize the Dsg3 peptide (amino acids [aa] 301–315 [RNKAEFHQSVISQYR]), and the transgenic T cells (Dsg3H1 T cells) from the mouse were, in fact, reactive to the peptide. Because the Dsg3H1 mouse expresses Dsg3 in the skin, only a part of $CD4^+$ T cells expressed the TCRβ6 chain, probably due to immunological tolerance. However, there has not been direct evidence that immunological tolerance functions against Dsg3. To demonstrate the existence, we made a bone marrow (BM) chimera mouse by transferring Dsg3H1 BM into a $Dsg3^{-/-}$ or wild-type (WT) mouse (Fig. 25.3d). In the recipient WT mouse, only a portion of $CD4^+$ T cells expressed the TCRβ6 chain, similar to the Dsg3H1 mouse itself. On the other hand, almost all $CD4^+$ T cells expressed the TCRβ6 chain in the recipient $Dsg3^{-/-}$ mouse. These results indicate that Dsg3-specific T cells can fully develop in the absence of Dsg3, but there is a developmental limitation in the presence of Dsg3; this clearly demonstrates the existence of immunological tolerance against Dsg3 in mice.

Upon making BM chimera mice, two different types of transgenic T cells became available: tolerized in the presence of Dsg3, and untolerized in the absence of Dsg3 (Fig. 25.3d). To understand the pathogenicity of these transgenic T cells, each type of T cell was transferred into $Rag2^{-/-}$ with $Dsg3^{-/-}$ B cells. The recipient mouse with the tolerized T cells started to show redness and become scaly in the skin on the face, limbs, and tail around 1 week after adoptive transfer. Histological examination of the lesion showed T-cell infiltration in the dermal–epidermal junction, liquefaction degeneration, and degenerated change of

keratinocytes. There was no acantholytic change in the recipient mouse or anti-Dsg3 IgG production, and IgG deposition on the keratinocyte cell surface was not detected. Transgenic T cells directly isolated from Dsg3H1 mice also induced the same skin phenotype in recipient $Rag2^{-/-}$ mice after adoptive transfer (Fig. 25.4c, d). These results demonstrated that tolerized transgenic Dsg3-specific T cells have the ability to induce the cellular immune response, characterized as interface dermatitis (Fig. 25.3d). Compared to the tolerized cells, untolerized transgenic T cells induced a different phenotype after transfer to $Rag2^{-/-}$ mice. These cells directly infiltrated into the dermal–epidermal junction and induced interface dermatitis, and also induced anti-Dsg3 antibody production and acantholytic blisters. These findings were also confirmed by a direct immunofluorescence study (Fig. 25.4e). Therefore, both interface dermatitis and acantholytic blisters were observed simultaneously. Combination of the two different phenotypes is one of the characteristic features in paraneoplastic pemphigus (PNP), in which the pathomechanism of the cellular immune response observed in PNP is completely unknown. However, this unexpected result suggests that interface dermatitis, seen in PNP, may result from the cellular immune response against Dsg3. In addition, some of the skin diseases showing interface dermatitis might be mediated through an autoimmune mechanism.

Although interface dermatitis is commonly observed in the skin pathology of various diseases, the molecular mechanism underlying the pathological change is poorly understood. A retroviral system made to transduce Dsg3H1 TCR in T cells was used to prepare WT, IFN-$\gamma^{-/-}$ and IL-$17a^{-/-}$ Dsg3-specific T cells. After adoptive transfer into $Rag2^{-/-}$ mice, WT and IL-$17a^{-/-}$ Dsg3-specific T cells induced interface dermatitis, but IFN-$\gamma^{-/-}$ Dsg3-specific T cells did not. Therefore, interface dermatitis induced by Dsg3-specific T cells was IFN-γ–dependent. Previously, interface dermatitis was considered a pathology mediated by $CD8^+$ T cells. However, these results clearly demonstrated that $CD4^+$ T cells alone can induce interface dermatitis, and that IFN-γ, but not IL-17a, is critical in the pathophysiology.

25.4 Concluding Remarks

This chapter focused on recent research progress of B- and T-cell studies in pemphigus. Among many autoimmune bullous diseases, pemphigus has been the most deeply dissected and has provided new concepts in the field of autoimmune diseases. However, the exact pathomechanism that answers the question as to why autoantibodies are produced is still unknown. We hope that further investigation of autoimmune bullous diseases will be helpful for understanding autoimmune diseases and establish promising treatments for patients.

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Chapter 26
Anaphylaxis

Eishin Morita

Abstract Anaphylaxis was first recognized and named in 1902 by Charles Robert Richet and Paul Portier. The definition of anaphylaxis was established with the consensus of several task forces in Europe and in the World Allergy Organization (WAO). Anaphylaxis is a rapid-onset systemic reaction involving multiple organ systems. The most frequent causes are foods, insect venom, and drugs. The mechanism of anaphylaxis is basically an IgE-mediated allergic reaction; mediators released from mast cells and basophils elicit the symptoms in various organs. The prevalence of anaphylaxis symptoms are as follows: urticaria and/or angioedema (85–90 %); flush (45–55 %); dyspnea and/or wheezing (45–50 %); swelling of the upper airways (50–60 %); vertigo, syncope, and/or hypotension (30–35 %); nausea, vomiting, diarrhea, and/or cramps (25–30 %); loss of consciousness (22 %); and rhinitis (15–20 %). In cases of food-dependent exercise-induced anaphylaxis (FDEIA), exercising and/or nonsteroidal anti-inflammatory drugs (NSAIDs) are common triggers of anaphylaxis. These triggers elicit symptoms within a few hours of eating the culprit food by enhancing allergen absorption from the gastrointestinal tract. The basic initial management of anaphylaxis is calling for assistance and injecting epinephrine intramuscularly. Lying supine with the lower extremities elevated, using supplemental oxygen and intravenous fluid resuscitation are also necessary.

Keywords Anaphylaxis • IgE • IgE-mediated allergic reaction • Mast cells • Basophils • Histamine • Food-dependent exercise-induced anaphylaxis (FDEIA) • Nonsteroidal anti-inflammatory drugs (NSAIDs)

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26.1 History of Anaphylaxis

The path to the discovery of anaphylaxis can be retraced to 1901 in the Principality of Monaco [1]. On the orders of Albert Grimaldi, Prince of Monaco, who had a perpetual passion for the sea, Charles Robert Richet and Paul Portier studied the adverse effects of stings by the tentacles of the gelatinous invertebrates, *Physalia*. They were able to confirm that *Physalia* tentacles contain a paralyzing poison, which they named as "hypnotoxin". On the basis of this observation, they extended their experiment to developing a vaccine against a toxin from the sea anemone, *Actinia sulcata*, which was later named "actinotoxin", by administering the toxin into dogs. The trial with dogs was astonishing, because all of the dogs collapsed and died within a few minutes after the repeat administration of doses as small as 0.1 mL/kg. In 1902, they reported their findings and coined the term "aphylaxis", which was subsequently changed to "anaphylaxis".

Anaphylaxis was formerly recognized as an IgE-mediated allergic reaction. However, the following definition of anaphylaxis was recently established as a result of the consensus of several task forces in Europe and in the World Allergy Organization (WAO): "a serious, life-threatening generalized or systemic hypersensitivity reaction" and "a serious allergic reaction that is rapid in onset and might cause death" [2].

26.2 Epidemiology and Symptoms of Anaphylaxis

26.2.1 Epidemiology

Anaphylaxis is not rare, and the rate of occurrence appears to be increasing [3]. Although numerous studies have estimated the occurrence rate of anaphylaxis, the true global occurrence rate from all triggers in the general population has not been established, mainly because patients are often seen by different medical specialists (e.g., emergency doctors, allergists, or other clinicians). The lifetime prevalence based on international studies is estimated to be 0.05–2 % [4]. The most frequent causes of anaphylaxis are foods, insect venom, and drugs [4]. The precise frequencies of the causes vary depending on age, geographical regions, and the data source (e.g., emergency doctors and allergists). Food allergens are the most frequent cause of anaphylaxis in children, and venom is the most frequent cause in adults. Among drugs, antibiotics, nonsteroidal anti-inflammatory drugs (NSAIDs), and biologic agents are the most common causes of anaphylaxis.

26.2.2 Symptoms

Anaphylaxis is a systemic reaction involving multiple organ systems in humans. A major characteristic of anaphylaxis is the rapid onset of symptoms, mostly within 1 or 2 h, after the allergen exposure. Symptoms involve the skin, mucosa, respiratory system, gastrointestinal tract, cardiovascular system, and central nervous system. The symptoms of anaphylaxis are summarized in Box 26.1. The prevalence of symptoms in anaphylactic reactions is as follows: urticaria and/or angioedema (85–90 %); flush (45–55 %); dyspnea and/or wheezing (45–50 %); swelling of the upper airways (50–60 %); vertigo, syncope, and/or hypotension (30–35 %); nausea, vomiting, diarrhea, and/or cramps (25–30 %); loss of consciousness (22 %); and rhinitis (15–20 %) [4]. Anaphylaxis is diagnosed on the basis of clinical findings. Box 26.2 shows the clinical criteria for diagnosing anaphylaxis established by the WAO [2].

The above-mentioned symptoms are sometimes followed by an asymptomatic period of 1 h or more and a subsequent return of symptoms without further exposure to the allergen; this is called a biphasic anaphylactic reaction. The reported incidence of the biphasic reactions ranges from 3 to 20 % in the literature [5].

Box 26.1: Symptoms of Anaphylaxis

Skin and mucosa

- Flushing, urticaria, angioedema, erythema, and itching (skin)
- Conjunctival erythema and tearing (eyes)
- Itching of lips, tongue, and palate, and swelling of lips, tongue, and uvula (oral mucosa)

Respiratory system

- Itching, congestion, rhinorrhea, and sneezing (nose)
- Itching and swelling (throat)
- Dyspnea sensation, cough, increased respiratory rate, wheezing, cyanosis, and respiratory arrest (chest)

Gastrointestinal tract

- Abdominal pain, nausea, vomiting, and diarrhea

Cardiovascular system

- Chest pain, tachycardia, hypotension, shock, and cardiac arrest

Central nervous system

- Vertigo, headache, and loss of consciousness

Box 26.2: Clinical Criteria for Diagnosing Anaphylaxis [2]
Anaphylaxis is highly likely when any one of the following three criteria is fulfilled,

1. Acute onset of an illness (minutes to several hours) with involvement of the skin, mucosal tissue, or both (e.g., generalized urticaria, itching or flushing, swollen lips-tongue-uvula)
   and at least one of the following,

   (A) Respiratory compromise (e.g., dyspnea, wheeze-bronchospasm, stridor, reduced peak expiratory flow (PEF), hypoxemia)
   (B) Reduced blood pressure or associated symptoms of end-organ dysfunction (e.g., hypotonia [collapse], syncope, incontinence)

   OR
2. Two or more of the following that occur rapidly after exposure to *a likely* allergen[1] for that patient (minutes to several hours),

   (A) Involvement of the skin–mucosal tissue (e.g., generalized urticaria, itch-flush, swollen lips-tongue-uvula)
   (B) Respiratory compromise (e.g., dyspnea, wheeze-bronchospasm, stridor, reduced PEF, hypoxemia)
   (C) Reduced blood pressure or associated symptoms (e.g., hypotonia [collapse], syncope, incontinence)
   (D) Persistent gastrointestinal symptoms (e.g., crampy abdominal pain, vomiting)

   OR
3. Reduced blood pressure after exposure to *known* allergen[2] for that patient (minutes to several hours)

   (A) Infants and children: low systolic blood pressure (age-specific) or greater than 30 % decrease in systolic blood pressure[3]

(continued)

[1] Or other trigger, for example, immunologic but IgE-independent, or nonimmunologic (direct) mast cell activation.

[2] For example, after an insect sting, reduced blood pressure might be the only manifestation of anaphylaxis; or, in a similar example, during allergen immunotherapy, after injection of a known allergen for that patient, generalized urticaria (only one body organ system affected) might be the only initial manifestation of anaphylaxis.

[3] Low systolic blood pressure for children is defined as less than 70 mmHg from 1 month to 1 year, less than (70 mmHg + [2 × age]) from 1 to 10 years, and less than 90 mmHg from 11 to 17 years. Normal heart rate ranges from 80 to 140 beats/min at age 1–2 years; from 80 to 120 beats/min at age 3 years; and from 70 to 115 beats/min after age 3 years. Infants are more likely to have respiratory compromise than hypotension or shock, and in this age group, shock is more likely to be manifest initially by tachycardia than by hypotension.

Box 26.2 (continued)

(B) Adults: systolic blood pressure of less than 90 mmHg or greater than 30 % decrease from that person's baseline

26.3 Mechanisms of Anaphylaxis

The pathogenesis of anaphylaxis includes immunologic and nonimmunologic reactions; the former is categorized as IgE-mediated and non-IgE-mediated reactions [2]. Nonimmunologic mechanisms include direct mast cell activation by factors such as physical exercise, ethanol, and medications. The IgE-mediated reaction has been thoroughly investigated. Exogenous allergens recognized by antigen-presenting cells in immune systems are introduced to helper T-lymphocytes (Th) as processed allergens. The Th cells subsequently differentiate into various types of effector cells such as Th1, Th2, Th9, and Th17 cells under the influence of several cytokines, chemokines, costimulatory signals, and regulatory T-cells. The expansion of allergen-specific Th2 cells results in the production of interleukin (IL)-4 and IL-13, which induce immunoglobulin class switching to IgE as well as the clonal expansion of naïve and IgE+ memory B-lymphocyte populations. The production of allergen-specific IgE is genetically influenced; this constitution is called atopic diathesis.

Allergen-specific IgE binds to high-affinity IgE receptors (FcεRI) on mast cells and basophils in a process called sensitization. Individuals exhibit no reactions during the sensitization stage. When IgE bound to FcεRI crosslinks with the bivalent or multivalent allergen, mast cells and/or basophils are activated. FcεRI comprises an α subunit, a β subunit, and two identical γ subunits, which are associated with nonreceptor protein tyrosine kinases (e.g., Lyn and Fin). The crosslinking of IgE initiates the activation of these kinases, resulting in a series of phosphate transfer events in mast cells and basophils. These events lead to the secretion of three categories of functional substances: (1) the extracellular release of preformed mediators in cytoplasmic granules, including proteases and vasoactive amines such as histamine; (2) the synthesis and release of lipid mediators (e.g., prostaglandin D_2, leukotriene C_4, and leukotriene B_4); and (3) the production of cytokines (e.g., tumor necrosis factor-α and IL-4).

Histamine was the first recognized and is the most important mediator of anaphylaxis. The actions of histamine in anaphylaxis are dominantly mediated by binding to H1 receptors on target cells such as the postcapillary venule endotherial cells, nerve fibers, and smooth muscle cells. The binding of histamine to the H1 receptor results in the dilatation and increased permeability of capillaries, pruritus, and smooth muscle contraction. Local histamine release in the tissues causes symptoms manifesting in the skin, mucosa, respiratory system, and gastrointestinal tract. Meanwhile, systemic histamine release causes symptoms manifesting in the

cardiovascular system and central nervous system (Box 26.1). Tissue mast cell activation is the main source of local histamine release, but basophil activation may be involved in systemic histamine release.

Histamine H2 receptor plays a major role in gastric acid secretion. The H2 receptor is suggested to be involved in the pathogenesis of anaphylaxis from findings obtained by a blocking study. A study of pretreatment with H1 and H2 antihistamines found that both H1 and H2 receptors mediate flushing, hypotension, and headache [6]. Although histamine H3 and H4 receptor are implicated in animal models of anaphylaxis, their roles in human anaphylaxis have not been studied [7–9].

Lipid mediators released from mast cells and basophils are other contributors to the pathogenesis of anaphylaxis. Prostaglandins and leukotrienes are synthesized de novo by the activation of these cells and considered to be mainly involved in late-phase reaction of anaphylaxis because of their pro-inflammatory effects: prostaglandin D_2 causes vasodilation and increases vascular permeability [10], leukotriene C_4 is a potent bronchoconstrictor [11–13], and leukotriene B_4 is a potent chemotactic factor for neutrophils and eosinophils [14].

Mast-cell–derived cytokines such as tumor necrosis factor-α and IL-4 possibly contribute to the pathophysiology of anaphylaxis via direct and/or indirect effects on target tissues. It remains to be determined whether IgG-mediated anaphylaxis exists in humans, although IgG-mediated pathways have been established in mouse anaphylaxis models [15, 16].

The pathogenesis of biphasic anaphylaxis may be mediated by the action of mast-cell–derived mediators. In the early phase of anaphylaxis, preformed mediators in the cytoplasmic granules, including proteases and histamine, play major roles in symptoms by increasing vascular permeability and smooth muscle contraction. In the late phase of anaphylaxis, lipid mediators (e.g., leukotriene B4) and cytokines play crucial roles in causing inflammatory cell infiltration; these cells include eosinophils, basophils, and lymphocytes, which are responsible for the symptoms observed in the second phase of the anaphylactic reaction.

26.4 Food-Dependent Exercise-Induced Anaphylaxis

Food-dependent exercise-induced anaphylaxis (FDEIA) is a special type of food allergy in which anaphylaxis occurs in response to physical exercise within a few hours of eating. The pathogenesis of FDEIA is basically an IgE-mediated immediate-type food allergy triggered by exercising. NSAIDs such as aspirin are another important trigger, and the ingestion of NSAIDs may precede eating the culprit food. The triggering factors of FDEIA include general conditions, alcohol, and atmospheric conditions. The most common culprit foods are wheat, other grains, and nuts in Western populations, and wheat and shellfish in Asian populations. A variety of culprit foods has been reported, including vegetables, fruits, nuts, eggs, mushrooms, corn, garlic, pork/beef, rice, and cow milk [17].

The general understanding of the mechanisms by which exercise induces the allergic reaction involves the enhancement of the absorption of food allergens from the gastrointestinal tract after ingesting the culprit food. Serum levels of food allergens (gliadin, one of the main wheat proteins) have been evaluated in the patients with wheat-dependent exercise-induced anaphylaxis during wheat-challenge tests using a gliadin-specific sandwich enzyme-linked immunosorbent assay [18, 19]. The results revealed immunoreactive gliadins in patient sera during positive challenge tests with exercise. The measured gliadin levels corresponded to allergic symptoms. The exercise-induced enhancement of gliadin absorption was even observed in healthy subjects in whom serum gliadin levels were monitored before and after eating wheat; this indicates that the exercise-induced enhancement of allergen absorption occurs not only in allergic patients, but in healthy subjects as well [18].

Symptoms are often triggered or precipitated by NSAID administration in patients with FDEIA [20–22]. This has been observed during challenge tests with aspirin even in patients with FDEIA who have no history of aspirin hypersensitivity or in the precipitation of symptoms by NSAIDs. NSAIDs induce symptoms possibly because they also enhance allergen absorption through the intestinal epithelium into circulating blood. In addition, serum gliadin levels have been reported to increase in combined challenge tests with wheat and aspirin [18, 19]. The aspirin-induced enhancement of allergic symptoms is possibly because cyclooxygenase is inhibited, in as much as the symptoms can be diminished by the coingestion of synthetic prostaglandin E1 [23].

26.5 Management of Anaphylaxis

Prompt assessment and management are critical [2]. The basic initial management of anaphylaxis is calling for assistance and injecting epinephrine intramuscularly (adrenaline: 0.01 mg/kg up to a maximum of 0.5 mg for adults and 0.3 mg for children). Epinephrine autoinjectors are used on patients with a high risk of anaphylaxis. These can be used by the patients themselves for self-medication. Lying supine with lower extremities elevated, using supplemental oxygen, and providing intravenous fluid resuscitation are also necessary. Second-line medications include H1 and H2 antihistamines, β-2 adrenergic agonists, and glucocorticoids.

To prevent the recurrences of anaphylaxis, strict avoidance of the specific cause is recommended after confirmation. Furthermore, immunomodulation therapy with relevant insect venom(s) is available to prevent the recurrence of stinging insect anaphylaxis [24–26].

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Chapter 27
Graft Versus Host Disease (GVHD)

Fumi Miyagawa and Stephen I. Katz

Abstract Graft-versus-host disease (GVHD), the major complication of allogeneic hematopoietic cell transplantation (HCT), remains lethal and limits the use of this therapy. Acute GVHD and chronic GVHD involve distinct pathological processes. Acute GVHD has strong inflammatory components, whereas chronic GVHD displays more autoimmune and fibrotic features. Acute GVHD begins with tissue damage to the recipient by conditioning regimens that release pro-inflammatory cytokines and gut bacteria and activate antigen-presenting cells (APCs). Following antigen presentation, a strong cytokine response is initiated, promoting greater antigen presentation and recruitment of effector T cells, and innate immune cells further contributing to the inflammatory cytokine milieu. Finally, effector T cells, NK cells, and pro-inflammatory cytokines will result in end-organ damage, clinically recognized as acute GVHD in the skin, gut, and liver. In contrast, one of the hallmarks to chronic GVHD includes damage to the thymus that is associated with the conditioning regimen and the acute GVHD resulting in decreased negative selection of alloreactive CD4 T cells. Dysregulation of B cells has also been implicated as another hallmark that leads to emergence of autoreactive B cells and production of autoreactive antibodies. It has also been proposed that Th2 cytokine pattern deviation may be involved that induces release of fibrogenic cytokines and macrophage activation followed by tissue fibroblast proliferation and activation. These will result in an autoimmune-like systemic syndrome that is mainly associated with fibroproliferative changes and that can occur in almost any organ in the body. In this chapter, we review the recent advances in understanding the pathophysiology of GVHD as well as its diagnosis.

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Keywords Graft-versus-host disease • Acute GVHD • Chronic GVHD • Allogeneic hematopoietic cell transplantation • HLA-incompatibility • Donor • Recipient • Host • Conditioning regimen

27.1 Introduction

Allogeneic hematopoietic cell transplantation (HCT) is the only curative option for many hematological malignancies. The number of HCTs continues to increase with more than 25,000 allogeneic transplantations performed annually. Allogeneic transplantation is in widespread use for hematologic malignancies, but is also increasingly used for nonneoplastic marrow failure syndromes, inborn errors of metabolism, autoimmune diseases, and immunodeficiency syndromes. GVHD accounts for the major cause of morbidity and non-relapse–related mortality associated with allogeneic HCT [23, 29, 53, 83]. The development and severity of GVHD depends on a number of transplant-related factors such as HLA-incompatibility, recipient age, female donor/male recipient, toxicity of the conditioning regimen, and hematopoietic graft source [7, 23, 53]. Nevertheless, the degree of HLA-mismatch between donor and recipient remains the single most important predictor of GVHD [62]. Whereas acute GVHD is induced by recognition of host tissues as foreign by immunocompetent donor T cells that are transferred along with the allograft, the pathogenesis of chronic GVHD is not as well understood. An unresolved challenge in the transplantation field is selectively to limit GVHD without abrogation of the desired graft-versus-tumor (GVT) effect.

Three requirements necessary for GVHD were proposed [6]. First, the transplanted graft must contain immunologically competent cells; second, the recipient must express tissue antigens that are not present in the transplant donor and thus be recognized as foreign; third, the recipient must be incapable of rejecting the transplanted cells. The immunocompetent cells are now well known to be mature T cells in the graft, which target the major histocompatibility complex (MHC) expressed on host tissues. The second requirement for GVHD, the expression of recipient tissue antigens not present in the donor, became the focus of intensive research with the discovery of MHC. Although MHC differences between donor and recipient are the most important risk factor for the induction of GVHD, GVHD still develops in recipients of MHC-identical transplants. In this setting, minor histocompatibility antigens are thought to be responsible for the development of GVHD. The third requirement for GVHD stipulates that the recipient must be immune compromised and thus unable to reject the donor cells; a normal immune system will usually reject T cells from a foreign donor and thus prevent GVHD. Immunocompromise commonly follows allogeneic HCT, as patients have usually received immune-ablative doses of chemotherapy and/or radiation before stem cell infusion [23, 29, 31, 53].

27.2 Clinical Manifestations

27.2.1 Acute GVHD

Traditionally, acute GVHD was defined to occur prior to day 100 posttransplantation, whereas chronic GVHD was said to occur after 100 days [23, 29, 53]. However, advances in HCT practice including the use of reduced-intensity conditioning (RIC) regimens and immune-modulating strategies such as donor lymphocyte infusions (DLI), have profoundly altered the presentation and natural history of both acute and chronic GVHD and bring previous definitions into question [53]. For instance, RIC may delay the onset of acute GVHD symptoms beyond the 100-day period (late-onset acute GVHD) [72], whereas manifestations of acute and chronic GVHD can be present simultaneously, for example, in patients treated with DLI (overlap syndrome). A recent National Institutes of Health classification reclassified acute and chronic disease definitions based on the consensus that clinical manifestations, and not the time to symptomatic onset after transplantation, determine whether the clinical syndrome of GVHD is considered acute or chronic ([32], Table 27.1). Nevertheless, extensive clinical data have been codified according to this classification and they are used throughout this chapter.

The clinical manifestations of acute GVHD occur in the skin, gastrointestinal tract, and liver. Total bilirubin levels, quantification of diarrhea volume, and extent and type of skin involvement are used to stage the disease ([23, 29, 53, 89], Table 27.2). Hepatic involvement is characterized by elevated total bilirubin. Gastrointestinal tract involvement presents as anorexia, abdominal pain, nausea/vomiting, and secretory diarrhea.

Skin is most commonly affected and is usually the first organ involved, often coinciding with engraftment of donor cells [28, 29]. Skin manifestations range from a mild, asymptomatic exanthema-like eruption to full-thickness skin loss resembling toxic epidermal necrolysis. Acute GVHD initially presents with erythematous-dusky macules and papules of the volar and plantar surfaces and ears that may rapidly become a diffuse morbilliform exanthema [23]. Very early involvement may manifest as erythema limited to hair follicles. Erythroderma may

Table 27.1 Categories of acute and chronic GVHD

Category	Time of symptoms after HCT or DLI	Presence of acute GVHD features	Presence of chronic GVHD features
Acute GVHD			
Classic acute GVHD	≤100 d	Yes	No
Persistent, recurrent, or late-onset acute GVHD	>100 d	Yes	No
Chronic GVHD			
Classic chronic GVHD	No time limit	No	Yes
Overlap syndrome	No time limit	Yes	Yes

Adapted from Filipovich et al. [32]

Table 27.2 Staging and grading of acute GVHD

Stage	Skin	Liver	Gut
1	Rash <25 % BSA	Bilirubin 2 mg/dL to <3 mg/dL	Diarrhea 500–1,000 mL/day or persistent nausea
2	Rash 25–50% BSA	Bilirubin 3–6 mg/dL	Diarrhea 1,000–1,500 mL/day
3	Rash >50% BSA	Bilirubin 6–15 mg/dL	Diarrhea >1,500 mL/day
4	Erythroderma w/ bullae formation	Bilirubin >15 mg/dL	Severe abdominal pain with or without ileus
Grade			
I	Stages 1–2	None	None
II	Stage 3	Stage 1	Stage 1
III		Stages 2–3	Stages 2–4
IV	Stage 4	Stage 4	

Adapted from Przepiorka et al. [89]

develop, and, in severe cases, spontaneous bullae with skin sloughing resembling toxic epidermal necrolysis may occur. Pruritus is variable and is not useful to distinguish acute GVHD from other causes of the rash. Mucous membranes can also become involved [23].

27.2.2 Chronic GVHD

Chronic GVHD is a multisystem disorder that may affect nearly any organ and is now known to have features resembling autoimmune and other immunologic disorders. The most common organs involved are the skin followed by oral mucosa, liver, and eyes [23, 53]. Risk factors for chronic GVHD include older recipient age, history of acute GVHD, the use of female donor for male recipient, use of DLI, use of unrelated or HLA-mismatched donors, and more recently, the use of growth-factor–mobilized peripheral blood leukocytes as opposed to marrow as a source of stem cells [12, 32]. As mentioned earlier, any manifestation of GVHD that was present (or continued) at 100 days after HCT or thereafter was arbitrarily defined as chronic GVHD even if the clinical manifestation was indistinguishable from that of acute GVHD. The current consensus is that acute or chronic disease definition is based primarily on clinical manifestations and histologic findings [32].

Chronic GVHD of the skin is remarkably variable in clinical presentation. In the past, an early lichenoid stage and a late sclerodermoid stage could be distinguished, however, in appreciation of the tremendous variability in clinical presentation, it is no longer useful to dichotomize chronic GVHD of the skin into either “lichenoid” or “sclerodermoid” categories [23]. Sclerotic or nonsclerotic (used to be lichenoid) are the terms that are now used [53]. Nonsclerotic cutaneous GVHD is characterized by epidermal changes that resemble lichen planus, keratosis pilaris, ichthyosis, poikiloderma, papulosquamous/psoriasiform/eczematous rash, dyspigmentation, or

acral erythema. Sclerotic changes may resemble lichen sclerosus, morphea, systemic sclerosis, or eosinophilic fasciitis [23, 53]. In contrast to systemic sclerosis, sclerotic involvement of the face, fingers, and toes (sclerodactyly) and Raynaud phenomenon is less common.

New consensus criteria for the diagnosis and staging of chronic GVHD have recently been developed by the National Institutes of Health Consensus Development Project [32] in which definitions of skin involvement as well as other organs have been proposed. According to these criteria, the following skin manifestations are diagnostic of chronic GVHD and therefore do not require a biopsy: poikiloderma, lichen planus-like eruptions, lichen sclerosus-like lesions, morphea-like sclerosis, and deep sclerosis/fasciitis. Various other clinical presentations are considered suggestive or distinctive, but are not sufficient to establish the diagnosis of chronic GVHD in the absence of a confirmatory biopsy or other organ manifestation. For instance, depigmentation is a distinctive feature for chronic GVHD and not seen in acute GVHD but is not sufficiently unique to be considered diagnostic of chronic GVHD [23, 32, 53]. A portion of the NIH criteria describing skin manifestations of chronic GVHD is shown in Table 27.3.

27.3 Histopathology

27.3.1 Acute GVHD

Early changes of GVHD consist of focal basal vacuolation and sparse superficial perivascular lymphocytic infiltrates with exocytosis of individual cells into the epidermis and follicular epithelium [113]. A histological grading system is well established for acute GVHD ([51, 63], Table 27.4). The hallmark feature of acute GVHD is the presence of necrotic keratinocytes accompanied by a sparse dermal lymphocytic infiltrate and basal vacuolar degeneration. In grade 1 disease, there is focal or diffuse vacuolization of the basal layer. In grade 2 lesions, spongiosis and dyskeratotic keratinocytes are identified, with some accompanied by two or more epidermal lymphocytes, a phenomenon known as satellite cell necrosis. The necrotic keratinocytes contain a pyknotic nucleus and eosinophilic cytoplasm. Grade 3 lesions are characterized by subepidermal cleft formation. In grade 4, there is complete separation of epidermis from dermis that correlates with clinical findings resembling toxic epidermal necrolysis. Early GVHD involvement with follicular papules correlates with degenerative changes limited to the hair follicle [34]. The presence of scattered eosinophils does not help differentiate between drug eruptions and GVHD [68]. A recent study demonstrated that a very high number of eosinophils (> average 16 eosinophils/10 high power fields) was necessary to rule out acute GVHD completely [110].

Table 27.3 Signs and symptoms of chronic GVHD

Organ or site	Diagnostic (Sufficient to establish the diagnosis of chronic GVHD)	Distinctive (Seen in chronic GVHD, but insufficient alone to establish a diagnosis of chronic GVHD)	Other features	Common (Seen with both acute and chronic GVHD)
Skin	Poikiloderma	Depigmentation	Sweat impairment	Erythema
	Lichen planus-like features		Ichthyosis	Maculopapular rash
	Sclerotic features		Keratosis pilaris	Pruritus
	Morphea-like features		Hypopigmentation	
	Lichen sclerosus-like features		Hyperpigmentation	
Nails		Dystrophy		
		Longitudinal ridging, splitting, or brittle features		
		Onycholysis		
		Pterygium unguis		
		Nail loss (usually symmetric; affects most nails)		
Scalp and body hair		New onset of scarring or nonscarring scalp alopecia (after recovery from chemoradiotherapy)	Thinning scalp hair, typically patchy, coarse, or dull (not explained by endocrine or other causes)	
		Scaling, papulosquamous lesions	Premature gray hair	
Mouth	Lichen-type features	Xerostomia		Gingivitis
	Hyperkeratotic plaques	Mucocele		Mucositis
	Restriction of mouth opening from sclerosis	Mucosal atrophy		Erythema
		Pseudomembranes		Pain
		Ulcers		

Adapted from Filipovich et al. [32]

27.3.2 Chronic GVHD

27.3.2.1 Nonsclerotic Manifestations

As in the case of acute GHVD, chronic cutaneous GVHD exhibits an interface dermatitis, lymphocyte satellitosis, and vacuolar changes at the basal cell layer.

Table 27.4 Histologic grading of acute GVHD

Grade	Histopathologic features
0	Normal skin or cutaneous disease unrelated to GVHD
1	Basal vacuolization of the dermal–epidermal junction
2	Basal vacuolization, necrotic epidermal cells, lymphocytes in the dermis and/or epidermis
3	Grade 2 plus subepidermal cleft formation
4	Grade 2 plus separation of epidermis from dermis

Adapted from Lerner et al. [63] and Cowen [23]

Apoptosis within the basilar or lower spinosum layers has been proposed to be the minimal histologic criteria for active GVHD. Acanthosis and wedge-shaped hypergranulosis resembling lichen planus may be seen. In many cases it may not be possible to distinguish acute and chronic epidermal involvement histologically [53, 98].

27.3.2.2 Sclerotic Manifestations

Sclerotic involvement of the papillary dermis may resemble lichen sclerosus with atrophy, hyperkeratosis, follicular plugging, and a pale homogenized appearance of the papillary dermis collagen. If epidermal changes of GVHD are not present, dermal fibrosis with thickened collagen bundles and loss of periadnexal fat involvement may be indistinguishable from idiopathic morphea/scleroderma. Subcutaneous and fascial involvement accordingly reveal changes in the fat septae and fascia, including thickening, edema, and fibrosis, with variable infiltration of lymphocytes, histiocytes, and eosinophils [53, 95, 98].

27.4 Pathophysiology

27.4.1 Acute GVHD

To understand the pathophysiology of acute GVHD, two principles need to be considered. First, acute GVHD reflects exaggerated but normal inflammatory mechanisms mediated by donor lymphocytes infused into the recipient where they function appropriately, given the foreign environment in which they find themselves. Second, the recipient tissues that stimulate donor lymphocytes have often been damaged by underlying disease, prior infection, and the transplant conditioning regimen [28]. As a result, these damaged tissues produce “danger” signals [70] including pro-inflammatory cytokines, chemokines, and increased expression of adhesion molecules, MHC antigens, and costimulatory molecules on host APCs that promote the activation and proliferation of donor immune cells

[43, 44, 115]. Animal models have been instrumental in understanding the pathophysiology of acute GVHD and based largely on these experimental models, the development of acute GVHD can be conceptualized in a three-phase process: (1) tissue damage to the recipient by the radiation/chemotherapy pretransplant conditioning regimen and activation of the APCs; (2) donor T-cell activation and clonal expansion; and (3) target tissue destruction [29, 31].

27.4.1.1 Phase I: Conditioning Phase

The first step of acute GVHD begins before donor cells are infused. Tissue damage from the recipient's underlying disease, infection, and/or the conditioning chemotherapy/radiotherapy leads to inflammatory responses through inflammatory cytokine production and host APC activation. Inflammatory cytokines (TNF-α and IL-1β) have been implicated in the pathogenesis of acute GVHD [43, 48, 49, 88, 115]. Total body irradiation (TBI) is particularly important because it activates host tissues to secrete inflammatory cytokines [43, 115] and induces damage to the gastrointestinal (GI) tract [43, 44]. Increasing evidence suggests that damage to the GI tract during acute GVHD plays a major pathophysiologic role in the amplification of systemic disease. It increases the translocation of inflammatory stimuli such as microbial products including LPS.

Clinical studies first suggested that a correlation between GVHD severity and radiation dose exists [18, 81] and that more severe GVHD occurs after conditioning regimens including radiation therapy than when only chemotherapy is used [19]. Large doses of TBI increase GVHD severity by amplifying the dysregulation of inflammatory cytokines [43]. TBI and allogeneic immune cells synergize to damage the GI tract, thereby permitting increased translocation of LPS into the systemic circulation. This damage, together with increased production of TNF-α by host cells after TBI that promotes further inflammation and additional GI tract damage [44] leads to an increase in the morbidity and mortality in patients with GVHD The GI tract is therefore critical to the propagation of the "cytokine storm" characteristic of acute GVHD.

LPS is a potent stimulator of inflammatory cytokine production, such as TNF-α and IL-1, which are important mediators of clinical [1, 48] and experimental GVHD [43, 45, 115]. The production of TNF-α by monocytes and macrophages is transduced by two signals. The first is a priming signal that may be provided by IFN-γ [82] and radiation [43]. The second is a triggering signal provided by bacterial products such as LPS [82]. Translocation of LPS across a damaged gut mucosa provides access to the systemic circulation where LPS triggers monocytes and macrophages primed by the effects of IFN-γ to release cytopathic amounts of inflammatory cytokines [82]. Together with natural killer (NK) and T cells, these cytokines mediate GHVD target organ damage. GVHD is associated with elevated serum levels of both TNF-α and LPS on day 7 after BMT [43]. The importance of LPS in GVHD has further been supported by a mouse study using strains that differ in their sensitivity to LPS as donors in an experimental BM transplant (BMT)

system showing that donor resistance to LPS reduces the development of acute GVHD [22]. In murine GVHD, it has been demonstrated that the administration of anti-TNF-α reduces cutaneous and intestinal lesions and mortality [88]. Increased levels of TNF-α have also been found in the serum of allogeneic bone marrow transplant recipients and have been correlated with the severity of GVHD [49]. Administration of recombinant human IL-1 receptor antagonist has been shown to improve acute GVHD and the response to therapy was associated with a reduction of TNF-α mRNA levels in blood mononuclear cells [1].

The Role of APC

The activation of host APCs by inflammatory cytokines that are induced by the underlying disease and the HCT conditioning regimen is thought to be the first step of acute GVHD [29, 31]. A benchmark study demonstrating the importance of APCs' origin (donor or host) in initiating GVHD was done by Shlomchik et al. They showed that host APCs initiate CD8-dependent GVHD by directly presenting host Ag to donor T cells in a mouse model across only minor histocompatibility antigens (miHAs) [97]. These host-derived APCs have been demonstrated to be dendritic cells (DCs), but not B cells [26]. Activation of host CD11c+DCs occurs rapidly after irradiation and could prime CD8 T cells before their disappearance [121]. Later after transplantation, host APCs are replaced by donor APCs and donor APCs intensify GVHD once the disease is initiated by host APCs [69]. These donor APCs were demonstrated to be CD11c+ cells that efficiently cross-present host minor histocompatibility antigens to CD8 T cells [109].

On the other hand, how antigen is presented within MHC class II to donor CD4 T cells in CD4-dependent GVHD is not well known. It has previously been shown that MHC class II-bearing host hematopoietic APCs, particularly DCs [26], can initiate CD4 T cell-dependent GVHD [26, 104] and conventional DCs are the major donor APC subset presenting allogeneic peptides and maintaining GVHD [67]. Moreover, the study exploring molecular mechanisms demonstrated that the induction and maintenance of GVHD is critically dependent on RelB, the NF-kB/Rel family transcription factor, within both host and donor CD11c+DCs [66]. However, recent studies have shown that host hematopoietic professional APCs within lymphoid organs may have only a limited capacity to induce GVHD, and host DCs may not be required [60]. In contrast, nonhematopoietic host APCs within target organs are sufficient to induce GVHD [60]. Similarly, in the case of H–Y minor Ag incompatibility, either the donor hematopoietic-derived or the host nonhematopoietic-derived APCs is sufficient for inducing GVHD [105].

The Role of Innate Immunity

The significance of intestinal bacterial microflora in the pathogenesis of acute GVHD is well documented [7, 31, 86]. Current data implicate the innate immune

response as being responsible for initiating or amplifying acute GVHD. Molecules such as bacterial lipopolysaccharide (LPS) that are released from the injured gut during the conditioning regimen activate innate immune receptors, including Toll-like receptors (TLRs), and cause a cytokine storm, which favors the development of acute GVHD [44]. A variety of studies has suggested a role for intestinal bacterial microflora in the pathogenesis of acute GVHD, including the demonstration, in germ-free or completely decontaminated rodents, that the absence or complete growth suppression of intestinal bacteria prevents the development of acute GVHD [55, 106]. Hence, intestinal decontamination using broad-spectrum antibiotics before transplantation has been introduced, by some, as standard practice [4, 5].

A crucial role of the LPS-TLR4 pathway in the pathophysiology of GVHD has been demonstrated [21]. After allo-HCT, the translocation of LPS and microorganisms from the bowel lumen through the damaged intestinal mucosa to the circulation can occur. It has been shown that during GVHD, LPS stimulates the secretion of TNF-α by macrophages leading to increased GVHD mortality [82]. Using a mouse BMT model, Ferrara and colleagues demonstrated that the transplantation of donor BM cells, which are resistant to LPS stimulation, results in less severe GVHD [22] by attenuating early intestinal damage mediated by TNF-α. They also found that LPS antagonism, administration of a lipid-A analogue into a mouse BMT model, reduced serum TNF-α levels, decreased intestinal histopathology, and resulted in reduction of GVHD, but did not alter T-cell activity to host antigens [21]. Although the importance of the LPS-TLR4 pathway during GVHD is well defined as above, experiments with TLR4-deficient mice show that no difference in GVHD morbidity and mortality was observed in recipient mice lacking TLR4 as compared with wild-type mice, suggesting that TLR4 signaling is not absolutely required for GVHD [11]. Similarly, mutations in TLR4 have been shown to reduce GVHD risk [54], whereas another study reported an increased risk [27]. Thus, an association between TLR4 and GVHD has not been clearly defined.

Recent studies also suggested the critical roles of other TLRs in the pathogenesis of GVHD. In a murine GVHD model, reduced systemic GVHD leading to improved survival was observed in TLR9−/− allo-BM transplant recipients [11]. One of the most direct lines of evidence for the effects of TLRs on GVHD was derived by applying a TLR7 agonist to mouse skin before inducing GVHD. This resulted in massive T-cell infiltrates and GVHD pathology only at the site of pretreated skin [15]. In contrast, the TLR5 ligand, flagellin, and NOD2 have been shown to have an inhibitory effect on GVHD. Administration of highly purified flagellin into allo-BM recipient mice reduced GVHD by reducing early donor T-cell activation and proliferation [52]. Finally, increased GVHD was observed in NOD2-deficient allo-BMT recipient mice suggesting that NOD2 regulates the development of GVHD through suppressing the function of APCs [87]. In the clinical setting, polymorphisms of the genes encoding NOD2 are associated with a higher GVHD incidence [50].

It is important to mention that despite the prominent role of the innate immune system in the pathogenesis of GVHD, T cells can still be activated and GVHD can still occur in the absence of appropriate TLR signaling [64].

27.4.1.2 Phase II: Activation Phase

This phase represents the core of the GVH reaction, where donor T cells proliferate and differentiate in response to host APCs. Inflammatory cytokines and microbial products generated in Phase I augment this activation at least in part by increasing the expression of costimulatory molecules [29]. Donor T cells are critical in the induction of acute GVHD because depletion of T cells from the bone marrow graft effectively prevents GVHD [58] but at the expense of an increase in marrow graft rejection and leukemic relapse [37]. Recent clinical studies confirm experimental data demonstrating that the severity of GVHD correlates with the number of donor T cells transfused [57].

MHC class II differences between donors and recipients stimulate CD4 T cells, whereas MHC class I differences stimulate CD8 T cells [59, 99]. In the majority of HLA-identical HCTs, both CD4 and CD8 subsets respond to minor histocompatibility antigens and can cause GVHD. Thus both donor CD4 and CD8 T cells have crucial roles in the pathogenesis of GVHD.

Acute GVHD appears to be primarily a Th1-driven process with massive release of IFN-γ, IL-2, and TNF-α and these cytokines have been implicated in the pathophysiology of acute GVHD [7, 29].

IL-2 production by donor T cells remains the principal target of many current clinical therapeutic and prophylactic approaches to GVHD. Monoclonal antibodies (mAb) against IL-2 or its receptor prevent GVHD when administered shortly after the infusion of T cells [30, 42]. Cyclosporine and FK506 dramatically reduce IL-2 production and effectively prevent GVHD [91]. But emerging data indicate an important role for IL-2 in the generation and maintenance of $CD4^+CD25^+$ regulatory T cells (Tregs), suggesting that prolonged interference with IL-2 may have an unintended consequence of preventing the development of long-term tolerance after allogeneic HCT [119].

IL-15 is another critical cytokine in initiating allogeneic T-cell division in vivo, which has similar biological activities to IL-2, and has been identified as an indispensable costimulator in an experimental mouse model of skin GVHD [77, 78]. In humans, elevated serum levels of IL-15 are associated with acute GVHD [61].

IFN-γ has multiple functions and can either amplify [79] or reduce GVHD [9, 116] depending on the timing of its production. IFN-γ can have immunosuppressive effects at early time points after HCT but can exacerbate disease via its pro-inflammatory properties at later stages [7, 29]. IFN-γ may amplify GVHD by increasing the expression of molecules including adhesion molecules, MHC proteins, and chemokine receptors [29]. It also primes macrophages to produce pro-inflammatory cytokines and nitric oxide (NO) in response to LPS [82]. By

contrast, IFN-γ may suppress GVHD by hastening the apoptosis of activated donor T cells [92].

Th2-type cytokines can reduce acute GVHD [112] as mice receiving donor Th2-type cells are protected from GVHD [33]. Moreover, donor T cells that lack the four classical Th2 cytokines (IL-4, 5, 9, and 13) enhance T-cell proliferative responses and aggravate GVHD [102].

Th17 cells have been shown to have a direct role in GVHD pathogenesis. It has been shown that IL-17$^{-/-}$ donor T cells augmented Th1 differentiation and exacerbated acute GVHD [117] and adoptive transfer of in vitro differentiated Th17 cells induced lethal acute GVHD [13], although other studies have shown that an absence of IL-17 production by donor cells markedly impairs the development of CD4-mediated acute GVHD [56].

Genetic polymorphisms in TNF-α, IL-10, and IFN-γ have been linked to increased risk and severity of GVHD [14, 65, 71].

Subpopulations of regulatory cells can prevent GVHD. CD4$^+$CD25$^+$ Treg cells can suppress the proliferation of conventional T cells and prevent acute GVHD in animal models when added to the donor inoculum containing alloreactive T cells [20, 46, 103]. Donor NK cells [2] and NKT cells of both the host and donor have been shown to suppress acute GVHD [120]. As well, CD3$^+$CD4$^-$CD8$^-$ double-negative regulatory T cells, which specifically suppress CD8 and CD4 T cells that are primed against the same alloantigen, inhibit GVHD [76, 118].

27.4.1.3 Phase III: Effector Phase

The final effector phase of acute GVHD is characterized by cell damage via cellular mediators including cytotoxic T lymphocytes (CTLs) and NK cells and soluble inflammatory mediators including TNF-α, IFN-γ, IL-1, and NO [29, 31]. Three cytolytic pathways have been identified as important to GVHD: the perforin/granzyme B pathway, the Fas/FasL pathway, and direct cytokine-mediated injury [8, 44]. The soluble and cellular mediators synergize to amplify local tissue injury and further promote inflammation and target tissue destruction.

Cellular Effectors

The cellular effectors of acute GVHD are primarily CTLs and NK cells [29]. Donor cells from perforin-deficient mice can mediate GVHD but the onset of clinical manifestations is significantly delayed [3]. The result using granzyme B (gzm)-deficient mice showed a significant impairment in class I-dependent GVHD mediated by gzm B−/− CD8$^+$CTL, whereas class II-dependent GVHD was not altered using gzm B−/− CD4$^+$ effectors, suggesting that granzyme B plays a significant role in acute GVHD mediated by CD8$^+$, but not CD4$^+$ CTL [39]. Similarly, when mutant mice deficient in FasL are used as donors, GVHD occurs in an attenuated fashion [3]. Interestingly, the Fas pathway is important in the development of

hepatic and cutaneous GVHD [3, 40]. When FasL-deficient mice are crossed with those having perforin knockout, the use of lymphocytes from these donors further diminishes but does not abrogate GVHD [8]. Currently, the Fas–FasL pathway is thought to be involved in skin and liver GVHD as well as systemic GVHD, whereas only some studies show evidence for involvement of the perforin–granzyme pathway in systemic GVHD and not target-organ GVHD [107].

Chemokines direct the migration of donor T cells from lymphoid tissues to the target organs where they cause damage. IFN-γ produced by effector T cells that express CCR2, CCR5, CXCR3, and CXCR6, induce production of interferon-inducible chemokines (CXCL9-11) in target organs. These chemokines play primary roles in directing the early migration of activated, CXCR3-expressing T cells after egress from lymphoid tissues. Following this initial phase of production of chemokine ligands, a second wave of production is induced from recruited donor T cells. These T cells produce CCR5-ligands such as CCL3-5 and effector cells continue to follow gradients of these chemokines to infiltrate target organs, leading to tissue pathology and clinical manifestations of GVHD [114]. The expression of chemokines and their receptors may help to explain the unusual cluster of GVHD target organs (skin, gut, and liver). Expression of α4β7 integrin and its ligand MadCAM-1 are also important for homing of donor T cells to Peyer's patches during intestinal GVHD [80, 108].

Inflammatory Effectors

In the effector phase of acute GVHD, inflammatory cytokines synergize with CTLs, resulting in amplification of local tissue injury and development of target organ dysfunction. A central role for inflammatory cytokines in acute GVHD was confirmed by murine experiments using bone marrow chimeras in which GVHD-target organ injury was induced, even in the absence of epithelial alloantigens, and mortality and organ injury were prevented by the neutralization of TNF-α and IL-1 [104]. TNF-α can be produced by both donor and host cells and it acts in three different ways: first, it activates APCs and enhances alloantigen presentation; second, it recruits effector cells to target organs via the induction of inflammatory chemokines; and third, it directly causes tissue necrosis [10, 44, 88]. The GI tract is particularly susceptible to damage from TNF-α, and plays a major role in the amplification and propagation of the cytokine storm characteristic of acute GVHD [44]. Several studies have shown that TNF-α plays a central role in intestinal GVHD [40, 41, 88]. In addition to the Fas–FasL pathway [40, 107], TNF-α is also important for skin GVHD.

27.4.2 Chronic GVHD

Less is understood about the pathophysiology of chronic GVHD but components of alloreactivity and autoimmunity are likely to be involved [7, 23, 53]. Defining the pathophysiology of chronic GVHD has been complicated by the absence of animal models that recapitulate all of the diverse characteristics of the disease, in contrast to acute GVHD where murine models of major and minor histocompatability mismatched HCT have provided a relatively comprehensive picture of its pathophysiology [16]. Disease-specific autoimmune models are often used based on the clinical similarities between chronic GVHD and autoimmune conditions such as systemic sclerosis, lupus-like diseases, and Sjogren syndrome. However, these animal models recapitulate only isolated organ involvement [7, 53].

Donor-derived alloreactive T cells have been proposed to play a role in the pathogenesis of both acute GVHD and chronic GVHD. Some believe that chronic GVHD is a Th2-type disease, whereas acute GVHD results from Th1 cells [7]. However, a large randomized trial has demonstrated that with T-cell depletion there is a reduced incidence of acute GVHD but not a reduced incidence of chronic GVHD [85]. In addition, despite effective prevention of acute GVHD with agents that primarily inhibit T cells (e.g., calcineurin inhibitors), the incidence of chronic GVHD has not decreased [91, 100], suggesting that chronic GVHD is not simply a continuation of acute GVHD and that immune mechanisms of chronic GVHD are distinct from acute GVHD.

The concurrent manifestation of autoimmunity, alloimmunity, and immunodeficiency observed in chronic GVHD indicate an impairment of central or peripheral immunologic tolerance. The thymus is damaged by prior chemotherapy, by the conditioning regimen, by acute GVHD, and by aging leading to impairment of thymic function [38, 111], which may contribute to dysregulation of central tolerance. Experimental data indicate that such thymic damage results in a loss of thymic negative selection [25, 36, 47] that results in the release of self-reactive T cells into the periphery that, in turn, causes chronic GVHD [36, 47, 93]. These results suggest that self-reactivity of donor T cells generated from hematopoietic stem cells via the recipient's thymus plays a role in chronic GVHD [93]. Taken together, these data suggest that the thymic-independent peripheral expansion of mature T cells is responsible for the development of acute GVHD because T-cell depletion of the donor BM reduces rates of acute GVHD [58], whereas thymic-dependent T cells play a role in mediating chronic GVHD.

Dysregulation of peripheral tolerance in chronic GVHD has also been addressed. As with acute GVHD, infusion of Tregs has been shown to ameliorate chronic GVHD in animal models [122]. In humans, however, available data have generated conflicting results. Whereas one phenotypic study suggested that patients with chronic GVHD had markedly elevated numbers of peripheral blood $CD4^+CD25^+$ Tregs [17], another study found that patients with active chronic GVHD have reduced frequencies of Tregs [123]. Furthermore, molecular studies demonstrated lower expression levels of the Treg-specific transcription factor *FOXP3* in patients with both acute and chronic GVHD [75].

There is now mounting evidence implicating B cells in the pathophysiology of chronic GVHD. Patients with chronic GVHD have a high incidence of detectable autoantibodies (e.g., antinuclear, double-stranded DNA) and autoimmune disease-related gene polymorphisms [84, 90, 96]. However, in contrast to classic autoimmune diseases, the autoantibodies detected in chronic GVHD generally do not correlate with organ-specific manifestations. Analysis of male patients who received stem cells from female donors demonstrated that antibodies to Y chromosome-encoded minor histocompatibility antigens (H–Y antigens) are generated after sex-mismatched allogeneic transplantation [74], and the presence of these antibodies has been correlated with the occurrence of chronic GVHD and maintenance of disease remission [73]. H–Y antigen elicited a coordinated B- and T-cell response but the specificity for recipient male cells was mediated by the B-cell response and not by donor T cells [124]. Also known is that soluble levels of B-cell activating factor (BAFF), a cytokine known to be crucial in the reconstitution and survival of B cells, correlate with chronic GVHD activity [35, 94]. Consistent with these findings, anti-B–cell therapy with anti-CD20mAb (rituximab) has been reported to be effective for steroid-refractory chronic GVHD with a decrease in allogeneic antibody titers [24].

The mechanisms responsible for chronic GVHD-induced fibrosis remain uncertain. It has been proposed that Th2 cytokine pattern deviation leads to the release of fibrogenic cytokines such as IL-2, IL-10, and TGFβ1 and the activation of macrophages that produce PDGF and TGFβ. These molecules induce proliferations and activation of tissue fibroblasts [7]. Stimulatory antibodies against the PDGF receptor have been identified in patients with extensive chronic GVHD [101].

27.5 Conclusions

Much has been learned about the mechanisms involved in the pathophysiology of GVHD. It is now clear that acute GVHD and chronic GVHD are not just part of a spectrum but involve different pathomechanisms. A better understanding of the mechanisms involving both acute and chronic GVHD will inform the use of better prevention and intervention modalities.

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Chapter 28
Rosacea in Skin Innate Immunity

Kenshi Yamasaki

Abstract Rosacea is a common and chronic inflammatory skin disease most frequently seen in groups of genetically related individuals. Although the symptoms of rosacea are heterogeneous, they are all related by the presence of characteristic facial or ocular inflammation involving both the vascular and tissue stroma. Until recently, the pathophysiology of this disease was limited to descriptions of a wide variety of factors that exacerbate or improve disease. Lesional skins of rosacea increase the susceptibility to environmental stimuli through TLR2 and consequently have aberrant cathelicidin antimicrobial peptides along with the increase of kallikrein 5, the cathelicidin-processing enzyme in epidermis. The molecular studies show a common link between the triggers of rosacea and the cellular response, and these observations suggest that an altered innate immune response is involved in disease pathogenesis. Understanding rosacea as a disorder of innate immunity explains the benefits of current treatments and suggests new therapeutic strategies for alleviating this disease.

Keywords Rosacea • Innate immunity • Toll-like receptor • Antimicrobial peptide • Cathelicidin • Serine protease • Kallikrein • Propionibacterium acnes • Demodex • Perioral dermatitis • Ultraviolet

28.1 Rosacea; Clinical Features

Rosacea is a common and chronic inflammatory skin disease that affects over ten million Americans [25]. Most individuals affected by rosacea are of northern European origin and up to one third have a family history of the disorder [25]. The disease affects mostly facial skin and is characterized by flushing, nontransient erythema, telangiectasia, papules, pustules, and inflammatory nodules. Secondary features that often occur include burning and stinging sensation of the face, occasional dermatitis or scaling of the face, edema, and complication of seborrheic dermatitis. In many sufferers, rosacea can be worsened or triggered by

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factors that initiate flushing, such as exercise, emotion, menopause, and alcohol [23].

The National Rosacea Society Expert Committee created a standard classification system for rosacea in 2002 [84] and grading system in 2004 [85]. The committee aimed to develop a standard system that can serve as an instrument to investigate the manifestation of rosacea for both clinician and researchers. They described four subtypes and one variant of rosacea. Subtypes of rosacea include erythematotelangiectatic rosacea (ETR), papulopustular rosacea (PPR), phymatous rosacea (PR), and ocular rosacea (OR). ETR is characterized by flushing and persistent central facial erythema. This may or may not be associated with telangiectasia. Patients may also report central facial edema, stinging or burning sensation, and roughness/scaling texture to the skin. PPR is characterized by persistent central facial erythema with transient papules or pustules [23, 84]. In phymatous rosacea, patients experience thickening of the skin, patulous follicles, and a nodular surface texture. PR is characterized by rhinophyma, the most common variant illustrated as an enlargement and irregular texture of the nose. This also can occur elsewhere on the face including cheeks, chin, forehead, and ears. On histology, there is sebaceous overgrowth and fibrosis [13, 23, 42]. Patients with OR often experience conjunctival redness, burning, stinging, dryness, and an ocular foreign body sensation. Clinically, they may have telangiectasia of the conjunctiva and lid margin, blepharitis, and conjunctivitis. The most serious sequelae of OR include punctuate keratitis and corneal ulceration. Some OR patients present with ocular symptoms prior to facial skin symptoms [23]. Granulomatous rosacea is a variant characterized by firm yellow, brown, red cutaneous papules found on cheeks and periorificial areas. Histologically there is granulomatous inflammation. Patients may exhibit only one subtype of rosacea or they may progress to other subtypes [23, 84].

Because the phenotypes of rosacea are clinically heterogeneous, rosacea studies were diversely conducted based on the findings in clinical manifestations, histology, and factors exacerbating the skin disorder. From the diverse findings, the pathophysiology of this disease has been poorly understood and limited to describing factors that exacerbate or improve this disorder. The multiple factors, which have been known to affect rosacea from clinical observation, lead to a trigger of the innate immune system. Hence, reports of consistent aberrant innate immune responses in rosacea explain the diverse findings on rosacea etiology and help to understand why the current therapies are effective. This chapter organizes the possible pathology of rosacea by connecting the known rosacea elements through the use of the innate immune system. This categorizes pathological mechanisms of rosacea in (a) innate immunity, (b) vascular changes, (c) reactive oxygen species, (d) ultraviolet radiation, and (e) microbes. These molecular events can now be linked to each other with our current knowledge of innate immunity.

28.2 Rosacea Molecular Pathology

28.2.1 Innate Immunity in Rosacea

A dysregulation of the innate immune system in rosacea would unify current clinical observations. In innate immunity, the pattern recognition system, which includes the TLR (Toll-like receptor) and NLR (nucleotide-binding domain and leucine-rich repeat-containing) families, respond to environmental stimuli such as UV, microbes, and physical and chemical trauma. Triggering the innate immune system normally leads to a controlled increase in cytokines and antimicrobial molecules in the skin [56, 79]. One of these antimicrobial molecules is a peptide known as cathelicidin antimicrobial peptide (CAMP) [30]. Some forms of CAMP were known to have a unique capacity to be both vasoactive and pro-inflammatory. Therefore, given the potential for a single molecule to affect both of the events that describe rosacea, analyses of cathelicidin in rosacea were conducted. Individuals with rosacea expressed abnormally high levels of cathelicidin [88]. Note that the cathelicidin peptides found in rosacea were not only more abundant but were different from those in normal individuals. These forms of CAMP promote and regulate leukocyte chemotaxis [24], angiogenesis [49], and expression of extracellular matrix components [34]. The presence of the vasoactive and inflammatory CAMP in rosacea was subsequently explained by abnormal production of local protease kallikrein 5 (KLK5), which controls the production of CAMP in epidermis [88, 90]. To confirm the importance of these observations and to test the hypothesis that abnormal CAMP could induce the signs of rosacea, these abnormal CAMP or the CAMP-producing enzymes were injected into the mouse skin. This rapidly resulted in skin inflammation resembling pathological changes in rosacea [88]. Azelaic acid is topically used for rosacea treatment and decreases CAMP and KLK5 in keratinocyte in vitro and mouse skin and rosacea patient in vivo studies, which proved why azelaic acid has benefit for rosacea [20]. Combined, these findings indicated that an exacerbated innate immune response induces abnormal CAMP, and that this then leads to the clinical findings of rosacea.

The innate immune system of the skin is programmed to detect microbes, tissue damage such as UV-induced apoptosis, or damage of the extracellular matrix [19, 80]. Sun exposure, dermal matrix changes, and microbes have been recognized as triggers and exacerbating factors of rosacea. Along with the aberrant CAMP expression, TLR2 expression is altered in rosacea skin [89]. Increase in TLR2 enhances skin susceptibility to innate immune stimuli and TLR2 stimuli lead to increased cathelicidin and kallikrein production from epidermal keratinocytes [67, 89]. Hence, combination of high cathelicidin and KLK5 induced by TLR2 generate aberrant and abnormal CAMP in rosacea. Interestingly, TLR2 involvement is also suggested in other disorders with resemblance to rosacea. Perioral dermatitis and glucocorticoid-inducing rosacea-like dermatitis are adverse events by topical steroid use on the face [22, 73, 83, 86]. Although the precise molecular mechanisms of the steroid-induced dermatitis is not determined, Shibata

et al. reported that glucocorticoid increases TLR2 expression in epidermal keratinocytes, and that *P. acnes* enhanced glucocorticoid-dependent TLR2 induction and cytokine production [70]. In acne vulgaris, TLR2-positive cells are rapidly recruited in the early lesion [48]. Thus, findings and accumulated knowledge on rosacea and dermatoses resembling rosacea in skin manifestations suggest the innate immune response in rosacea has gone awry. For these reasons the events worsening rosacea trigger innate immunity, and rosacea patients are more susceptible to environmental stimuli that do not cause reactions in normal patients.

28.2.2 Vascular Changes in Rosacea

Most rosacea patients have telangiectasia and flushing episodes, thus leading to a common hypothesis that vascular hyperreactivity and increased blood flow play a role in the susceptibility to this disease. Studies have demonstrated a measurable increase in blood flow in skin lesions of patients with rosacea [39, 71]. Some factors that trigger flushing such as emotional stress, spicy food, hot beverages, high environmental temperatures, and menopause worsen rosacea [16], thus supporting the hypothesis of vascular hyperreactivity in rosacea. Resolution of erythema and flushing by topical α-adrenergic receptor agonist application also supports the hypothesis that vascular hyperreactivity is major factor of rosacea pathology [33, 76].

Elevated expression of vascular endothelial growth factor (VEGF), CD31, and lymphatic endothelium maker D2-40 are observed in the skin of patients with rosacea [37]. VEGF proliferate vascular endothelial cells as well as increase permeability of vessels. CD31 is platelet/endothelial cell adhesion molecule (PECAM1 in gene symbol), and anti-CD31 antibody recognizes the endothelial cells. Anti-D2-40 monoclonal antibody identifies a 40 kDa O-linked sialoglycoprotein and has also been demonstrated to label lymphatic endothelium but not vascular endothelium. Thus, elevated expression of VEGF, CD31, and D2-40 in rosacea demonstrates rosacea skins have more stimulants to increase vascular and lymphatic endothelial cells.

Sun and UV exposure exacerbate rosacea, and UV irradiation induces VEGF in human keratinocytes and skin [15]. UVB induces cutaneous angiogenesis that is histologically similar to the telangiectasia seen in rosacea histopathology [11]. In skin, epidermal keratinocytes are a major source of angiogenic factor VEGF (vascular endothelial growth factor) and FGF2 (fibroblast growth factor 2, also known as basic FGF) [9, 27]. UVB increases VEGF and FGF2 secretion from human keratinocytes and expression in mouse epidermis [11, 15, 53].

From the aspect of innate immunity, cathelicidin is one of the triggers of hypervascularity in rosacea. Injection of CAMP LL37 in mouse skin induced vasodilatation [88]. Application of LL37 resulted in neovascularization in a rabbit model of hind-limb ischemia, and the angiogenesis by LL37 is mediated by formyl peptide receptor-like 1 (FPRL1), a G-protein coupled receptor expressed on

endothelial cells [49]. LL37 also transactivates epidermal growth factor receptor (EGFR) and downstream signaling in epithelial cells [65, 81]. EGFR signaling induces VEGF in epidermal keratinocytes [27]. Thus, cathelicidin induces endothelial cell changes through several signaling pathways; directly to endothelial cells and indirectly through keratinocyte activation.

28.2.3 Reactive Oxygen Species in Rosacea

Reactive oxygen species (ROS) has been discussed in rosacea pathology and served as the molecular explanation of why rosacea medications are effective. Tetracyclines, azelaic acid, metronidazole, and retinoids inhibit ROS generation in neutrophils [3, 4, 58, 92]. The molecular action of these medicines used for rosacea provoked the hypothesis of ROS involvement in rosacea pathology. Erythromycin and azithromycin, the other effective medicine for rosacea treatment, have been shown to have antioxidant effects [8, 40]. ROS levels were examined in skin biopsy samples from rosacea and healthy individuals, and confirmed higher ROS activity in rosacea lesional skin than healthy controls [8, 60]. The decrease of ROS in rosacea skin was also observed after azithromycin treatment [8], suggesting rosacea treatments affect ROS activity and supporting the hypothesis of ROS involvement in rosacea pathology.

Although the precise localization of ROS is not determined in rosacea skin, UV radiation generates ROS and activates cellular signaling in keratinocytes [61, 62]. ROS is a mediator of innate immune signaling and activates cellular signaling and induces chemokine production by TLR2 in monocytes [51, 91] and cytokine production by TNFα in human keratinocytes [93]. ROS simulates fibroblast and alters matrix metalloproteinases (MMP) and the tissue inhibitor of metalloproteinase (TIMP) expression. UVA radiation increased MMP-1, and ROS increased MMP-2 mRNA and suppressed TIMP-1 in human dermal fibroblast [46]. In a three-dimensional culture, normal human dermal fibroblasts increased MMP-1 and MMP-2 mRNA expression by ROS, whereas both proalpha1(I) and proalpha1(III) collagen mRNA production were suppressed by ROS. Thus, ROS production by innate immune stimuli and UV irradiation caused vascular and dermal matrix damage via upregulation of matrix metalloproteinases [59, 66, 87]. Increased ROS activity in rosacea skin would enhance inflammatory reactions by abnormal and damaged dermal matrix, which may permit accumulation and prolonged retention of inflammatory cells, cytokines, and chemokines.

28.2.4 Ultraviolet in Rosacea

UV and sun exposure are known to cause a flushing response and appear to worsen the clinical symptoms of rosacea [16]. As discussed above, UV irradiation could

induce erythema in the skin by increasing expression of angiogenic factors and by degenerating extracellular matrix. Innate immunity is also involved in UV-mediated cytokine and matrix metalloproteinase expression in keratinocytes. Myeloid differentiation factor 88 (MyD88), an essential adaptor molecule for Toll-like receptors (TLR) family signaling, increases expression in UV-irradiated human culture keratinocytes as well as photoaged human skin [52]. Overexpression of dominant negative form of MyD88 prevented UV-induced expressions of IL-6 and MMP-1 in human keratinocytes, whereas overexpression of dominant positive form of MyD88 increased IL-6 and MMP-1 expression. Combined with ROS involvement in chemokine production by TLR2 stimuli [51, 91], TLRs/MyD88 signaling would be part of the link between UV irradiation to skin inflammation. The future studies of photoaging in animals lacking TLRs signaling molecules will be of great interest.

28.2.5 Proteases in Rosacea

MMPs digest dermal matrices such as collagens, fibronectin, elastin, and the like, and balances between MMPs and their inhibitor TIMPs dictate dermal components and vascular remodeling [44]. MMP2 and MMP9 increase was immunohistochemically observed in granulomatous rosacea [41], and MMP-8 (collagenase 2) and MMP-9 (gelatinase B) activities are higher in the fluid of ocular rosacea than in normal subjects [2, 54, 74]. MMPs are inducible by UV irradiation and ROS stimulation in keratinocytes and fibroblasts [46, 52]. The supports of protease involvement in rosacea pathology are the evidence that tetracyclines, which are effective for rosacea treatment, inhibit several matrix metalloproteinases (MMP) and serine proteases [1, 64, 75]. Thus, the effective rosacea treatments might be partially dependent on their antiprotease properties.

In vitro study showed that doxycycline inhibits keratinocyte MMP and in turn inhibits keratinocyte proteolytic activity of tryptic kallikrein-related peptidases, which leads to cathelicidin activation [45]. Serine protease kallikrein 5 (KLK5, also known as *stratum corneum* tryptic enzyme SCTE) is a representative tryptic kallikrein-related peptidase in keratinocytes. KLK5 is the processing enzyme of cathelicidin, and high KLK5 expression and hyperprotease activity are observed in rosacea skin, which is inducible by TLR2 stimuli [88, 90]. KLK5 expresses in the upper epidermis (granular to cornfield cell layer) in normal skin, and rosacea skin expresses KLK5 in the entire epidermis. KLK5 also digest corneodesmosome proteins desmocollin 1 and desmoglein 1 in epidermis and are supposed to affect desquamation of epidermal keratinocytes [18, 26]. Hence high KLK5 and serine protease activity may increase skin sensitivity by enhanced desquamation of the cornfield layer. KLK5 also efficiently digest the extracellular matrix components including collagens type I, II, III, and IV, fibronectin, and laminin [57]. Considering the high KLK5 expression in basal cells of rosacea epidermis, KLK5 would have

roles in skin inflammatory reactions in rosacea by affecting dermal matrix and vascular remodeling.

28.2.6 Microbes in Rosacea

Two microbes have been discussed in rosacea pathology: *Demodex folliculorum* and *Helicobacter pylori*. *D. folliculorum*, a mite that lives within sebaceous follicles, has been implicated as a trigger of rosacea since histological studies revealed inflammation of the pilosebaceous follicle units. Studies have shown increased density of the mites in patients with rosacea compared with control patients [14, 17, 31, 32]. Lacey et al. isolated *Bacillus oleronius* from *D. folliculorum* and identified the antigens reacting to sera from rosacea individuals but not from control individuals [50]. The extracts of the *B. oleronius* stimulate proliferation of mononuclear cells from patients with rosacea suggesting that rosacea individuals are exposed to the *B. oleronius* molecules and that *B. oleronius* from *D. folliculorum* induces inflammation in rosacea. Interestingly, they identified heat shock proteins (HSP) and a lipoprotein in the antigenic molecules of *B. oleronius*. HSP and lipoproteins from microbes are also known to be stimulants for TLRs [21, 36]. This report supports the hypothesis of mite and pilosebaceaous unit involvement in rosacea. Further studies are required to examine if these *B. oleronius* molecules evoke an innate immune reaction or if rosacea is caused by an adaptive immune reaction against *B. oleronius* and *D. folliculorum*.

Correlation of *H. pylori* infection and rosacea is controversial and inconsistent in clinical observation [5, 28, 43, 63, 78]. Several reports showed seropositivity to *H. pylori* in rosacea individuals [38]. Eradication therapy for gastric *H. pylori* infection showed a preferable outcome for rosacea symptoms though it is not clear if the improvement of rosacea is due to *H. pylori* eradication [12, 35, 82]. *H. pylori* produces ROS [7, 29, 55] and rosacea individuals showed higher ROS including NO (nitric oxide) in plasma than controls [10, 38]. *H. pylori* induces cytokine release through TLR2 and TLR4 in gastric epithelial cells [47, 72]. Thus ROS and cytokines released by TLRs stimuli in organs other than skin may be mediators that worsen rosacea by *H. pylori* infection. However, concrete molecular evidence is still required to support the involvement of *H. pylori* in rosacea pathology.

28.3 Conclusion

The discovered role of cathelicidin and proteases in rosacea pathology creates further broad questions about the origins of this disease. The factors that promote cathelicidin production include innate immune molecules, which connect between clinical and molecular observations in rosacea. Microbes and environmental

changes, such as sun and UV exposure, are sensed by innate immune systems through pattern recognition molecules. The innate immune systems would enhance and be enhanced by cytokine, ROS, antimicrobial peptide, and proteases, which lead histological changes observed in rosacea. The multiple factors may heap up to cause rosacea clinical manifestations, whereas individual susceptibility to the factors is highly counted to cause rosacea. Although there is much work to do to understand the correlation between innate immunity and other rosacea factors such as neural factors [6, 68, 69] and fibrosis [77], these new associations give us clues to further understanding of the mechanisms responsible for the disease. Note that these advances also provide informed strategies for the optimal treatment of the clinical findings. The current ideas of "rosacea as a disease of innate immunity" unite the clinical observations of rosacea features and exacerbation factors to its molecular mechanisms and, it is hoped, give perception and imagination for basic research on the treatment of rosacea.

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Chapter 29
Cutaneous Lymphomas

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Abstract Primary cutaneous lymphomas represent a group of lymphatic malignancies arising primarily in the skin. They represent the second most common form of extranodal non-Hodgkin lymphomas and are characterized by heterogeneous clinical, histological, immunological, and molecular features. Their variety reflects the complexity of the lymphocytic populations of the skin and the different levels of malignant transformation. They thus include primary cutaneous T-cell lymphomas, primary cutaneous B-cell lymphomas, and a rare cutaneous blastic plasmacytoid dendritic cell neoplasm. Most of the primary cutaneous lymphomas are indolent neoplasms, confined to the skin for a long period of time without significant influence on life expectancy and therefore do not require systemic therapy in the beginning. Systemic immunomodulatory treatment is generally reserved for advanced stages and tries to counteract Th2-skewing cytokine dysbalance or to target lymphoma T or B cells specifically.

Keywords Cutaneous lymphomas • Cutaneous T-cell lymphomas • Cutaneous B-cell lymphomas • Immune interventions

29.1 Introduction

Primary cutaneous lymphomas represent a heterogeneous and dynamic group of malignancies that derive from transformed immune cells in the skin. The first published description of a cutaneous lymphoma (mycosis fungoides) was made by Jean-Louis Alibert in the nineteenth century [5]. Since then it took almost 150 years to acknowledge primary cutaneous lymphomas as an independent entity. In 2005 published WHO-EORTC classification of primary cutaneous lymphomas reached an agreement for characteristic features of the different primary cutaneous

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lymphomas and other hematologic neoplasms that frequently manifest in the skin and provide a solid basis for diagnosis and treatment, which has led to its wide acceptance [66]. Based on WHO-EORTC classification, these rare lymphomas were also included in the recent fourth edition of WHO classification of tumors of hematopoietic and lymphoid tissues [56].

Primary cutaneous lymphomas represent the second most common form of extranodal non-Hodgkin lymphomas and are characterized by heterogeneous clinical, histological, immunological, and molecular features. Their variety reflects the complexity of the lymphocytic populations of the skin and the different levels of malignant transformation. By definition primary cutaneous lymphomas represent clonal accumulation of lymphocytes in the skin and include primary cutaneous T-cell lymphomas, primary cutaneous B-cell lymphomas, and a rare separate entity of cutaneous blastic plasmacytoid dendritic cell neoplasms [32, 56, 66]. An important distinction between primary and secondary cutaneous lymphoma is that the former are restricted to the skin at the time of initial diagnosis [66], whereas secondary cutaneous lymphomas have an obligatory extracutaneous origin with secondary involvement of the skin. Most of the primary cutaneous T- and B-cell lymphomas remain indolent and confined to the skin for a long period of time, not significantly influencing the overall survival and in the majority of cases not requiring systemic treatment [35, 55].

The goal of this chapter is to give an overview of the most common primary cutaneous lymphomas, focus on their immunological peculiarities, and discuss the possible therapy interventions targeting these immunological aspects.

29.2 Cutaneous T-Cell Lymphomas

Primary cutaneous T-cell lymphomas (CTCL) comprise a heterogeneous group of extranodal non-Hodgkin lymphomas with various clinical and histological manifestations that arise from malignant transformation of skin-homing T cells (CLA^+/$CCR4^+$) [22, 33, 66]. In a recent large study based on data from 13 institutions and a total of 6,230 CTCL cases, an overall annual CTCL incidence of approximately 7 new CTCL cases per million persons was calculated, and the total overall CTCL survival was 78.3 % [37].

Mycosis fungoides (MF) is the most frequent one and accounts for approximately 60 % of all primary CTCL and for 1 % of all non-Hodgkin lymphomas [36]. Sézary syndrome (SS) is a less frequent erythrodermic and leukemic CTCL (L-CTCL) variant. Primary cutaneous CD30+ T-cell lymphoproliferative disorders (e.g., anaplastic large cell lymphoma and lymphomatoidpapulosis) make up 20 % of CTCL. Three percent are comprised of other rare CTCL: subcutaneous panniculitis-like T-cell lymphoma (α/β and γ/δ subtype), extranodal NK/T-cell lymphoma, hydroa vacciniforme-like T-cell lymphoma, and the primary cutaneous peripheral T-cell lymphoma, not otherwise specified [50]. The WHO-EORTC (World Health Organisation-European Organization for Research and Treatment

of Cancer) classification of cutaneous T cell and NK/T cell lymphomas updated in 2005 gives an overview of the variety of primary CTCL [66].

Although early-stage MF and SS have been considered to represent different stages in a disease continuum, differing molecular profiles and responses to therapy have provided new evidence that MF and SS may be distinct diseases [54]. Campbell, Clark, and colleagues recently suggested that the different clinical and biological behavior of SS and MF likely reflects the fact that the malignant T cells in these two CTCL variants arise from different memory T-cell subsets. Strong evidence indicates that malignant T cells in SS (leukemic CTCL) are of central memory T-cell phenotype ($CD62L^+/CCR7^+$), which means that they actively recirculate through the skin, blood, and lymph nodes. This is consistent with the clinical presentation of SS with diffuse erythema of the skin, peripheral blood disease, and lymphadenopathy. Furthermore, T cells from MF skin lesions are of effector memory T-cell phenotype. Effector memory T cells are sessile in the skin and do not recirculate through the blood, and this would explain the tendency of MF plaques to remain in fixed anatomic locations for many years [6, 10].

Although likely of different biologic origin, MF and CTCL still share myriad common features. Malignant T cells in MF and SS are mostly $CD4^+$ and only seldom $CD8^+$ T helper cells with characteristic morphology. The cerebriform nuclei are their classical morphogical hallmark and enable detection of malignant cells in blood smears and structurally well-preserved paraffin sections (Fig. 29.1). However, yet at a high-power magnification, CTCL cells are difficult to be detected via conventional laboratory methods. Clonality, CD158κ expression, or loss of CD26 and/or CD7 expression, as well as T-cell scatter profile on flow cytometry are characteristic of malignant T cells, however, a specific biomarker or specific

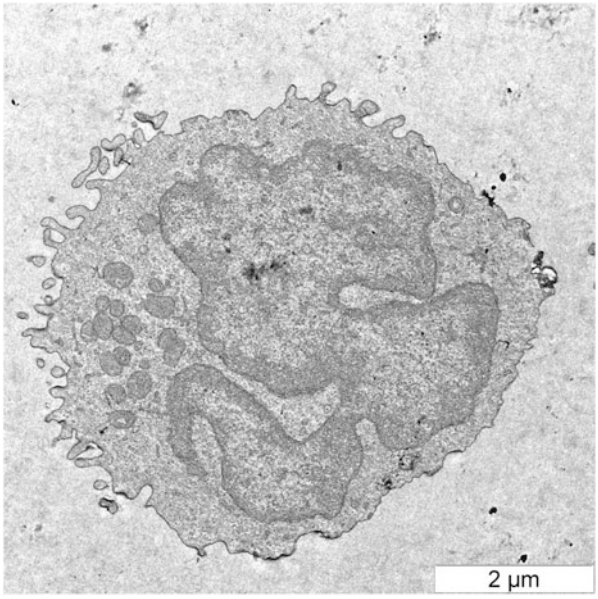

Fig. 29.1 Electron microscopy of a malignant T cell in a patient with Sézary syndrome with characteristic prominent polychromatic cerebriform nucleus. *Scale bar* 2 µm

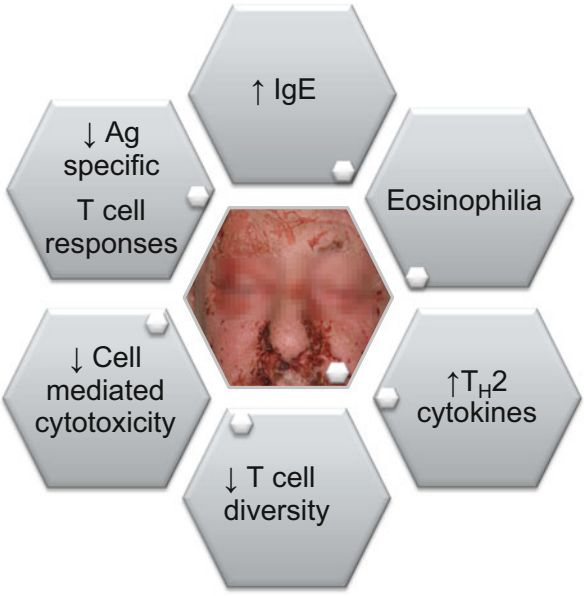

Fig. 29.2 Overview of immunological abnormalities in mycosis fungoides and Sézary syndrome patients

cell surface molecule that reliably identifies them has not yet been unequivocally defined [9, 15, 47].

From an immunological perspective, patients with MF and SS have clinical abnormalities characteristic of Th2 mediated immunologic processes, including decreased antigen-specific T-cell responses, impaired cell mediated cytotoxicity, peripheral eosinophilia, and elevated levels of serum IgE [46]. Notably, the malignant T cells themselves are the main source of extreme excess of Th2 cytokines, and further enforce a global Th2 bias and suppress protective Th1 responses in CTCL individuals [27].

Additionally, increased IL-31 production in malignant T cells has recently been described to correlate with CTCL pruritus [45]; other important cytokines such as IFN-γ, IL-17, or TNF-α tend to be decreased, which likely accounts for the progressive impairment of cellular immunity and notorious susceptibility to infections in MF and SS individuals [33] (Fig. 29.2).

Activated dermal dendritic cells bridge innate and adaptive immune responses and are critical for tumor immune surveillance in the skin. Recently, Schwingshackl and colleagues analyzed the distribution and maturation status of different dendritic cell subsets in cutaneous lesions of MF and SS. Surprisingly, the numbers of dendritic cells are significantly increased within the skin infiltrate of both MF and CTCL; however, these dendritic cells are functionally largely immature. This phenomenon might be associated with induction of tumor tolerance or, on the contrary, may represent an on-going tumor immune surveillance [52]. Albeit critical observations on dendritic cells–T cells interactions in CTCL individuals are limited, activation status of the skin innate immune system might be a key factor for disease progression in CTCL [69, 70].

29.3 Cutaneous B-Cell Lymphomas

Primary cutaneous B-cell lymphomas (CBCL) are a heterogeneous group of lymphoproliferative disorders primarily arising in the skin that differ in biological behavior and prognosis [66]. The three most prevalent CBCL entities are part of the WHO classification of tumors of hematopoetic and lymphoid tissues as well as WHO-EORTC classification of cutaneous lymphomas: primary cutaneous marginal zone B-cell lymphoma (PCMZL), primary cutaneous follicle-center lymphoma (PCFCL), and primary cutaneous diffuse large B-cell lymphoma, leg type (PCDLBCL-LT), which account for >90 % of CBCL [56, 66]. In contrast to cutaneous T-cell lymphomas that can affect large skin areas, CBCL mainly form circumscribed skin lesions, such as singular or multiple papules and nodules. Different types seem to favor different anatomic sites, such as the upper back and arms in PCMZL, head and neck region for PCFCL and lower legs in PCDLBCL-LT [24].

Both PCFCL and PCLBCL-LT are large B-cell lymphoma with yet significantly different clinical courses and prognoses. The five-year survival rate of PCFCL is 95 % [53], and that of PCDLBCL-LT is ~50 % emphasizing its aggressiveness [26, 66]. Tumor cells in PCDLBCL-LT are mainly centroblasts with few admixed reactive elements. Tumor cells in PCFCL consist of follicle center cells with predominantly centrocytic differentiation. PCFCL can have a follicular, follicular and diffuse, or diffuse growth pattern. In contrast to nodal and secondary cutaneous follicular lymphomas, they do not express bcl-2 [8]. Gene expression profiling of PCFCL and PCDLBCL-LT revealed similarities to germinal center B-cell–like (GCB) and activated B-cell–like (ABC) nodal DLBCLs, respectively [29]. Likewise, immunohistochemical analysis could confirm overlaps between PCFCL and GCB [18, 71]. HGAL, B7-DC, MRC, CD130, and CXCR4, which are expressed in germinal center cells from normal tonsil were shown to be positive in PCFCL, but not in PCDLBCL-LT. On the other hand, most of PCLBCL-LT express MUM-1/IRF4 protein whereas PCFCL do not, which is associated with ABC nodal DLBCL [29]. Furthermore, PCDLBCL-LT also express cytoplasmic IgM with or without IgD in contrast to PCFCL. Array-based comparative genomic analysis also revealed differences in chromosomal alterations between PCFCL and PCDLBCL-LT [11].

PCMZL is a very indolent CBCL type with a 5-year disease-specific survival of almost 100 % [66]. Tumor cells of PCMZL consist of small B cells resembling marginal zone cells, lymphoplasmacytoid cells, and plasma cells. Characteristically, plasma cells can be observed particularly in the periphery of the tumor infiltrates. Neoplastic lymphocytes express B-cell markers (CD20, CD79a) and bcl-2, but are negative for CD5, CD10, and bcl-6. Infiltrating plasma cells typically display monoclonal expression of κ-and λ-Ig–light chains. The infiltrate may also include eosinophilic granulocytes, with moderate/marked tissue eosinophilia found almost only in Asian cases [58]. In the last WHO classification, PCMZL is assigned to the group of extranodal marginal zone lymphomas of the mucosa-associated lymphoid tissue (MALToma). However, PCMZL is distinguishable from other

extracutaneous MALTomas on various levels. Unlike other extranodal MALTomas, the translocations t(11;18) and t(14;18)(q32;q21) can be detected in only a small proportion of PCMZL. PCMZL express IgG, IgA, and IgE and thus differ from other extranodal MALTomas, which mostly express IgM. PCMZL with plasmacytic differentiation frequently express IgG4, whereas only 1 out of 120 noncutaneous marginal zone lymphomas expressed IgG4 [3]. Recently, PCMZL was recognized to encompass two subgroups [17, 64]. The more frequent one shows the presence of a class-switched immunoglobulin heavy chain and a distinct T-cell microenvironment with Th2-like cytokines opposite to Th1-like cytokines that characterize many extracutaneous MALTomas. In this subgroup, PCMZL are distinctive because the B cells frequently lack expression of CXCR3, a receptor for interferon-γ–induced chemokines, usually present on activated T cells . If PCMZL are nevertheless expressing CXCR3, they also express IgM and are in some cases associated with *Borrelia burgdorferi* infection [17].

An association with *Borrelia burgdorferi* infection and CBCL development has been reported in European cases [25, 38], which has certain parallels to *Helicobacter pylori*-associated MALTomas of the gastrointestinal tract [20]. Large geographic differences appear to exist, as an association with a *Borrelia* infection has not been observed in all European countries [48], the United States [68], and Asia [41]. On the other hand, Bahler et al. analyzed the immunoglobulin heavy chain variable genes from eight US cases of PCMZL and found the usage of V(H) genes and motifs that suggest antigen triggering as growth stimulator [1]. Furthermore, numerous CD3+ T cells and CD123+ plasmacytoid dendritic cells were described in PCMZL implying antigen presence [39]. Clusters of CD123+ plasmacytoid dendritic cells (PDC) are observed in all PCMZL and the majority of cutaneous B-cell pseudolymphomas and minority of PCFCL, whereas PCDLBCL-LT were negative. Although the role of PDC in PCMZL remains unclear, the high number of PDC may indicate an infectious cause or cross-reacting autoantigens as the primary trigger for PCMZL, as PDC migrate to nonlymphoid tissues in response to inflammatory or infectious stimuli. These findings indicate that a clonally restricted response to chronic antigenic stimulation could play a role in the pathogenesis of PCMZL [39].

Similar to cutaneous T-cell lymphomas (CTCL), a significantly increased number of mast cells is observed in CBCL lesions and those infiltrations are correlated with disease progression [51]. BCA-1 (CXCL-13) and its receptor CXCR5 are constantly expressed in CBCL, but not in healthy skin and CTCL. BCA-1 is also expressed in B-cell pseudolymphoma, and its expression pattern differs between CBCL and B-cell pseudolymphoma. BCA-1 expression is restricted to CD22+ B-cells in CBCL, whereas BCA-1 is expressed in CD35+ dendritic cells in B-cell pseudolymphoma [44]. Expression of programmed death-1 (PD-1) on T cells appears to have prognostic significance in some types of nodal B cell lymphomas [7]. Recently, PD-1 expression, restricted to tumor infiltrating T cells (TILs), was also shown in CBCL [43]. It appears that PCMZL and PCFCL harbor higher numbers of PD-1 positive TILs than PCDLBCL-LT, which in turn may also reflect better prognosis of the former. These data are in line with gene expression profiling

studies on nodal follicle center lymphoma describing a more favorable clinical outcome in patients with a T-cell activation or subgroup signature [23].

In conclusion, CBCL represent a highly heterogeneous disease group that reflects morphologic and functional diversity of B-cells homing into the skin arising after malignant transformation.

29.4 Immunologic Therapeutic Interventions in Cutaneous Lymphomas

In the last decade, immense progress has been made in the biological therapy of primary cutaneous T- (CTCL) and B-cell (CBCL) lymphomas. It is, however, important to note that the majority of cutaneous lymphomas (CL) has an indolent nature and thus does not require aggressive treatment at the beginning. Studies have shown that early aggressive therapies do not improve the prognosis of patients with CTCL as compared with conservative treatment of topical therapies [31]. These and other data have led to a paradigm change in the therapy of CL, where cytotoxic therapies are not used as first-line treatment of indolent CL types. In early stages, the quality of life can be significantly impaired because of cosmetic concerns and itch, whereas advanced stages are often accompanied by immune dysfunctions leading to increased risk of infection and secondary malignancies. Hence, modern therapy algorithms consider the disease stage in order to achieve the best possible, sustainable, and life quality-preserving remission while reducing toxicity.

The simplest immunomodulatory skin-directed approach for CL lesions limited to the skin encompasses intermediate- and high-potency topical corticosteroids, with potency adapted to the infiltration of the lesion [2, 49]. Topically applied corticosteroids exert a multitude of inhibitory effects on both T and B cells which are well known [57] and result in efficient regression of skin lesions of different CL. Phototherapy is another effective skin-directed modality in the treatment of CTCL. Narrow-band ultraviolet B (UVB) light with a wavelength of 311 nm is becoming more popular over psoralen and UVA (PUVA) therapy, as it requires no premedication with photosensitizing psoralen and has fewer side effects, especially in terms of photoaging and UV-associated carcinogenesis. Apart from induction of multiple immunomodulatory molecules, UVB light induces DNA damage with changes in the cell cycle leading to lymphocyte apoptosis [4]. PUVA, on the other hand, not only downregulates expression of skin-homing molecules of epidermotropic malignant T-cells; it is far more potent than UVB in inducing apoptosis of T-lymphocytes in the skin. Although both therapies can be employed with equal success, narrow-band UVB appears to be superior in cases with limited disease [2, 67]. A further development in the field of topical immunomodulators is imiquimod, a small molecule compound that activates Toll-like receptor (TLR) 7 and induces production of myriad pro-inflammatory cytokines [12]. By activating plasmacytoid dendritic cells that express TLR7, imiquimod activates cytokine

production, dominated by interferon (IFN) α, which counteract immune dysbalance in different CL types and promote antitumor immunity [61]. This effect could be potentiated by immunostimulatory activity of the isostearic acid, a major component of its cream vehicle [65].

Systemic immunomodulatory treatment is reserved for advanced stages or for therapy-resistant lymphoma lesions. For this purpose, various recombinant Th1-skewing cytokines, such as IFN-α and IFN-γ are currently routinely used either via intralesional or systemic route to treat these lymphomas [2, 12, 19, 67]. The goal is to reverse lymphoma-induced Th2-potentiated immunosuppression thereby stimulating antitumor immune effectors. To circumvent systemic toxicities, which can be a limiting factor in the treatment with IFNs, an adenovirus containing a human IFN-γ gene was constructed and tested for intralesional application in various CLs, showing overall good tolerability and higher response rates in CBCL than in CTCL [14, 16, 60]. Interestingly, patients who fail to respond to IFN-based treatments appear to have "IFN resistance," that is, defects in the IFN signaling pathway [61, 62]. These defects can be successfully targeted by replicating measles virus that easily lyses lymphoma cells (i.e., oncolytic virus therapy) as they are no longer able to mount an efficient antiviral response [13, 28]. TLR stimulation can also be achieved systemically by employing oligonucleotide CpG-7909 that acts as a TLR9 agonist and induces a similar plethora of cytokines as imiquimod. Promising results have been shown in CTCL patients [34], and further studies are on the way not only in T- but also in B-cell malignancies [40]. In advanced stages of CTCL with leukemic blood involvement, extracorporeal photopheresis (ECP) can be used alone or combined with IFN-α. ECP is a method where circulating mononuclear cells are separated by a leukapheresis-based technique, mixed with psoralen and reinfused to the patient. This therapy option, representing PUVA treatment of blood, is thought to induce apoptosis of circulating malignant lymphocytes and to stimulate antitumor response through release of tumor antigens and generation of dendritic cells from monocytes [67].

Development of monoclonal antibodies (mAbs) targeting surface molecules on T and B cells is a fast growing addition to the repertoire of immunotherapeutic options in CL. In CTCL, targeting CD4 by zanolimumab and CD52 by alemtuzumab shows variable success depending on the lymphoma type treated [2]. Denileukin–diftitox is a fusion protein, which consists of diphtheria toxin fragments conjugated to interleukin 2. After binding to interleukin-2–receptor this molecule is internalized, where it leads to inhibition of protein synthesis and apoptosis [21]. Its activity as a single agent or in combination with chemotherapy has been shown in CTCL patients who are CD25 positive [30]. Brentuximab–vendotin is a conjugate of an antibody against CD30 with methyl-auristatin E, a tubulin destabilizing agent, that can be selectively used for CD30+ CL [63].

In CBCL, expression of CD20 is mandatory for the treatment with rituximab, a human–mouse chimeric antibody against CD20. Rituximab can be used either via an intralesional or systemic route and has shown impressive responses in CBCL [19]. Anti-CD20 antibodies can also be conjugated with various radionuclides, such as yttrium (^{90}Y-ibritumomab tiuxetan) or iodine (^{131}I-tositumomab) and applied

with or without chemotherapy in relapsed patients or patients with refractory disease [59]. Several additional antibodies targeting other molecules on B cells, such as CD22 (epratuzumab), CD19 (Medi-551), and their toxin conjugates inotuzumab-ozogamicin (CD22) and SAR3419 (CD19) come from the field of nodal lymphomas and may be of interest in CBCL. Bispecific T-cell–engager molecules, also known as BiTEs, are another novelty and represent single-chain dual-specific antibodies that target both CD3 on the surface of T cells and a specific antigen on the surface of the malignant B cell in order to induce cell lysis. One BiTE with specificity to CD19, blinatumomab (MEDI-538), has demonstrated high clinical activity in B-cell leukemia and lymphoma patients [59].

Lastly, it should not be left unsaid that not only immunotherapeutics are engaged in immune modulation, but also other compounds used for CL, such as histone deacetylase inhibitors, may have profound immunomodulatory properties through regulation of innate and adaptive immune responses [42]. If all therapies fail, allogeneic stem cell transplantation may become the last resort, particularly in CTCL, inasmuch as autologous stem cell transplantation has yielded disappointing results. Immune interventions in CL have and are working well, emphasizing strong immunogenicity of these tumors and giving hope that the time will come where a therapeutic intervention will not only stabilize but be able to cure the disease.

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Chapter 30
Photodermatology: Therapeutic Photomedicine for Skin Diseases

Akimichi Morita

Abstract Natural sunlight has beneficial effects for various skin conditions and immunoregulatory functions. Phototherapy utilizes the beneficial effects and immunoregulatory functions of natural sunlight. Phototherapy is used for refractory skin disease when topical steroid treatment is not effective. Ultraviolet light (UV) phototherapy using broadband UVB (290–320 nm) and narrowband UVA (311–313 nm) is a well-established treatment for refractory skin disease, such as psoriasis. UV phototherapy has two primary modes of action: apoptosis induction and immune suppression. Narrowband UVB depletes pathogenic T cells by inducing apoptosis and induces regulatory T cells. UVB, psoralen and UVA, and UVA-1 (340–400 nm) are useful treatments of refractory skin diseases, and can be used in conjunction with topical steroids. Selective wavelength phototherapies are used to minimize the carcinogenic risks of UV exposure. UVA-1 effectively penetrates the dermal layers, and is thus superior to UVB, which is mainly absorbed by the epidermis. UVA-1 induces both early and late apoptosis, whereas UVB induces only late apoptosis, making UVA-1 phototherapy particularly effective for treating pathogenically relevant cells, leading to immediate and long-lasting remission. Excimer light (308 nm) therapy effectively targets affected skin without undue exposure of other areas and increases the levels of T regulatory cells. Fewer treatments and a lower cumulative UVB dose are other advantages of excimer light; the greater carcinogenic risk is ameliorated by the reduced number of treatments needed. Intensive studies of phototherapy effects have led to several improvements in the design and protocols, providing several options to patients with skin disease.

Keywords Apoptosis • Excimer light • Narrowband UVB • Regulatory T cells • TH17 • UVA-1

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30.1 Introduction

Ultraviolet light (UV) is divided into UVA (320–400 nm), UVB (290–320 nm), and UVC (200–290 nm). Although the shorter wavelengths of UVC are filtered by the upper layer of the atmosphere and the ozone layer, UVA and UVB wavelengths reach the earth's surface. Natural sunlight has beneficial effects for skin conditions and potent immunoregulatory functions, and is sometimes recommended for psoriasis patients. The immunomodulatory effects of heliotherapy likely underlie the therapeutic effects of photomedicine using UVA and UVB for skin diseases.

UVB radiation suppresses local and systemic immune responses in a mouse model of contact hypersensitivity [16, 17]. In a human study, 20 patients with moderate to severe psoriasis were subjected to controlled sun exposure [32]. Both $CD4^+$ and $CD8^+$ T cells were significantly reduced in the epidermis and dermis of lesional skin. In contrast, dermal $FOXP3^+$ regulatory T cells were relatively increased, but the increase was not statistically significant. In the peripheral blood, skin-homing cutaneous lymphocyte-associated antigen T cells were significantly decreased after only 1 day in the sun and in vitro stimulated peripheral blood mononuclear cells exhibited a reduced capacity to secrete cytokines, that is, tumor necrosis factor-α, interleukin (IL)-12p40, IL-23p19, and IL-17a, after 16 days [32]. These findings demonstrate that sun exposure induces a rapid reduction in local and systemic inflammation, and suggest that natural sunlight has a beneficial wavelength for inducing immunosuppression in patients with immunologic disorders.

30.2 Action Spectra of UV Phototherapy

UV phototherapy using broadband UVB (290–320 nm) and narrowband UVB (311–313 nm) are established treatment modalities for refractory skin diseases, such as psoriasis, atopic dermatitis, and vitiligo [12, 13]. Although the detailed mechanisms that underlie successful treatment of these refractory skin diseases remain unclear, the effectiveness of these phototherapies is considered to depend on their respective action spectra. The action spectrum of UV phototherapy for treating psoriasis was examined in 1980, using a monochromator to produce radiation between 254 and 313 nm [22]. Shorter wavelengths (254, 280, and 290) were not phototherapeutic for psoriasis. Longer wavelengths (296, 300, and 304 nm), however, effectively induced the erythema reactions needed to clear psoriasis, with suberythemogenic exposure of 313 nm resulting in complete clearing in all subjects. These findings suggest that wavelengths greater than 296 nm are more effective phototherapy for psoriasis and led to the successful development of narrowband UVB therapy for clinically effective treatment of psoriasis [12]. Narrowband UVB therapy uses a very narrow wavelength band of 311–312 nm.

30.3 Dual-Action Mechanisms of UV Phototherapy: Apoptosis and Immune Suppression

Within the UVB spectrum (290–320 nm), narrowband UVB (311 nm) phototherapy has become more popular for irradiating refractory lesions [12]. In addition to its efficacy in treating mild and moderate stages of atopic dermatitis, narrowband UVB is particularly effective for treating psoriasis, resulting in faster lesion clearance, fewer excessive erythema episodes, and longer periods of remission [38]. For psoriasis, the efficacy of narrowband UVB (311–313 nm) as compared to broadband UVB (290–320 nm) irradiation is attributed to the ability of 311-nm narrowband UVB to deplete skin-infiltrating T cells more effectively from the epidermis and dermis of psoriatic plaques [21]. Narrowband UVB has two modes of action: induction of apoptosis and induction of antigen-specific immunosuppression [21, 31] (Fig. 30.1). The narrowband UVB-induced depletion of pathogenically relevant T cells is due to the induction of apoptosis [21].

30.3.1 Regulatory T Cells (Treg) Induction

Narrowband UVB therapy generally induces a relatively long remission period of approximately 4–6 months in patients with psoriasis. The induction of apoptosis, however, might be only partially responsible for this relatively long remission period. The role of Treg should also be considered, as narrowband UVB radiation suppresses local and systemic immune responses in a model of contact hypersensitivity [31]. Tregs have an immune regulatory function and play a key role in peripheral tolerance [27, 30]. Peripheral T cells from patients undergoing UVB phototherapy exhibit a $CD4^+CD25^+$ T-cell profile [15]. Induction of Treg cells following UV irradiation is associated with UV-induced DNA damage, which induces Langerhans cells to move from the skin into the draining lymph nodes, and IL-12 can induce DNA repair and limit the number of UV-damaged Langerhans cells in the draining lymph nodes [28]. It is thus possible that UV-induced DNA damage alters cutaneous antigen-presenting cells and enhances their ability to activate Treg cells. UV irradiation increases the proportion of fluorescein isothiocyanate-bearing dendritic cells within the draining lymph nodes [19] that exhibit deficient maturation and deficient T-cell priming [18]. UV irradiation, however, also promotes the generation of $CD4^+CD25^+Foxp3^+$ T cells within the draining lymph nodes following immunization [29]. Among these $CD4^+CD25^+Foxp3^+$ T cells are cells with an antigen-specific regulatory function in vivo [5]. The findings from these experiments suggest that $CD4^+CD25^+Foxp3^+$ T cells, that is, Treg, are responsible for the immunosuppressive effect.

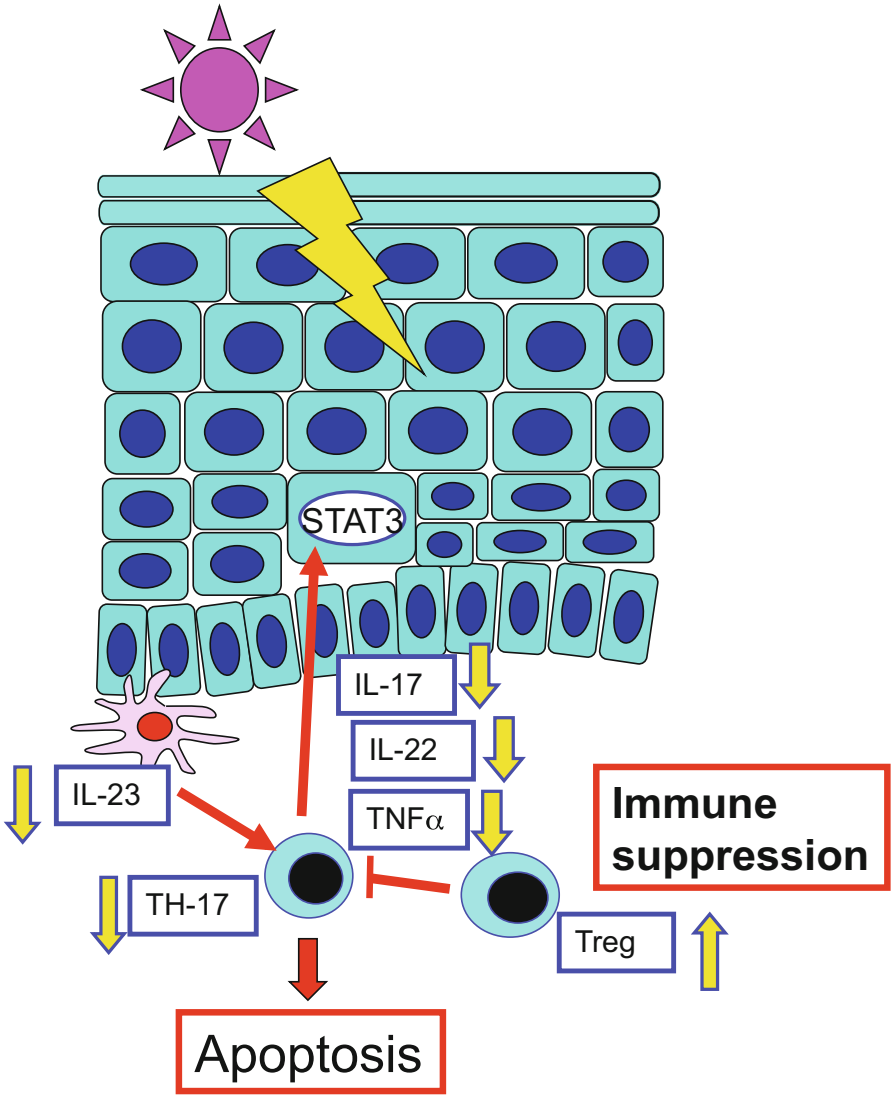

Fig. 30.1 Dual action mechanisms for phototherapy-apoptosis and immune suppression

30.3.2 Dysfunctional Treg in Psoriasis Is Restored by Phototherapy

In psoriasis, there is a functional defect in Treg suppressor activity that is not associated with a decrease in the number of $CD25^+$ Treg in the peripheral blood [34]. The quantity and quality of Treg might be related to the pathogenesis of psoriasis. In our previous study, there was no difference in the percentage of Treg

between psoriasis patients and healthy controls [26]. In another report, the frequencies of circulating Treg were higher in patients with severe psoriasis than in normal controls [40]. We analyzed the number of Treg in 68 patients and 20 controls. Mean Treg levels were not significantly higher in patients than in normal healthy controls. Based on our two independent studies, the number of Treg in psoriasis patients is similar to that in healthy controls [3].

We demonstrated that bath-psoralen UVA (bath-PUVA) therapy induced circulating $Foxp3^+$ Treg in 10 patients with psoriasis who were first treated with phototherapy [26]. We analyzed the Treg levels in 68 patients before and after phototherapy. Although Treg levels were not increased by phototherapy in any of the 68 patients, Treg levels in patients with less than 4.07 % Treg, defined as the mean of the controls, were significantly increased. We further assessed the Treg function before and after phototherapy. To confirm the previous report that psoriasis patients have dysfunctional Treg, we assayed the Treg function. The Treg functional ratio was significantly lower in psoriasis patients than in age-matched controls. The Treg functional ratio was significantly increased and the Treg function was restored to almost normal levels. These findings suggest that successful phototherapy not only increased the Treg number, but also restored the Treg function [3].

30.3.3 Imbalance of Th17 Cells and Treg Is Normalized by Phototherapy

Th17 cells producing IL-17, IL-22, and tumor necrosis factor-α are pathogenically relevant to psoriasis. The imbalance of Th17 cells and Treg is thought to contribute to the pathogenesis of psoriasis. In a clinical study, 14 patients with moderate to severe psoriasis were treated with narrowband UVB. Narrowband UVB suppressed the IL-23/IL-17 pathways, including IL-12/23p40, IL-23p19, IL-17, and IL-22, in normalized plaques, but not in nonresponsive plaques [9]. In another study, gene expression profiling was performed using epidermal RNA from lesional and nonlesional skin undergoing narrowband UVB phototherapy. The Th17 pathway was downregulated during narrowband UVB phototherapy in psoriatic epidermis [24]. In our study, Th17 levels were compared before and after narrowband UVB ($n = 18$) and bath-PUVA ($n = 50$). Th17 levels were not decreased by narrowband UVB and bath-PUVA in the 68 patients. Patients with more than 3.01 % Th17, defined as the mean +1 SD of controls, were defined as the high-Th17 population. Th17 levels in the high-Th17 population were significantly reduced by phototherapy. Our findings indicated that successful phototherapy restored Th17 levels back to normal levels in the high-Th17 population [3].

Serum levels of both IL-17 and IL-22 were significantly increased in psoriasis patients compared to healthy volunteers. Phototherapy significantly decreased serum levels of both IL-17 and IL-22 in psoriasis patients. Furthermore, the percent

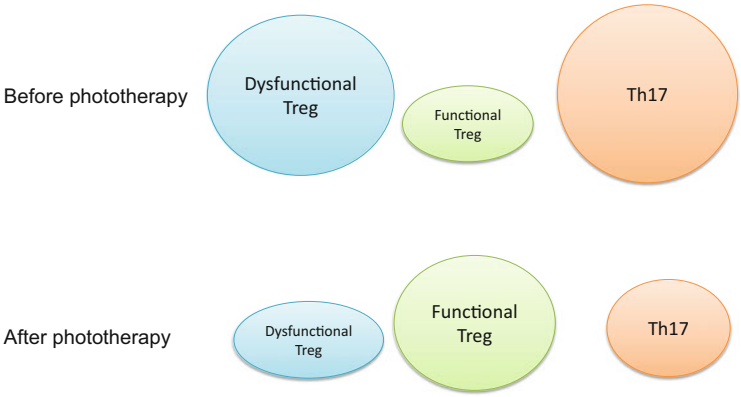

Fig. 30.2 Imbalance of Treg and TH17 in the pathogenesis of psoriasis. After phototherapy, Treg function is restored and Th17 levels in the circulating blood are normalized

reduction of the Psoriasis Area and Severity Index was correlated with serum levels of IL-6, but not IL-17 or IL-22, before phototherapy, suggesting that psoriasis patients with high serum IL-6 levels are more susceptible to phototherapy. Based on this study, phototherapy might suppress serum IL-17 and IL-22 levels by inhibiting the IL-6 induced generation of Th17 [14].

These findings indicate that phototherapy induces a decrease in Th17 and an increase in Treg in the peripheral blood of patients with psoriasis, thereby resolving the TH17 and Treg imbalance in these patients (Fig. 30.2). Treg induction is a target for phototherapeutic efficacy. The detailed mechanisms of Treg induction/restoration, however, remain unclear. Moreover, it is necessary to investigate whether Treg proliferates in lymph nodes or lesional skin. Monitoring Treg function might help to establish a more effective phototherapy regimen in a clinical setting.

30.4 Practical Phototherapy for Atopic Dermatitis: Narrowband UVB, PUVA, and UVA-1

Phototherapy is used for refractory skin disease when topical steroid treatment is not effective. PUVA therapy has been used effectively for general-purpose treatment for 30 years throughout the world. Psoralen is administered orally, topically, or by bath. Due to its efficacy and safety, bath-PUVA therapy is a standard treatment among phototherapies. Narrowband UVB (311 nm) therapy and UVA-1 (340–400 nm) therapy are also applied for the treatment of refractory skin diseases. These phototherapies employ selective wavelengths to reduce the risk of carcinogenicity and increase their efficacy [12]. For example, the first line of treatment for atopic dermatitis comprises topical steroid and moisturizer treatment, as well as instruction regarding lifestyle influences and the removal of any known causes of

skin irritation. Phototherapy is used as a second-line therapy when adverse reactions occur and control is difficult with topical steroid treatment. Phototherapy can result in long-term remission, and it may also be possible to reduce the class of steroid used. In addition, with phototherapy, the topical steroid dosage can be reduced even in cases of resistance to topical therapy alone. For moderate and chronic cases, narrowband UVB can be combined with topical steroid treatment [11].

UVA-1 phototherapy uses a wavelength of 340–400 nm. The exposure dose can be relatively high, leading to erythema, due to removal of the short wavelength, UVA-2 (320–340 nm). For acute and severe cases, UVA-1 phototherapy can be used as monotherapy. UVA-1 therapy may be as effective or even more effective than PUVA therapy for the treatment of atopic dermatitis.

Narrowband UVB phototherapy has proved to be an ideal modality for maintenance therapy once high-dose UVA-1 has been used in the initial phase of management of an acute severe exacerbation of atopic dermatitis [11]. If high-dose UVA-1 therapy is not available, severe atopic dermatitis should be controlled prior to the start of phototherapy by aggressive topical steroid therapy or systemic immunosuppressive modalities, such as cyclosporin A.

30.5 Immunologic Effects of UVA-1 Phototherapy

Photoimmunologic mechanisms responsible for the therapeutic effectiveness of UVA-1 therapy in atopic dermatitis have been investigated. Phototherapy of atopic dermatitis employing UVA-1 (340–400 nm), which effectively penetrates the dermal layers of human skin and thus has the potential to affect intradermal T cells directly, is superior to short-wavelength UVB radiation [10], which is almost exclusively absorbed by the epidermis. Accordingly, successful UVA-1 therapy of atopic dermatitis is associated with a significant downregulation of the in situ expression of T-helper-cell–derived cytokines as well as a significant reduction in the number of intradermal $CD4^+$ T cells [8]. We demonstrated that UVA-1 phototherapy induces apoptosis in T-helper cells present in the eczematous skin of atopic dermatitis patients [20]. UVA-1 irradiation-induced apoptosis in human atopen-specific T-helper cells from atopic dermatitis was mediated by the FAS/FAS-ligand system and induced through the generation of singlet oxygen [20]. To assess whether UVA-1 phototherapy of atopic dermatitis patients induces apoptosis in skin-infiltrating T-helper cells, sequential biopsies were obtained from the eczematous skin of five patients before and after UVA-1 radiation. Before therapy, numerous $CD4^+$ cells were present intradermally in lesional skin. Already after the first UVA-1 radiation exposure, $CD4^+$ apoptotic cells were detected. Subsequent UVA-1 treatments led to further increases in the number of apoptotic cells, and a decrease in the total number of $CD4^+$ cells. After 10 exposures, the total number of intradermally located $CD4^+$ T-cells was significantly diminished, and the remaining cells almost all showed signs of apoptosis.

The mechanisms by which UVA-1 and UVB irradiation induce T-cell apoptosis differ markedly. In general, UVA-1 irradiation can cause preprogrammed cell death (early apoptosis), which is protein synthesis-independent, as well as programmed cell death (late apoptosis), which requires de novo protein synthesis [6]. In contrast, UVB irradiation (and also PUVA treatment) exclusively induces late apoptosis [7]. UVA-1 radiation causes both early and late apoptosis, with UVA-l-R-induced singlet oxygen generation being the initiating event, leading to T-cell apoptosis [20]. Singlet oxygen production induces the expression of Fas-ligand molecules on the surface of UVA-1–irradiated T cells. The key role of singlet oxygen in eliciting early apoptosis in human T cells has been corroborated in an independent study using Jurkat cells. UVA-1 radiation/singlet oxygen is postulated to act on the mitochondria and induce Jurkat cell apoptosis by opening the megachannels and by decreasing the mitochondrial membrane potential [7]. The capacity to induce early apoptosis in mammalian cells seems to be highly specific for UVA-1 radiation and singlet oxygen. From a phototherapeutic point of view, this qualitative difference suggests that UVA-1 phototherapy is superior to UVB or PUVA therapy for skin diseases in which the induction of apoptosis in pathogenically relevant cells is of critical importance. The unique properties of UVA-1 radiation have also stimulated interest in its therapeutic use for patients with cutaneous T-cell lymphoma [23]. In vitro studies indicate that malignant T cells are exquisitely sensitive to UVA-1 radiation-induced apoptosis [39].

Immunohistochemical studies of biopsy specimens obtained from patients with atopic dermatitis undergoing UVA-1 therapy indicate that, in addition to T cells and keratinocytes, epidermal Langerhans cells and dermal mast cells represent target cells for UVA-1 radiation. UVA-1 therapy, in contrast to UVA/UVB therapy, reduces not only the relative number of immunoglobulin E-bearing Langerhans cells in the epidermis, but also the number of dermal $CDla^{+}$ Langerhans cells and mast cells. The latter observation prompted the use of UVA-1 therapy in the treatment of patients with urticaria pigmentosa, in which immediate and long-lasting remissions from cutaneous and systemic symptoms are achieved [33].

30.6 Immunologic Effects of 308-nm Excimer Light Therapy

Excimer light (308 nm) therapy is another option for phototherapy, particularly for the treatment of psoriasis. This method can effectively target the affected area (targeting therapy) and prevent unwarranted exposure of normal skin [1, 2]. Moreover, compared to narrowband UVB, excimer light effectively treats resistant and localized psoriasis lesions with fewer treatments and a lower cumulative UVB dose [1]. The mean number of treatments using an excimer lamp is approximately one-third that of using narrowband UVB phototherapy [9]. Although shorter wavelengths may damage DNA and present greater risks of erythema and

carcinogenesis, such long-term risks might be outweighed by the comparative advantages of fewer necessary treatments and lower cumulative UVB doses.

Pustulosis palmaris and plantaris (PPP) shares many immunologic characteristics with psoriasis, but may have a different genetic background. The proportion of Th17 among the peripheral blood mononuclear cells is 2.52 ± 0.811 % (mean $\pm$ SD) in healthy controls and 3.23 ± 1.45 % in PPP patients. The proportion of Th17 in PPP patients is significantly higher than that in healthy controls. PPP patients have significantly fewer Treg (5.69 ± 1.86 %) than healthy controls (7.10 ± 1.78 %). Th17 is inversely correlated with Treg [35]. In our study, we treated patients with PPP by excimer light (308 nm) with a filter to cut off wavelengths below 297 nm. Twenty patients with PPP were recruited and treated once a week for a total of 30 sessions. Levels of Th17 cells and Treg in the peripheral blood in patients with PPP were also evaluated. Th17 levels after excimer therapy were not significantly different from those at baseline. In contrast, Treg levels after excimer therapy were significantly higher than those at baseline [4]. The increased level of Treg might be related to the efficacy of excimer light for PPP.

30.7 Future Perspectives

Several studies have been performed to understand the photobiologic mechanisms underlying the effects of phototherapy. Based on these investigations, phototherapy is now more rationally designed providing more options available for irradiation protocols and devices. Biologics are highly effective for the treatment of psoriasis. Other refractory skin diseases may also be treated with biologics in the near future. The long-term safety of these biologic therapies, however, is not known, and they are expensive [25]. Phototherapy takes advantage of wavelengths from natural sunlight, which, despite its well-known deleterious effects, such as premature skin aging and cancer, has also long been known to have beneficial effects on skin disease. Sunlight exposure has been historically recommended to maintain health and to treat disorders, and natural sunlight comprises beneficial wavelengths, such as 311-nm narrowband UVB. Several studies are ongoing to investigate various wavelength-dependent effects on both skin disease and the underlying immunomechanisms [36, 37].

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Chapter 31
Collagen Vascular Disease

Yoshihide Asano

Abstract Collagen vascular diseases (CVDs) are a heterogeneous group of multisystem autoimmune disorders characterized by the presence of autoantibodies. It is generally accepted that the initiation and progression of CVDs, including lupus erythematosus, systemic sclerosis, and dermatomyositis, are caused by the complex interplay between environmental and intrinsic factors. The presence of autoantibodies and autoantigens derived from dying cells, such as keratinocytes in cutaneous lupus erythematosus and dermatomyositis and endothelial cells in systemic sclerosis, is a common pathological feature shared among these diseases, which leads to the activation of interferon-dependent signalings because immune complexes consisting of autoantibodies and autoantigens promote type I interferon production especially from plasmacytoid dendritic cells. Type I interferon potentially induces autoimmunity by activating innate and adaptive immunity. In addition, interferon directly induces apoptosis as well as vascular damage, two histologic hallmarks of CVDs in the skin. Note that recent progress provides new insights into the better understanding of the disease-specific pathological process connecting the initial events, cell death of keratinocytes or endothelial cells, with the progressive tissue damage via innate and adaptive immune responses. In this chapter, the role of skin immunology in the pathogenesis of cutaneous lupus erythematosus, scleroderma, and dermatomyositis is overviewed.

Keywords Cutaneous lupus erythematosus • Systemic sclerosis • Dermatomyositis • Interferon • Ro52 • Ultraviolet radiation • Transforming growth factor-β • Autoantibody • Glycosaminoglycan

31.1 Introduction

Collagen vascular diseases (CVDs) are a heterogeneous group of multisystem autoimmune disorders characterized by the presence of autoantibodies. CVDs affect multiple organs with a wide range of clinical presentations and their skin

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lesions are generally characterized by the following common features: (i) inflammatory tissue damage, (ii) tendency to chronicity with acute exacerbations, and (iii) favorable response to high doses of systemic corticosteroids and/or immunosuppressive agents. Clinically important cutaneous manifestations of CVDs comprise cutaneous lupus erythematosus (CLE), scleroderma, dermatomyositis (DM), and vasculitis. Because vasculitis is characterized by inflammation targeting various sizes of blood vessels and is mediated by blood-vessel–specific immune responses rather than skin-specific ones, the role of skin immunology in the pathogenesis of CLE, scleroderma, and DM is overviewed in this chapter.

31.2 Role of Type I Interferon in the Developmental Process of Cutaneous Lupus Erythematosus, Scleroderma, and Dermatomyositis

The developmental process of skin lesions in each CVD is largely different, but the activation of interferon (IFN) signaling pathways is a common pathological feature shared among CLE [8], systemic sclerosis (SSc) [32], and DM [72]. This notion is plausible because immune complexes (ICs) consisting of autoantibodies and autoantigens from dying cells promote IFN-α production from plasmacytoid dendritic cells (pDCs) [67], leading to the induction of IFN-inducible genes from neighboring cells. IFN can induce autoimmunity in several ways: (1) maturation of antigen-presenting dendritic cells (DCs) capable of activating T cells and breaking tolerance to self-antigens; (2) upregulation of MHC molecules as well as autoantigens; (3) activation of effector cells, including natural killer (NK) cells and cytotoxic T cells; and (4) promotion of B-cell differentiation and autoantibody production. In addition, IFN directly induces apoptosis as well as vascular damage, two histologic hallmarks of CVDs in the skin. As described in the following sections, the expression levels of IFN-inducible genes are elevated in skin lesions of CVDs, indicating a possible contribution of the IFN-dependent signaling pathways to the developmental process of skin lesions in CVDs.

31.3 Cutaneous Lupus Erythematosus

Lupus erythematosus (LE) is a chronic autoimmune disease that has a highly variable range of clinical presentation and course. CLE solely manifests dermatological symptoms, whereas systemic LE (SLE) is a life-threatening disease that commonly affects any organ system, including the skin, joints, and cardiovascular and central nervous systems. Except nonspecific cutaneous manifestations of LE, typical CLE lesions are classified into three subtypes, including acute, subacute, and chronic CLE, that share common histological features characteristic of this

disease. The most typical clinical manifestation of acute CLE is a butterfly rash occurring over the bridge of the nose. Subacute CLE represents annular erythema or psoriasis-like scaling erythematous plaques. In chronic CLE, discoid LE (DLE) is the most frequent type and chilblain lupus is its variant. The most prominent histological features of CLE are keratinocyte death and variable inflammation around the dermoepidermal junction. The initial trigger leading to the development of CLE has been believed to be apoptosis of epidermal keratinocytes especially caused by ultraviolet radiation (UVR). This notion is supported by the clinical observation that CLE lesions are preferentially distributed in sun-exposed areas such as face, nose, ears, and neck.

The mechanism underlying the developmental process of CLE has been well studied using experimental photoprovocation. In healthy individuals, the increase in apoptotic keratinocytes is observed only 24 h after UVR exposure and unviable cells completely disappear within 72 h [37], indicating that appropriate phagocytosis efficiently removes apoptotic cells and prevents the release of pro-inflammatory mediators from them. In contrast, apoptotic keratinocytes accumulate up to 72 h after UVR exposure in lupus patients as a result of clearance deficiency [37], which is an intrinsic defect of this disease [46]. When clearance fails, apoptotic cells enter the stage of secondary necrosis. The release of intracellular danger signals from the dying cells potentially plays a crucial role in the breaking of immune tolerance in CLE, but the danger signals from secondary necrotic cells still remain elusive because apoptosis consumes most of well-known danger signals derived from primary necrotic cells. Recent studies have identified danger signals from secondary necrotic cells, including high mobility group Box 1 (HMGB1) associated nucleosomes [69], caspase-cleaved or granzyme B-cleaved autoantigens [54], and uric acid forming monosodium urate crystals in the extracellular space [36]. The detailed mechanism by which these danger signals activate immune cells is largely unknown due to the little information on their cognate receptors, but they activate NFκB and inflammasome in immune cells [12], resulting in the recruitment of monocytes, macrophages, neutrophils, and DCs; production of pro-inflammatory cytokines and chemokines; and upregulation of costimulatory molecules.

Ro52 plays a key part in a series of the pathological events following the initial injuries of epidermal keratinocytes. In CLE lesions, the expression levels of Ro52 are markedly elevated in the cytoplasm of injured keratinocytes, including apoptotic cells and secondary necrotic cells, regardless of the triggers inducing keratinocyte injury [49]. Ro52 is an IFN-inducible E3 ubiquitin ligase and regulates the activity and stability of IFN regulatory factors (IRF) 3, 5, 7, and 8 via ubiquitination and subsequent degradation [13, 15, 24, 25, 35]. Upregulated expression of Ro52 promotes the ubiquitination of these IRFs and suppresses the IFN-dependent signaling pathways. On the other hand, Ro52 promotes apoptosis as shown in a lymphoma-derived B-cell line overexpressing Ro52 [15]. The expression of Ro52 is induced in epidermal keratinocytes injured by UVR exposure within 24 h [49], leading to the suppression of IFN-dependent signaling pathways and the promotion of apoptosis. Because Ro52 binds to the Fc part of any IgG with

unexpectedly high affinity comparable to that of the bacterial superantigen protein A [53], Ro52 derived from dying keratinocytes forms ICs with IgG. Furthermore, anti-Ro52 antibody, which is frequently detected in lupus patients, recognizes the Ro52 antigen, resulting in the formation of massive ICs as detected at the dermoepidermal junction by the lupus band test.

ICs containing nuclear constituents might be recognized by pDCs and amplify type I IFN secretion, especially IFN-α [67]. IFN-α generally induces the expression of Ro52 in immune cells [15, 65] and suppresses the ongoing inflammation by inhibiting their proliferation and promoting their apoptosis. Supporting this notion, Ro52 is highly expressed in infiltrating immune cells of psoriasis, lichen planus, and atopic dermatitis as well as CLE [13, 49], and Ro52-deficient mice show uncontrolled inflammation in response to minor skin injury leading to an LE-like condition [13]. In CLE lesions, Ro52 is expressed at high levels particularly in $CD4^+$ T cells, $CD8^+$ T cells, and macrophages [15, 70]. The presence of anti-Ro52 antibody, which neutralizes E3 ubiquitin ligase activity of Ro52 [14], and a genetic polymorphism of Ro52 [17] potentially leading to its altered expression and/or function may explain a part of the mechanism underlying the dysregulated inflammatory process of CLE.

Pro-inflammatory mediators derived from injured and dying keratinocytes activate macrophages and DCs and promote phagocytosis of cell debris and ICs including autoantigens. DCs may present autoantigens to naïve, potentially self-reactive, T and B cells in the lymph node. An attack of the adaptive immune system via autoreactive antibody and effector T cells induces keratinocyte injury and amplifies inflammation. Upregulated expression of CXCL9, 10, 11, and 12 indicates the contribution of Th1 immune response to the development of CLE lesions [16, 40, 44, 70]. In addition to Ro52, genetic polymorphisms are also reported in IRF5 [20, 29] and IRF7 [18]. Therefore, the impairment of IFN-dependent signaling pathways due to altered expression and/or function of Ro52, IRF5, and IRF7 may be a possible predisposing factor for LE.

Based on the available data described above, the most probable trigger for the onset of LE may be the interplay between environmental factors inducing apoptotic keratinocytes, such as UVR exposure, and intrinsic factors, such as clearance deficiency and altered expression and/or function of multiple genes involved in the regulation of the innate and adaptive immune responses.

31.4 Systemic Sclerosis

Systemic sclerosis (SSc) is a multisystem connective tissue disease characterized by immune abnormalities, vasculopathy, and resultant fibrosis of skin and certain internal organs. Although the pathogenesis of SSc still remains unknown, genomewide association studies have implicated polymorphisms in the HLA locus and other immune-associated genes as risk factors for the development of SSc [1, 52, 73], suggesting that the immune system is a central mediator of this

disease. Consistently, autoantibody production usually precedes the earliest dermal pathological changes, such as alterations in endothelial cell (EC) function and ultrastructure of the microvasculature [50, 63]. Following and/or in parallel with these vascular changes, inflammatory mononuclear cells (MNCs) infiltrate into perivascular regions [31] and further promote EC damage, leading to the development of vasculopathy characteristic of SSc, such as reduction in the number of capillaries, thickening of arteriolar walls, and intimal fibrosis of small arteries. In parallel with the progression of vasculopathy, dermal fibroblasts are activated especially in the perivascular areas [60]. Further activation of dermal fibroblasts due to their intrinsic abnormalities, such as autocrine TGF-β signaling (described below), and tissue hypoxia eventually results in the establishment and maintenance of extensive skin sclerosis. These sequential pathological events of SSc suggest that the microvascular endothelium is one of the major targets of the immune reaction and a potential trigger for subsequent inflammatory changes and the development of fibrosis.

Although the detailed mechanism causing the initial EC damage remains largely elusive, several lines of evidence suggest the contribution of γδT cells and antiendothelial cell antibodies (AECAs) to this pathological process. In the early edematous phase of the disease, the perivascular infiltrate consists primarily of $CD3^+$ T cells, with $CD4^+$ T cells predominating over $CD8^+$ T cells [43]. Note that there are a number of γδT cells infiltrating in the perivascular areas as well. The majority of γδT cells in the skin as well as in peripheral circulation and bronchoalveolar lavage fluid express the Vδ1 chain in SSc patients, whereas the majority of circulating γδT cells are $V\delta2^+$ in healthy subjects [19]. Furthermore, almost all of the peripheral $V\delta1^+$ γδT cells in SSc patients are positive for the activation marker CD49d [19], which mediates adherence of these cells to ECs by interacting with vascular cell adhesion molecule-1. Considering that circulating γδT cells from early diffuse cutaneous SSc (dcSSc) patients favorably bind to ECs and exert a great cytotoxicity against them [30], γδT cells potentially contribute to an immune-mediated damage to the endothelium in the early pathological process of SSc. Another potential mediator of EC damage is a subset of autoantibodies against heterogeneous antigens on ECs, that is, AECAs, which are detectable in serum of 44–84 % of SSc patients [26, 55, 56]. AECAs induce apoptosis of human dermal microvascular ECs through antibody-dependent NK cell cytotoxicity via the Fas pathway, but not via the perforin/granzyme pathway [62]. Because the cytotoxic effect of AECAs is not observed against human umbilical vein endothelial cells [62], AECAs-dependent vascular injury may at least partially explain the reason why EC damage in early SSc selectively occurs in microvasculature.

EC damage generally induces the expression of cell adhesion molecules and chemokines and promotes the infiltration of inflammatory cells to the perivascular areas. Of note, the comparison of gene expression profiles between peripheral blood mononuclear cells from SSc patients and those from healthy controls indicate overexpression of a cluster of gene-encoding molecules that target these cells to the endothelium in those from SSc patients [66]. Because serum levels of cell adhesion molecules correlate with the severity of tissue fibrosis and/or vascular

complications to a variable degree [27, 28, 39, 61, 64], the cell adhesion molecule-mediated interaction between T cells and ECs/other target cells may play a vital role in the development of vascular and fibrotic involvement of SSc.

Tissue fibrosis is the most prominent clinical feature of SSc, which is achieved by fibroblast activation following the initial vascular and immunological events. In the acute inflammatory stage of the disease, fibroblasts expressing type I and type III procollagen are predominantly located around small blood vessels with surrounding infiltration of MNCs [60], suggesting that these immune cells are activated through their interaction with the injured endothelium and subsequently stimulate fibroblasts and enhance their collagen production. In this disease stage, a majority of infiltrating T cells, including $V\delta1^+$ γδT cells, express activation markers, including the early activation marker CD69 [31]. Inasmuch as CD69 regulates cell contact interaction between T cells and other cells, including fibroblasts, activated T cells may affect the activation status of SSc dermal fibroblasts. Notably, the response to activated T cells in a coculture system is different between normal and SSc dermal fibroblasts. In normal dermal fibroblasts, collagen production is suppressed by Th1-polarized cells through membrane-associated IFN-γ [11] and by Th2-polarized cells through membrane-associated TNF-α [10], which overcome a pro-fibrotic effect of IL-4. In contrast, increased collagen synthesis of SSc fibroblasts is more resistant to Th1-polarized cell-mediated suppression and completely unresponsive to Th2-polarized cells [10, 11]. Given Th2 immune polarization during the early active stage of dcSSc (as described below), unresponsiveness to Th2-polarized cell-mediated suppression may contribute to fibroblast activation in early stage of dcSSc. As well as direct interaction, cytokines secreted by activated T cells are involved in the regulation of ECM deposition by fibroblasts [9]. It has been suggested that Th1 cytokines, including IFN-γ, generally decrease ECM deposition, whereas Th2 cytokines, including IL-4, IL-6, IL-10, and IL-13, increase it [9]. A series of studies suggests that the Th1/Th2 paradigm largely contributes to tissue fibrosis of SSc. In the early stage of dcSSc with progressive skin sclerosis, serum levels of IL-6 and IL-10 are significantly elevated, and their levels are decreased to normal levels in the late stage of dcSSc with the improvement of skin sclerosis [58]. Another Th2 cytokine, IL-4, keeps normal levels in the early stage of dcSSc, but is decreased as well in the late stage of dcSSc. In contrast, serum levels of IL-12, a key Th1 cytokine, are decreased in the early stage of dcSSc, then gradually increase in parallel with disease duration and finally reach significantly higher levels than normal controls in the late stage of dcSSc with the resolution of skin sclerosis [42]. Of note, the levels of maximal serum IL-12 levels throughout the disease course inversely correlate with mortality in early dcSSc with diffuse cutaneous involvements. Thus, immune polarization in SSc generally shifts from Th2 to Th1 in parallel with disease duration, whereas the sustained Th2 immune polarization closely associates with exacerbation of the disease. As for Th17 cytokines, the expression levels of IL-17A, but not IL-17F, are increased in the skin of early dcSSc. Furthermore, IL-17A suppresses collagen production in normal dermal fibroblasts, but not in SSc dermal fibroblasts at least partly due to the

loss of IL-17 receptor type A [47]. Collectively, Th1/2/17 paradigm regulates tissue fibrosis in SSc.

TGF-β is a key growth factor regulating the activation status of dermal fibroblasts in SSc patients. Although the expression pattern of TGF-β in the lesional skin of SSc is still controversial, TGF-β expression levels generally seem to be higher in patients with early active disease, but weak or undetectable in patients with sclerotic disease. A series of studies regarding the expression profiles of the three isoforms of TGF-β have elucidated the following findings: (i) expression of TGF-β1 and TGF-β2 is most prominent around dermal vessels and is associated with infiltrating MNCs; (ii) staining for all three isoforms of TGF-β is detected in the ECM; (iii) TGF-β mRNA or protein is detected in fibroblasts, particularly those located closely to infiltrating MNCs; and (iv) TGF-β2 transcripts colocalize with α1 (I) collagen mRNA and are found in perivascular distribution and surrounded by infiltrating MNCs [22, 38, 51]. Given that the nature of TGF-β is determined by the state of activation and differentiation of the target cells and the presence and concentration of other cytokines and growth factors, TGF-β potentially promotes inflammation by recruiting leukocytes through regulation of cell adhesion molecule expression and creation of a chemokine gradient, by activating leukocytes, and by inducing various pro-inflammatory cytokines and other mediators in an early stage of the disease. In the sclerotic stage, by contrast, SSc dermal fibroblasts are constitutively activated with the pro-fibrotic phenotype quite similar to that of normal fibroblasts treated with TGF-β1 even though the expression of TGF-β is weak or undetectable in the skin [5]. This observation suggests that once activated as a result of vascular and immunological events SSc fibroblasts establish a self-activation system at least partially via autocrine TGF-β signaling. The increased expression of latent TGF-β receptors, including integrin αVβ3, αVβ5, and thrombospondin-1, contribute to this process in SSc dermal fibroblasts [2–4, 6, 45]. These receptors recruit and activate latent TGF-β on the cell surface and efficiently increase the concentration of active TGF-β around SSc fibroblasts. Therefore, dermal fibroblasts may be constitutively activated by autocrine TGF-β in SSc lesional skin.

Another subset of infiltrating immune cells in the lesional skin of early SSc is B cells and plasma cells, which are occasionally, but not frequently seen [50]. Note that a cluster of genes associated with B cells is strongly expressed in lesional and nonlesional skin of SSc patients [71]. Taken together with the evidence that hypergammaglobulinemia, autoantibody production, and CD19 overexpression in B cells are seen in SSc [57], B cells may play a critical part in the pathogenesis of SSc.

Autoantibodies in SSc are classified into two groups: autoantibodies directed against nuclear antigens (topoisomerase I, centromere, RNA polymerase I/III, etc.), and autoantibodies with putative pathogenic roles. Of note, antinuclear antibodies specific to SSc, such as antitopoisomerase I antibody and antinucleolar antibodies, react with nuclear antigens derived from apoptotic cells (e.g., apoptosis of ECs by AECAs) and these ICs induce the production of IFN-α from circulating pDCs [32], which potentially induces autoimmunity in several ways (as already described).

The other group of autoantibodies with putative pathogenic roles includes antibodies against fibrillin-1, MMP-1, MMP-3, and PDGR receptor. Antifibrillin-1 antibodies are detectable in more than 50 % of SSc patients and can activate fibroblasts and stimulate release of TGF-β [74]. Antibodies to MMP-1 and MMP-3 may also occur in a high proportion of patients, preventing the degradation of excessive collagen [48, 59]. A putative pathogenic autoantibody to PDGF receptor has been recognized in SSc patients, and implicated in collagen gene overexpression by fibroblasts [7]. Therefore altered B-cell function may be a key link between autoimmunity and tissue fibrosis.

Thus, accumulating evidence suggests the central role of the immune system in the development of vasculopathy and tissue fibrosis in SSc. However, this notion is still largely based on several hypotheses. At this moment, the lack of animal models recapitulating all three cardinal features, including immune abnormalities, vasculopathy, and fibrosis, has hindered further progress in understanding the pathogenesis of this disease.

31.5 Dermatomyositis

DM is an idiopathic inflammatory myopathy characterized by proximal muscle weakness, muscle inflammation, and inflammatory skin rash. DM skin lesions include Gottron's papules (violaceous papules overlying the dorsal interphalangeal, metacarpophalangeal, elbow, or knee joints), violaceous poikiloderma (atrophic, hypo/hyperpigmented macules with telangiectasia around the anterior neck, chest, shoulders, back, and buttocks), a periorbital heliotrope rash, and periungual telangiectasias. Histopathology of DM skin lesions features an inflammatory infiltrate of $CD4^+$ and $CD8^+$ T cells, pDCs, and neutrophils, along with basal keratinocyte vacuolar alteration with apoptotic keratinocytes, endothelial cell damage, and mucin deposition.

On histological examination, cutaneous lesions of DM are mostly indistinguishable from CLE lesions. However, evidence demonstrated the difference in the pathological process inducing apoptosis of epidermal keratinocytes between DM and CLE. Granzyme B released by $CD8^+$ T cells plays a primary role in CLE lesions, whereas the Fas/Fas-ligand death receptor pathway is relatively dominant for keratinocyte apoptosis in DM skin lesion [21, 68]. Furthermore, the IFN signature is also different between CLE lesions and DM skin lesions. As already described, IFN-α is deeply associated with the developmental process of CLE lesions. In contrast, there is a closer correlation of IFN-inducible genes with transcript levels of IFN-β and IFN-γ, compared with those of IFN-α in DM skin lesions [72]. Consistently, the number of pDCs, a main producer of IFN-α, does not correlate with the downstream IFN response gene MxA expression in DM skin lesions [41]. The difference in the mechanism of vascular injury is also shown between CLE lesions and DM skin lesions. Complement deposition and overexpression of serglycin, a regulator of leukocyte adhesion, are detected in the

blood vessels of DM skin lesions, but not in CLE lesions [34]. Thus, these distinct molecular events are present behind the similar histological changes of CLE and DM skin lesions.

Recently, a hypothesis linking mucin deposition with the developmental process of Gottron's papules has been reported. Glycosaminoglycans in DM skin lesions, especially Gottron's papules, contain large amounts of chondroitin-4-sulfate (C4S), a molecule with both pro-inflammatory and anti-inflammatory properties, as well as increased levels of CD44 variant 7 (CD44v7), a ligand for C4S, and its binding partner, osteopontin (OPN). Note that mechanical stretching induces the expression of CD44v7 and IFN-γ, but not IFN-α or IL-1α, induces the expression of OPN in cultured dermal fibroblasts. Furthermore, mechanical stretching of fibroblasts, particularly in combination with OPN treatment, augments the adhesion of human monocytic cell line THP-1 cells in a process dependent on CD44v7 [33]. These results provide a new hypothesis explaining the preferred localization of rash overlying joints and support the previous data that IFN-γ, rather than IFN-α, plays a critical role in DM skin lesions.

As described above, recent studies have provided useful clues to understand the mechanism underlying the developmental process of cutaneous lesions in DM. Similar to LE and SSc, a variety of autoantibodies are detectable and their clinical associations have been clarified in DM [23], but the role of autoantibodies in the pathogenesis of this disease still remains unknown. Furthermore, the mechanism leading to keratinocyte and vessel damage is much less clear in DM than in LE and SSc. These are largely attributable to the lack of animal models recapitulating selected aspects of DM.

31.6 Conclusions

It is generally accepted that the initiation and progression of CVDs, including LE, SSc, and DM, are caused by the complex interplay between intrinsic and environmental factors. The presence of autoantibodies and autoantigens derived from dying cells, which are typical factors associated with intrinsic abnormality and environmental influence, respectively, is a common pathological feature shared among these diseases. Recent progress has provided new insights into the better understanding of the disease-specific pathological process connecting the initial events, such as cell death of keratinocytes or ECs, with the progressive tissue damage. In order to make further progress in the understanding of the pathogenesis of CVDs and in the development and testing for therapies against selected aspects of them, further studies, including the generation of animal models recapitulating all clinical features, are required in the future.

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