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Invited review article

Novel insights into cutaneous immune systems revealed by *in vivo* imaging

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MPM, Multiphoton microscopy; DC, Dendritic cell; MC, Mast cell; CHS, Contact hypersensitivity; LC, Langerhans cell; dDC, Dermal dendritic cell; dLN, Draining lymph node; YFP, Yellow fluorescence protein; LTB4, Leukotriene B4; CXCL2, Chemokine (C-X-C motif) ligand 2; iSALT, Inducible skin-associated lymphoid tissues; PD-1, Programmed death-1; CTLA-4, Cytotoxic T-lymphocyte-associated protein 4; Treg, Regulatory T cell

ABSTRACT

In vivo imaging is a novel experimental approach for biological research. Multiphoton microscopy (MPM), a type of fluorescence microscopy, is a new tool for *in vivo* imaging analysis. MPM allows observation of both tissue structures and cell behaviors or cell–cell interactions in living animals in real time. Skin is an ideal tissue for MPM analysis as it is directly accessible to the microscope. In the skin, immune cells cooperate to maintain skin homeostasis or to exert immune responses against foreign antigens. *In vivo* imaging by MPM analysis provides precise information on cell dynamics in the skin, and has significantly expanded our knowledge of the cutaneous immune system. In this review, we will discuss recent insights related to the mechanisms of allergic skin inflammation that have been revealed by MPM analysis.

Introduction

The skin contains various kinds of immune cells, such as dendritic cells (DCs), T cells, and mast cells (MCs). Their dynamic interactions are essential for the maintenance of homeostasis and also for the induction/regulation of cutaneous immune responses.[1,2] For example, DCs continuously migrate and scan antigens in the skin. When DCs capture antigens, they present them to effector T cells, which then activate and produce various cytokines to eliminate the antigens. Here, to exert their effector functions to an appropriate extent and with correct timing, their motility and the duration of interaction must be tightly regulated spatiotemporally. If not, these immune responses may cause unnecessary tissue damages as a result of excessive T cell activations, or they may instead fail to protect the host from the antigens due to inadequate T cell activations.

Until recently, cell dynamics analysis has mainly been performed by *in vitro* systems. For example, chemotaxis chamber assay is frequently performed to investigate cell migration ability *in vitro*. However, it remains unknown whether such cell dynamics in a culture system actually reflect *in vivo* cell behaviors, since tissue conditions, such as collagen fiber structures, blood supply, oxygen concentration and cell–cell interactions, significantly affect cell dynamics *in vivo*. Immunohistochemical analysis is the conventional method used to analyze cell localization and behaviors in tissues. Although this analysis can extract *in vivo* information, it provides a static picture of a certain specific moment in the

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continuous flow of biological phenomena, and it provides limited information related to cell dynamics. For the accurate evaluation of cell movement and cell–cell interaction *in vivo*, direct observation of the cells in a living animal is necessary.

Multiphoton microscopy (MPM) is a type of fluorescence microscopy that excites fluorophores with long-wavelength photons. Since the excitation of fluorophores with multiphotons occurs only on focal planes, the resolution of MPM is very high. In addition, long-wavelength photons penetrates deep into tissues with low phototoxicity, which enables long time-lapse observation with minimal tissue damages. Moreover, some tissue structures, such as collagen fibers and elastins, are visualized without fluorescent labeling by multiphoton excitation. Based on these characteristics, MPM is a useful tool for *in vivo* imaging with subcellular resolutions. Using this technique, we have investigated immune cell dynamics in various skin conditions.[3–7] In this review, we will introduce how MPM analysis has expanded our knowledge of the cutaneous immune systems in allergic inflammation, with a focus on contact dermatitis.

Contact dermatitis

Contact dermatitis, such as metal allergy and plant allergy, is a kind of delayed-type hypersensitivity in skin, and is a common allergic skin disease affecting approximately 15–20% of the world's populations.[8] Contact hypersensitivity (CHS) is a frequently used mouse model of contact dermatitis. Chemicals that induce contact dermatitis and CHS are small compounds called haptens.[9,10] Haptens binds to self-proteins, which become immunogenic neo-antigens.[10] Haptens or haptenized self-proteins activate innate immune cells, such as keratinocytes, MCs, and macrophages, which produce various chemical mediators, and induce two important phases in CHS: the sensitization phase and the elicitation phase (Fig. 1). In the sensitization phase, skin DCs capture antigens (hapten-self complex), and migrate to draining lymph nodes (dLNs) to induce differentiation and proliferation of antigen specific T cells (mainly Th1 and Tc1 cells). When the same hapten enters the skin, the antigen-specific T cells are activated in the skin by antigen-captured cutaneous DCs. The activated T cells produce various cytokines/chemokines and induce skin inflammation.[11] This inflammatory phase is called the elicitation phase. Using the CHS model, we have analyzed how DC and T cell dynamics are regulated in the skin during allergic inflammation.[12–14]

DC dynamics after hapten application

At least three DC subsets have been identified in the skin: Langerhans cells (LCs; located in the epidermal layer) and two dermal DC (dDC) populations. These two dDC subsets express different patterns of surface molecules and are classified as langerin-positive (or CD103-positive) DCs and langerin-negative (or CD11b-positive) dDCs.[2] In the sensitization phase, dDCs, especially langerin-positive dDCs, are considered to be the essential cell populations that mediate the sensitization,[15–17] although other dDC subsets have the ability to exert the functions.[18–22]

CD11c-YFP mice, which express yellow fluorescence protein (YFP) under the transcriptional control of mouse integrin alpha X (CD11c), are frequently used for the visualization of DCs by MPM.[23] LCs and dDCs are clearly visualized in CD11c-YFP mice (Fig. 2). In the steady state skin, dDCs exhibit active motility with polarized morphology.[4,24,25] After hapten application, dDCs exhibit a

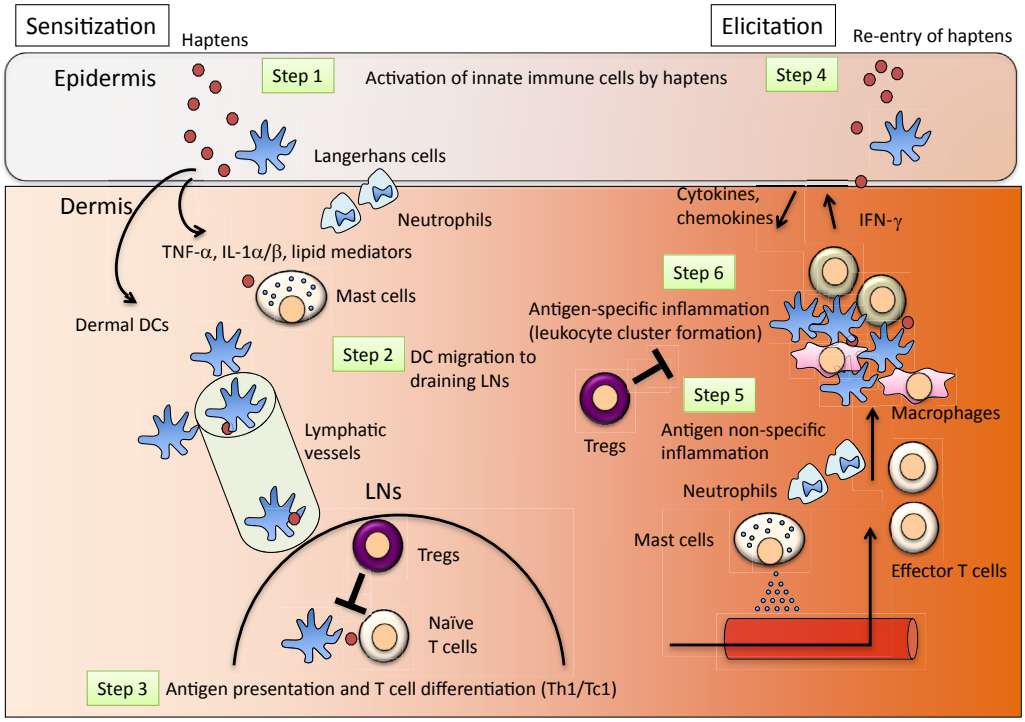

Fig. 1. Overview of the immunological mechanisms of CHS. Step 1. Haptens activate innate immune cells (e.g. keratinocytes, mast cells) and induce the production of various chemical mediators. Step 2. Antigen-captured activated DCs migrate to the dLNs. Step 3. Migrated DCs present the antigen to naïve T cells, which in general differentiate them to Th1 and Tc1 cells. Step 4. Haptens generally induce subtle inflammation by activating innate immune cells, and recruit neutrophils. Haptens also activate macrophages, which lead to leukocyte cluster formation, which is called iSALT. Step 5. Leukocytes including antigen-specific effector T cells are recruited to form iSALT. Step 6. The antigen-specific effector T cells are activated in the skin by antigen-captured dermal DCs, which induce antigen-specific inflammation. Activation of effector T cells mainly occurs in leukocyte clusters, iSALT. Tregs play inhibitory roles in both the sensitization and elicitation phases.