

Update of Immune Events in the Murine Contact Hypersensitivity Model: Toward the Understanding of Allergic Contact Dermatitis

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Allergic contact dermatitis (ACD) is one of the most common skin diseases, consisting of sensitization and elicitation phases. With the advancement of technology and the discovery of new types of immune cells, our knowledge of the immunological mechanisms of contact hypersensitivity (CHS) as a murine model of ACD has expanded significantly in the past decade. For example, by introducing regulatory T cells, CD4⁺ T-helper 17 cells, and Langerin-positive dermal dendritic cells, the initiation and termination mechanism of CHS has been revealed. In addition, the role of mast cells in CHS, long a matter of debate, has become apparent by developing conditional mast cell-deficient mice. Moreover, the role of the innate immunity system, such as that of Toll-like receptor signaling, has made a breakthrough in this field. In this review, we will integrate the recent advancement of immunological mechanisms of both the sensitization and elicitation phases of CHS into the classic view, and we will discuss updated mechanisms on its development and future directions.

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INTRODUCTION

Allergic contact dermatitis (ACD) is one of the most common skin diseases, affecting 15–20% of the general population worldwide (Peiser *et al.*, 2012). It is classified as a delayed-type hypersensitivity response, and murine contact hypersensitivity (CHS) is one of the most frequently used animal models

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 in both mice and humans, and the important roles of those cell subsets in ACD have been implicated from studies on CHS. In addition, the discovery of Langerin-positive dermal dendritic cells (DCs) questioned the relevance of epidermal Langerhans cells (LCs) as key antigen-presenting cells in cutaneous immune responses, although the existence of DCs in humans has not been revealed yet. As a result, some key dogmas on the development of ACD have been changing drastically. In this review, we summarize the recent reports of CHS, integrate recent advances into the classic view of CHS, and discuss the updated mechanisms of CHS development.

SECTION 1: EVENTS IN SENSITIZATION PHASE

Haptens, skin barrier function, and keratinocytes

Most of the chemicals that induce CHS are small compounds called haptens, which typically have a molecular mass of <500. Haptens require chemical interactions with proteins to elicit adaptive immune responses (Lepoittevin and Karlberg, 1994; Lepoittevin, 2006). Upon hapten application, keratinocytes are activated and produce various chemical mediators, such as tumor necrosis factor α , IL-1 β , and prostaglandin E₂, which promote the migration and maturation of skin DCs (Cumberbatch and Kimber, 1995; Cumberbatch *et al.*, 1997; Kabashima *et al.*, 2003).

The skin's barrier function prevents the entry of allergens into the body. The first line of defense is formed by the stratum corneum, the uppermost layer of the epidermis. In the stratum corneum, filaggrin (FLG), a late epidermal differentiation protein, has a pivotal role in barrier function. It has been reported that loss of function variant of *FLG* leads to impaired skin barrier function and is a strong predisposing factor for atopic dermatitis (Palmer *et al.*, 2006). *FLG* mutations may also increase susceptibility to haptens, because mutations within the *FLG* gene were reported to represent a predisposition to contact allergy in both mice (Moniaga *et al.*, 2010) and humans (Novak *et al.*, 2008; de Jongh *et al.*, 2008). On the other hand, other reports failed to find a clear association between contact allergy risk and *FLG* mutations (Lerbaek *et al.*, 2007; Schnuch *et al.*, 2010); therefore, further studies are needed to confirm the association between *FLG* mutation and contact dermatitis.

Tight junctions act as a second barrier to block antigen penetration into the skin. These junctions are composed of

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Abbreviations: ACD, allergic contact dermatitis; ATP, adenosine triphosphate; CHS, contact hypersensitivity; DC, dendritic cell; dDC, dermal DC; LC, Langerhans cell; MHC, major histocompatibility complex; NLR, NOD-like receptor; NOD, nucleotide-binding oligomerization domain; Ox, oxazolone; Th17, T-helper 17; TLR, Toll-like receptor; TNCB, trinitrochlorobenzene; Treg, regulatory T cell

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several proteins, such as claudins and occludins, in the stratum granulosum. Protein antigens are blocked from penetrating the body by this barrier. It has recently been reported that activated LCs can elongate their dendrites above this barrier to take up protein antigens (Kubo *et al.*, 2009) and that LC deficiency leads to reduced clinical symptoms and antigen-specific IgE production in an ovalbumin-induced AD model (Nakajima *et al.*, 2012), suggesting the important role of LCs in mediating sensitization with protein antigens. However, because haptens are composed of molecules that are small enough to penetrate the dermis across the tight junction, all cutaneous DC subsets may encounter haptens.

Roles of cutaneous DC subsets in sensitization

Until recently, LCs have been considered to be central cells for antigen presentation in the sensitization phase of CHS because (1) they have potent antigen-presenting ability *in vitro*, (2) they exist abundantly in the epidermis, and (3) their artificial removal by drugs, such as gliotoxin application to the skin, results in impaired CHS response (McMinn *et al.*, 1990). However, novel depletion systems of LCs (Langerin-diphtheria toxin receptor knock-in mice) have revealed that Langerin⁺ dermal DCs (dDCs), but not LCs, may have a crucial role in sensitization (Kissenpfennig *et al.*, 2005; Bursch *et al.*, 2007; Wang *et al.*, 2008). We and others have, however, recently reported that these two populations work in a compensatory manner to initiate sensitization (Honda *et al.*, 2010a; Noordegraaf *et al.*, 2010). Consistently, Batf3-deficient mice that lack Langerin⁺ dDCs exhibited a normal CHS phenotype (Edelson *et al.*, 2010), suggesting the compensation of its function by other DCs.

It has also been reported that LCs have a regulatory role, rather than a stimulatory role, during sensitization in CHS. Research on another LC depletion system that uses the human *Langerin* promoter combined with diphtheria toxin A (the active subunit of diphtheria toxin (Kaplan *et al.*, 2005)) revealed that congenitally LC-depleted mice exhibited an exacerbated CHS response, suggesting that LCs may have a suppressive function in CHS by producing IL-10 (Igyarto *et al.*, 2009).

Thus, although the function of LCs in sensitization remains controversial, Langerin⁺ dDCs probably exert stimulatory effects during sensitization. The function of Langerin[−] dDCs in sensitization has not yet been investigated intensively; however, Langerin[−] dDCs also appear to have stimulatory functions in sensitization, because ablation of both LCs and Langerin⁺ dDCs before sensitization impairs CHS response but is unable to abrogate it completely (Bursch *et al.*, 2007; Wang *et al.*, 2008; Honda *et al.*, 2010a).

As antigen presentation is required for both the sensitization phase and the elicitation phase of CHS, it is important to assess the relevance of DC subtypes in both phases separately. We previously demonstrated that the depletion of skin DCs in hapten-sensitized mice enhanced the effector phase of CHS (Grabbe *et al.*, 1995). This suggests the existence of some DC subsets that have a regulatory function in the elicitation phase.

In certain situations, it has been reported that LCs and Langerin⁺ dDCs have completely distinct roles. For example, in a gene-gun immunization model, LCs mediated the production of Th2-type Ig, IgG1, whereas Langerin⁺ dDCs mediated Th1-type Ig, IgG2a (Nagao *et al.*, 2009). In a cutaneous candida infection model, LCs induced Th17 cells, whereas Langerin⁺ dDCs promoted Th1 cells (Igyarto *et al.*, 2011). In addition, LCs induced Tregs in UV- or skin graft-induced immunosuppression (Yoshiki *et al.*, 2009, 2010; Schwarz *et al.*, 2010). Thus, LCs may have both positive and negative immunological functions in a context-dependent manner.

Innate immune activation

As mentioned earlier, full activation of DCs is essential for the establishment of sensitization with haptens. However, the molecular mechanisms that explain how haptens activate DCs remain unknown. Martin *et al.* have intensively investigated the involvement of the innate immunity system in hapten-induced DC activation, and they have reviewed this aspect comprehensively (Martin *et al.*, 2011).

Innate immunity is activated through pattern recognition receptors, such as membrane-associated Toll-like receptors (TLRs) and cytosolic nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs). DCs are activated through the cooperation of these two pathways (Martin *et al.*, 2011). Among TLRs, TLR2 and TLR4 have a critical role in DC maturation in CHS induced with trinitrochlorobenzene (TNCB), oxazolone (Ox), and FITC (Martin *et al.*, 2008). The endogenous ligands for these receptors might be generated by hapten-induced degradation of extracellular matrix via reactive oxygen species from DCs (Bruchhausen *et al.*, 2003; Matsue *et al.*, 2003; Mehrotra *et al.*, 2005), because haptens can induce the production of reactive oxygen species in DCs *in vitro* (Soltes *et al.*, 2006; Stern *et al.*, 2007; Naik and Dixit, 2011). For example, biglycan, a derivative from extracellular matrix, is a possible candidate for the endogenous TLR2/4 ligand and an activator of NACHT-, LRR-, and pyrin (NALP) 3 inflammasome of NLRP3 (Schaefer *et al.*, 2005; Babelova *et al.*, 2009). Hyaluronic acid is also degraded by reactive oxygen species, and blocking of the hyaluronic acid degradation significantly reduced sensitization for CHS, suggesting the degraded hyaluronic acid as another endogenous activator of TLR2/4 signaling in CHS (Martin *et al.*, 2008).

Nickel (Ni²⁺) is one of the most frequent causes of contact dermatitis in humans, although it rarely occurs in mice. Earlier studies have reported that coadministration of adjuvant, such as complete Freund's adjuvant or lipopoly-saccharide, efficiently induced Ni²⁺ allergy in mice, suggesting the important role of TLR4 signaling in efficient sensitization with Ni²⁺. Very recently, Schmidt *et al.* (2010) reported that Ni²⁺ 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 Ni²⁺. These reports indicate the crucial roles of TLR4 in Ni²⁺ allergy in both mice and humans.

Haptens also activate keratinocytes in an NLR-dependent manner. Among the NLR family, NLRP3 controls the production of proinflammatory cytokines through activation of caspase-1. Without NLRP3 or its adaptor protein ASC (Sutterwala *et al.*, 2006; Watanabe *et al.*, 2007a, 2008), the production of IL-1 β and IL-18 from keratinocytes was inhibited, which resulted in impaired DC migration and T-cell priming and led to impaired CHS (Antonopoulos *et al.*, 2001; Nakae *et al.*, 2003; Antonopoulos *et al.*, 2008).

The adenosine triphosphate (ATP) and purinergic receptor P2X7-dependent pathways have been reported as being crucial for NLRP3 inflammasome activation by contact allergens. ATP is the main energy carrier within cells. Stressed, damaged, or dying cells release ATP into the extracellular space, which interacts with purinergic receptors on different immune and structural cells. Triggering of P2X7, a kind of ligand-gated ion channel (Surprenant and North, 2009), by ATP induces the activation of the NLRP3 inflammasome through K⁺ efflux from the cell (Martinon *et al.*, 2009). Loss of ATP-P2X7 signaling results in the failure of NLRP3 activation, which leads to impaired sensitization capacity of DCs and absence of CHS in P2X7-deficient mice (Weber *et al.*, 2010). Indeed, contact allergens, such as TNCB and Ox, induce the release of ATP from skin cells *in vivo* (Martin *et al.*, 2011). In addition to NLRP3, NLRP12 regulates sensitization in CHS. NLRP12 is expressed on DCs and neutrophils, and a lack of NLRP12 resulted in the impaired migration of skin DCs to draining lymph nodes (dLNs) and neutrophil infiltration into the skin, leading to severely impaired CHS (Arthur *et al.*, 2010).

Together, these hapten-induced irritant effects through innate immune systems are essential for the activation of cutaneous antigen-presenting cells (Figure 1) and thereby

determine the allergenic potential of a hapten during the sensitization phase. During the elicitation phase of CHS, they induce the activation of skin endothelial cells and thereby initiate the inflammatory response via antigen nonspecific mechanisms.

Activation of mast cells

The role of mast cells in CHS has been controversial. In some studies, mast cell-deficient mice exhibited reduced inflammation in TNCB-induced CHS (Askenase *et al.*, 1983; Biedermann *et al.*, 2000). Other studies reported undiminished CHS induced with TNCB or DNFB (Galli and Hammel, 1984; Mekori and Galli, 1985). Furthermore, a recent publication reported that mast cells have regulatory roles through the production of IL-10, as mast cell-deficient mice exhibited enhanced urushiol and DNFB-induced CHS (Grimbaldeston *et al.*, 2007). 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⁻sh/W⁻sh}). 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.

Recently, a novel mast cell ablation system was established by two independent groups (Dudeck *et al.*, 2011; Otsuka *et al.*, 2011). In these mice, mast cells can be conditionally depleted through the administration of diphtheria toxin. Both groups reported that mice depleted of mast cells exhibited reduced CHS induced with FITC or Ox (Dudeck *et al.*, 2011) and also with DNFB or Ox (Otsuka *et al.*, 2011). In addition, mast cell-specific deletion of IL-10 did not result in exacerbated CHS. Without mast cells, skin DC migration/maturation and T-cell priming in the sensitization phase were

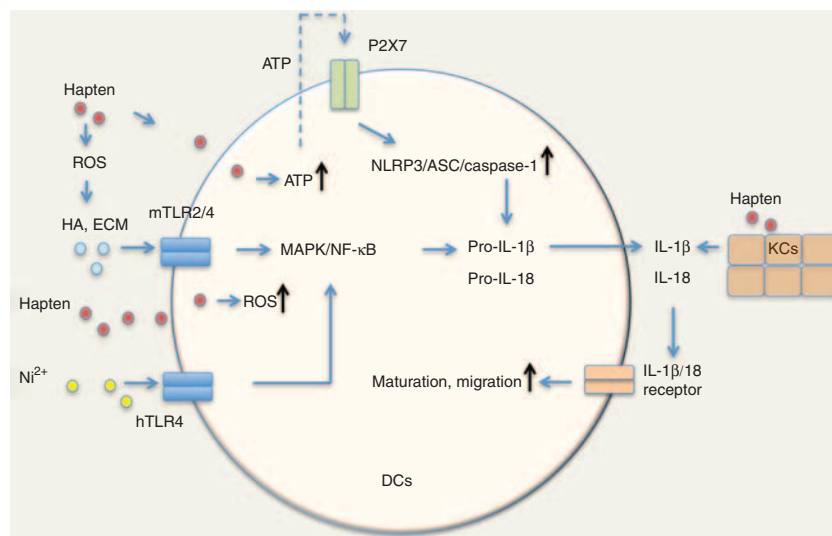


Figure 1. Activation of the innate immune system by contact allergens (haptens, Ni²⁺). Dendritic cells (DCs) are activated by haptens directly or indirectly through degraded hyaluronic acid (HA)-TLR2/4 signaling, which induces pro-IL-1 β and pro-IL-18 transcription. Adenosine triphosphate (ATP)-P2X7 signaling activates NLRP3, which results in caspase-1 activation and the production of the biologically active form of IL-1 β and IL-18. These cytokines induce DC maturation and migration toward draining lymph nodes; and they contribute to the sensitization process. IL-1 β and IL-18 from neighboring cells such as keratinocytes (KCs) are also important in this process. ASC, apoptosis-associated speck-like protein containing a caspase recruitment domain; ROS, reactive oxygen species.

impaired. Mast cells stimulated DCs via ICAM-1 or lymphocyte function-associated antigen 1 interaction and by membrane-bound tumor necrosis factor α on mast cells. 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 (Dudeck *et al.*, 2011).

To date, it remains unknown why there is such a discrepancy between the reports using stem cell factor 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-1 α and IL-1 β (Swope *et al.*, 1994; Yu *et al.*, 2009). In addition, because of the congenital absence of mast cells in *Kit^{W/W^v}* and *Kit^{W-sh/W-sh}* mice, a compensatory mechanism may exist, such as the repopulation of the skin with basophils (Piliponsky *et al.*, 2010). Therefore, *Kit^{W/W^v}* and *Kit^{W-sh/W-sh}* mice may not necessarily be appropriate to evaluate the exclusive roles of mast cells.

SECTION 2: EVENTS IN ELICITATION PHASE

Antigen-nonspecific inflammation: keratinocytes, mast cells, and neutrophil activation

The mechanism of inflammatory cell infiltration in the elicitation phase has been explained using both antigen-nonspecific and -specific inflammatory signals (Grabbe *et al.*, 1996; Grabbe and Schwarz, 1998). Following antigen-nonspecific inflammation, antigen-specific T cells are recruited to the inflammatory sites and are activated by the antigen, which causes antigen-specific inflammation. Keratinocytes, neutrophils, and mast cells are the main factors to create the antigen-nonspecific inflammation at elicitation. First, haptens stimulate keratinocytes to produce proinflammatory cytokines such as IL-1 β and tumor necrosis factor α in an NLR-dependent manner (Sutterwala *et al.*, 2006; Watanabe *et al.*, 2007a). 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 histamine (Dudeck *et al.*, 2011), which helps neutrophils to 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 neutrophil recruitment (Biedermann *et al.*, 2000; Honda *et al.*, 2009). The initial neutrophil recruitment is proposed to be essential for subsequent T-cell infiltration, because the depletion of neutrophils reduces CD8⁺ T-cell infiltration and leads to impaired CHS (Dilulio *et al.*, 1999; Engeman *et al.*, 2004). Indeed, one of the immunosuppressive mechanisms of the action of topical steroids in CHS may be mediated through its inhibition of neutrophil and macrophage functions, not by T-cell or keratinocyte functions (Tuckermann *et al.*, 2007).

Overall, the aforementioned mechanism creates the first round of antigen-nonspecific inflammation, which is an important step for subsequent antigen-specific inflammation. Indeed, when the concentration of haptens is not high enough to provoke this antigen-nonspecific inflammation, no CHS reaction occurs (Grabbe *et al.*, 1996) (see Figures 1 and 2).

Antigen-specific inflammation: T-cell activation

Following the antigen-nonspecific inflammation, T cell-mediated antigen-specific inflammation is initiated. When T cells infiltrate into the skin, they are activated by cutaneous antigen-presenting cells, and they 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 (Egawa *et al.*, 2011), and inhibition of CD86 expression by siRNA resulted in reduced inflammation (Ritprajak *et al.*, 2008), suggesting that effector T cells are activated locally by hapten-carrying antigen-presenting cells.

Cytokines produced by activated T cells then stimulate skin-resident cells, which leads to further recruitment of T cells, amplifying the inflammation. Each T-cell subset (i.e., Th1/T-cytotoxic (Tc) 1, Th2, and Th17/Tc17) activates the skin-resident cells differently and forms their specific type of inflammation. For example, the expression level of CXCL9 in wild-type mice and IL-17 receptor-deficient mice in DNFB-induced CHS skin lesions was comparable, whereas the expression level in IFN- γ receptor-deficient mice was marginal, indicating the importance of IFN- γ signaling for CXCL9 induction. In contrast, CXCL1 was strongly expressed in IFN- γ receptor-deficient mice, whereas its expression was significantly reduced in IL-17 receptor-deficient mice (He *et al.*, 2009). The importance of each cytokine in the development of CHS will be discussed again in the latter part of this section.

Kinetics of histological change and chemokine expression

In TNCB-induced CHS, capillary vasodilation occurs within 3 hours after the challenge, and neutrophils accumulate in the skin around 6 hours later. Lymphocytes then start to infiltrate into the skin within 12 hours after the challenge, and massive infiltration of lymphocytes and to a lesser extent of neutrophils constitutes the inflammatory cells 24 hours after the challenge (Mitsui *et al.*, 2003, 2004). Differential and sequential expressions of multiple chemoattractant factors, mainly chemokines, regulate such sequential cell infiltration patterns. Mitsui *et al.* (2003) have analyzed the kinetic profiles of chemokine mRNA expression using microarray analysis, and they classified the chemokines into four groups based on their kinetic pattern. At the early time point (3 hours post challenge), the expression level of neutrophil-recruiting chemokines, such as CXCL1, is increased, and that of lymphocyte-recruiting chemokines, such as CXCL9 and CXCL10, is increased around 6–9 hours post challenge and sustained until at least 24 hours post challenge, which corresponds well with the time course of the histological findings (Mitsui *et al.*, 2003).

In human ACD, on the other hand, the cell infiltration and chemokine expression patterns are slightly different from

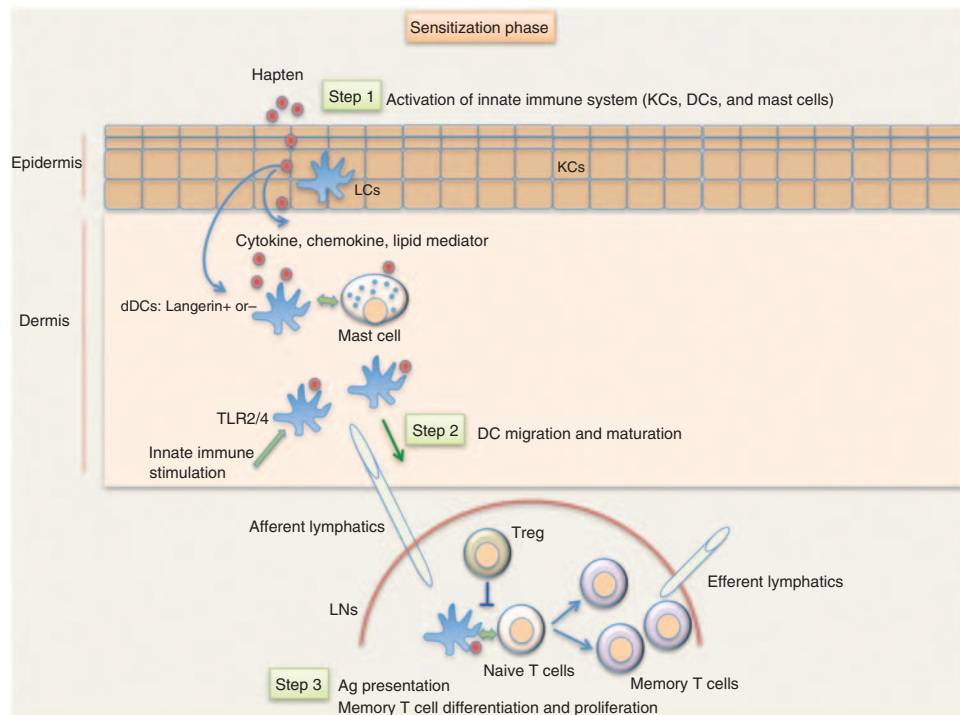


Figure 2. A schematic view of the sensitization phase. Step 1: Haptens activate keratinocytes (KCs) and mast cells directly or indirectly through innate immune systems. The activated KCs and mast cells produce various chemical mediators, which activate cutaneous dendritic cells (DCs). Step 2: The activated DCs capture antigens, start maturation, and migrate to the dLNs via afferent lymphatics. Step 3: Migrated DCs present antigen to naive T cells in dLNs. Antigen-specific clones differentiate and proliferate into effector T cells. Tregs affect DC function and have a suppressive role in effector T-cell generation. dDC, dermal DC; dLNs, draining lymph nodes; LC, Langerhans cell; LN, lymph node; Tc, T cytotoxic; Th, T helper; Treg, regulatory T cell.

those in CHS. Goebeler *et al.* (2001) analyzed the kinetics of cell infiltration and chemokine mRNA expression in patients after a patch test. CCL2 was detected 6 hours after the challenge, followed by CCL5 and CCL22 expressions within 12 hours. Thereafter, expression of CXCL9, CXCL10, CCL17, and CCL18 reached maximum levels at 72 hours (Goebeler *et al.*, 2001). The expression levels of neutrophil-attracting chemokines, such as CXCL1 and CXCL2, were weak. Although the histology did not exhibit significant changes 6 hours after the antigen challenge, inflammatory cells began to appear 12 hours after the challenge and remained for at least 96 hours. CD3⁺ cells were the most dominant type of cells at all time points. The second most dominant cells were CD68⁺ macrophages, and neutrophils were relatively fewer than other cell types. It remains to be elucidated whether neutrophils are prerequisites for subsequent cell infiltration in ACD. In addition, the role of macrophages at the elicitation phase remains unclear and should be pursued in future studies.

Keratinocytes are the most important source of chemokine production. The activated keratinocytes produce multiple chemokines, such as CXCL1, CXCL2, CXCL9, CXCL10, CCL8, CCL17, and CCL27 (Flier *et al.*, 2001; Karsak *et al.*, 2007; Mori *et al.*, 2008; Honda *et al.*, 2009). CXCL10 is a ligand for CXCR3, which is strongly expressed on Th1 cells. Blockade of CXCL10 and CXCL10-deficient mice exhibited reduced TNCB-induced CHS in C57BL/6 mice (Nakae *et al.*, 2003) or DNFB-induced CHS in 129Sv/C57BL/6 F1 mice,

indicating the important role of CXCL10-CXCR3 signaling for T-cell infiltration. The CCL27-CCR10 and CCL17/22-CCR4 axes constitute another important mechanism for T-cell recruitment to skin (Reiss *et al.*, 2001; Homey *et al.*, 2002; Wang *et al.*, 2010). Although the blocking effect of these chemokines was reported differently depending on the model used, as shown in Table 1, this is likely related to the dominant T-cell subsets induced under each experimental condition. Neutrophil-recruiting chemokines also have important roles, because a blockade of CXCL1 or a deficiency of its receptor (CXCR2) leads to reduced CHS (Dilulio *et al.*, 1999; Cattani *et al.*, 2006) induced by TNCB and/or DNFB. Infiltrated T cells then stimulate the neighboring cells to produce cytokines and chemokines, which create a positive feedback loop of T-cell infiltration and local chemokine production. The activated T cells, as well as other inflammatory cells, contribute to dermal edema and epidermal spongiosis, which peaks 24–48 hours after hapten challenge. It has been proposed that IFN- γ -producing T cells trigger Fas-induced apoptosis of keratinocytes (Trautmann *et al.*, 2000) and that cytokine-activated keratinocytes produce hyaluronan and decrease E-cadherin expression, which leads to spongiosis (Ohtani *et al.*, 2009).

Effector cells: CD4⁺ and CD8⁺ T cells

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

Table 1. Summary of the roles of chemokines and chemokine receptors in CHS

Hapten	Chemokine/chemokine receptor	Mouse strain	CHS response	Reference	Proposed phase of involvement/note
TNCB	Anti-CXCL10 antibody	C57BL/6	Impaired	Nakae <i>et al.</i> (2003)	Elicitation phase
Ox	Anti-CXCL1 antibody	BALB/c	Impaired	Dilulio <i>et al.</i> (1999)	Elicitation phase
	CXCR2 KO	BALB/c	Impaired	Cattani <i>et al.</i> (2006)	
	Anti-CCL27 antibody	BALB/c	Impaired	Wang <i>et al.</i> (2010)	Elicitation phase
	CCL27 Tg	C57BL/6	Normal	Kagami <i>et al.</i> (2008)	
	Anti-CCL17 antibody	BALB/c	Normal	Wang <i>et al.</i> (2010)	Elicitation phase
	Anti-CCL22 antibody	BALB/c	Normal	Wang <i>et al.</i> (2010)	Elicitation phase
	Anti-CCL17/22 antibody	BALB/c	Impaired	Wang <i>et al.</i> (2010)	Elicitation phase
	Anti-CXCL1 antibody	BALB/c	Impaired	Dilulio <i>et al.</i> (1999)	Elicitation phase
DNFB	CXCR2 KO	BALB/c	Impaired	Cattani <i>et al.</i> (2006)	
	CXCL10 KO	129sv/C57BL/6 F1	Impaired	Dufour <i>et al.</i> (2002)	
	Anti-CCL27 antibody	BALB/c	Impaired	Homey <i>et al.</i> (2002)	Elicitation phase
	Anti-CCL27 antibody	C57BL/6	Normal	Reiss <i>et al.</i> (2001)	Elicitation phase
	CCR4 KO	C57BL/6	Normal	Reiss <i>et al.</i> (2001)	Elicitation phase
	CCR4 KO plus anti-CCL27 antibody	C57BL/6	Impaired	Reiss <i>et al.</i> (2001)	Elicitation phase
	CCL27 Tg	C57BL/6	Normal	Kagami <i>et al.</i> (2008)	
	Anti-CXCL1 antibody	BALB/c	Impaired	Takeshita <i>et al.</i> (2004b)	Elicitation phase
FITC	Anti-CXCL2 antibody	BALB/c	Impaired	Takeshita <i>et al.</i> (2004b)	Elicitation phase

Abbreviations: CHS, contact hypersensitivity; Ox, oxazolone; TNCB, trinitrochlorobenzene.

are important in the elicitation of CHS. Gocinski and Tigelaar (1990) first revealed the distinct function of T-cell subsets by performing antibody depletion experiments. Through depletion of CD8⁺ T cells in the elicitation phase, the CHS response was reduced, whereas depletion of CD4⁺ T cells in either the sensitization phase or the elicitation phase resulted in an enhanced and prolonged response, suggesting that CD8⁺ T cells have effector functions, whereas CD4⁺ T cells have regulatory roles (Gocinski and Tigelaar, 1990; Bour *et al.*, 1995; Xu *et al.*, 1996; He *et al.*, 2009). However, the simultaneous depletion of both CD8⁺ and CD4⁺ T cells at the elicitation phase further reduced the CHS response, suggesting that CD4⁺ T cells contain both effector and regulatory populations (Gocinski and Tigelaar, 1990; Wakabayashi *et al.*, 2005).

The establishment of gene-targeting mice that lack CD4⁺ (major histocompatibility complex (MHC) class II-deficient mice) and CD8⁺ (MHC class I-deficient mice) T cells is another approach for investigating the role of T-cell subsets in CHS. In MHC class I-deficient mice, the CHS response was completely absent, whereas MHC class II-deficient mice developed enhanced reactions (Bour *et al.*, 1995). In another report, however, MHC class II-deficient mice exhibited reduced CHS (Takeshita *et al.*, 2004a). CD4-deficient mice, which have no CD4⁺ T cells (Tyznik *et al.*, 2004; Saint-Mezard *et al.*, 2005), exhibited an impaired CHS response (Kondo *et al.*, 1996; Wang *et al.*, 2000). In adoptive transfer experiments, the transfer of either CD8⁺ or CD4⁺ T cells

induced CHS (Gautam *et al.*, 1991; Vocanson *et al.*, 2006; He *et al.*, 2009). Taken together, these studies indicate that CD8⁺ T cells mainly have proinflammatory effector functions, whereas CD4⁺ T cells have both proinflammatory and anti-inflammatory functions that are dependent on their cytokine production pattern or subset, as we discuss in the next section.

Effector cytokines in the elicitation phase: the role of Th1/Tc1, Th2/Tc2, and Th17/Tc17

CD4⁺ Th cells and CD8⁺ 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. Th1/Tc1 cells are characterized by the secretion of IFN- γ ; Th2/Tc2 cells by IL-4, IL-5, and IL-13; and Th17/Tc17 cells by IL-17A and IL-22 production (Weaver *et al.*, 2007). As high levels of IFN- γ and an accumulation of Th1/Tc1 cells were observed at the elicitation sites, it was previously believed that CHS was mainly mediated by Th1/Tc1 and regulated by Th2 cells (Grabbe and Schwarz, 1998). However, as each hapten may have its own properties to induce a dominant type of Th/Tc subset, and the type of mouse strain used can also influence the results, we have to consider these factors when interpreting the role of each T-cell subset in CHS. Table 2 shows a summary of previous reports discussing the role of IFN- γ , IL-4, and IL-17 in CHS. Typical haptens used in CHS include TNCB, DNFB, Ox, and FITC. Among them, TNCB, DNFB, and Ox have generally been regarded as Th1 IFN- γ -

Table 2. Summary of the roles of cytokines in CHS

Hapten	Cytokine	Mouse strain	CHS response	Reference	Proposed phase of involvement/note
TNCB	IFN- γ KO	C57BL/6	Impaired	Mori <i>et al.</i> (2008)	Elicitation phase
	IFN- γ KO	C57BL/6	Normal	Nakae <i>et al.</i> (2003)	
	IFN- γ KO	BALB/c	Normal	Nakae <i>et al.</i> (2003)	
	IFN- γ receptor KO	129sv	Normal	Saulnier <i>et al.</i> (1995)	Reduced inflammatory cell infiltration
	r-IL-4	BALB/c	Impaired	Biedermann <i>et al.</i> (2001)	Elicitation phase
	Transfer of Th2 cells	BALB/c	Impaired	Biedermann <i>et al.</i> (2001)	Elicitation phase
	r-IL-4	C3H/HeN	Normal	Gautam <i>et al.</i> (1992)	Sensitization phase
	r-IL-4	CeH/HeN	Impaired	Gautam <i>et al.</i> (1992)	Elicitation phase
	Anti-IL-4 antibody	C3H/HeN	Normal	Gautam <i>et al.</i> (1992)	Sensitization phase
	Anti-IL-4 antibody	C3H/HeN	Increased	Gautam <i>et al.</i> (1992)	Elicitation phase
	Anti-IL-4 antibody	CBA/J	Normal	Salerno <i>et al.</i> (1995)	Sensitization phase
	Anti-IL-4 antibody	CBA/J	Impaired	Salerno <i>et al.</i> (1995)	Elicitation phase
	IL-4 KO	C57BL/6	Impaired	Dieli <i>et al.</i> (1999)	
	r-IL-4	C57BL/6	Increased	Dieli <i>et al.</i> (1999)	Elicitation phase
	STAT6 KO	C57BL/6	Impaired	Yokozeki <i>et al.</i> (2000)	Sensitization
	Anti-IL-4 antibody	BALB/c	Increased	Asada <i>et al.</i> (1997)	
	IL-17AKO	129/C57BL/6 F1	Impaired	Nakae <i>et al.</i> (2002)	Sensitization
Ox	IFN- γ receptor KO	129Sv	Normal	Saulnier <i>et al.</i> (1995)	Reduced inflammatory cell infiltration
	IL-4 KO	BALB/c	Normal	Traidl <i>et al.</i> (1999)	
	IL-4 KO	129J	Normal	Berg <i>et al.</i> (1995)	
	IL-4 KO	C57BL/6	Normal	Berg <i>et al.</i> (1995)	
	IL-4 KO	C57BL/6	Normal	Dieli <i>et al.</i> (1999)	
	STAT6 KO	C57BL/6	Impaired	Yokozeki <i>et al.</i> (2000)	
DNFB	IFN- γ receptor KO	C57BL/6	Normal	Reeve <i>et al.</i> (1999)	
	IFN- γ receptor KO	C57BL/6	Impaired	He <i>et al.</i> (2009)	Elicitation phase
	Anti-IFN- γ antibody	C57BL/6	Normal	He <i>et al.</i> (2006)	Elicitation phase
	IFN- γ KO	C57BL/6	Impaired	Mori <i>et al.</i> (2008)	Elicitation phase
	STAT6 KO	C57BL/6	Impaired	Yokozeki <i>et al.</i> (2000)	
	STAT6 KO	C57BL/6	Increased	Takeshita <i>et al.</i> (2004a)	
	IL-4 KO	C57BL/6	Impaired	Weigmann <i>et al.</i> (1997)	Late phase reaction
	IL-4 KO	BALB/c	Impaired	Traidl <i>et al.</i> (1999)	
	IL-13 KO	C57BL/6	Increased	Herrick <i>et al.</i> (2003)	
	IL-17A KO	129/C57BL/6 F1	Impaired	Nakae <i>et al.</i> (2002)	
	IL-17R KO	C57BL/6	Impaired	He <i>et al.</i> (2009)	Elicitation phase
	Anti-IL-17 antibody	C57BL/6	Increased	He <i>et al.</i> (2009)	Elicitation phase
FITC	IFN- γ R2 KO	C57BL/6/129sv	Impaired	Lu <i>et al.</i> (1998)	
	r-IFN- γ	BALB/c	Impaired	Takeshita <i>et al.</i> (2004a)	Sensitization phase
	Anti-IL-4 antibody	BALB/c	Impaired	Takeshita <i>et al.</i> (2004a)	Sensitization phase
	IFN- γ KO	C57BL/6	Impaired	Mori <i>et al.</i> (2008)	Elicitation phase
	STAT6 KO	C57BL/6	Impaired	Takeshita <i>et al.</i> (2004a)	

Abbreviations: CHS, contact hypersensitivity; Ox, oxazolone; Th, T helper; TNCB, trinitrochlorobenzene.

inducing haptens, whereas FITC is regarded as inducing Th2 IL-4, possibly owing to the adjuvant effects by dibutyl phthalate, which is used as a standard solvent for FITC (Larson *et al.*, 2010).

However, as shown in Table 2, several conflicting results have been reported even among the same hapten-mouse strain combination, especially with regard to the role of IFN- γ and IL-4. For example, a normal ear-swelling response was demonstrated in IFN- γ -deficient mice in both a C57BL/6 and a BALB/c background in TNCB-induced CHS (Nakae *et al.*, 2003). However, another group reported that IFN- γ -deficient mice in a C57BL/6 background exhibited reduced ear swelling in CHS induced by three different haptens (TNCB, DNFB, and FITC) (Mori *et al.*, 2008). They concluded that IFN- γ acted on keratinocytes to produce Th1 chemokines, which lead inflammatory cell infiltration into the skin during the elicitation phase of CHS (Mori *et al.*, 2008). As for IL-4, administration of an IL-4-blocking antibody exacerbated (C3H/HeN) (Gautam *et al.*, 1992) or decreased (CBA/J) (Salerno *et al.*, 1995) in TNCB-induced CHS. Deficiency of the STAT6 that transmits IL-4/13 signaling resulted in reduced (Yokozeki *et al.*, 2000) or increased (Takeshita *et al.*, 2004a) ear swelling in DNFB-induced CHS in a C57BL/6 background. It has been reported that IL-17 has a stimulatory role in both the sensitization phase (Nakae *et al.*, 2002) and the effector phase (He *et al.*, 2009), and no contradictory results have been reported so far. As IL-17 is produced by many other cells, such as $\gamma\delta$ T cells and neutrophils, it would be interesting to investigate the contribution of IL-17 produced by each cell population in the development of CHS.

Although there is no clear explanation of why such a discrepancy exists, experimental data on the relative importance of T-cell subsets for the elicitation of CHS appear to depend on the experimental model used. Indeed, the relevant factors that determine the polarization of the emerging antigen-specific T cells during the priming/sensitization phase of CHS may be the microenvironment in which the antigen is first presented (skin vs. other body sites; normal vs. inflamed skin) and the type of antigens and the genetic background of the individual. Moreover, ongoing immune responses against microbiota in the animal housing facility may also affect the type of T-cell responses generated in this context. Similarly, the skin microenvironment may also regulate which T-cell subsets are activated during the effector phase of CHS.

Other mechanisms in antigen-specific inflammation: involvement of B cells and natural killer T cells

B cells and natural killer T cells may also contribute to the development of CHS, although their relative importance to this type of immune response remains unclear. It has been demonstrated that IL-4-producing invariant natural killer T cells in the liver were activated by hapten application (Campos *et al.*, 2006a, 2006b) and promoted the migration of peritoneal B1-like B cells to lymphoid organs and their production of hapten-specific IgM. In the elicitation phase, hapten-specific IgM can bind to the haptens and activate a complement to generate C5a (Tsuji *et al.*, 2002). C5a then stimulates mast cells and platelets, which release serotonin

and tumor necrosis factor α , leading to local endothelial activation and subsequent inflammation (Tsuji *et al.*, 2000; Tamagawa-Mineoka *et al.*, 2007).

Regulation of inflammation: Regulatory T cells and B cells

Evidence has accumulated regarding the regulatory mechanisms of Tregs in CHS (Honda *et al.*, 2011). Transfer of Tregs before elicitation suppresses the ear-swelling response, for which several different suppression mechanisms have been proposed. The first is that IL-10 from Tregs inhibits leukocyte influx into the skin by downregulating E/P-selectin expression in blood endothelial cells (Ring *et al.*, 2006). The second is through an adenosine-mediated mechanism. Tregs generate adenosine by the degradation of ATP through CD39/73, which is strongly expressed on Tregs. Adenosine can act as a suppressor of leukocyte activation and can also block leukocyte influx into the skin by downregulating E/P-selectin expression in blood endothelial cells (Ring *et al.*, 2009). However, ATP is not just a substrate for the generation of adenosine, it also activates the immunosuppressive function of Tregs through its receptor P2X7 (Ring *et al.*, 2010a). Tregs from IL-10-deficient mice had poor ability to produce adenosine and were not activated by ATP, despite the equivalent expression of CD39/73 and P2X7 (Ring *et al.*, 2011); this suggests that the impaired suppression ability of IL-10-deficient Tregs is not solely attributable to the loss of IL-10 production. Transfer of Tregs during the sensitization phase also suppresses CHS. Tregs in lymph nodes acquire an activated phenotype through ATP, and the activated Tregs establish gap junctions with DCs in lymph nodes, which causes a reduction in the capacity of DCs to stimulate CD8⁺ T cells (Ring *et al.*, 2010b).

Although the exogenous transfer of Tregs can suppress inflammation, the extent to which the endogenous Tregs contribute to the suppression of CHS was unknown. To address this issue, Treg-specific depletion *in vivo* is required. As Foxp3 is a transcriptional factor for Tregs and exists intracellularly, depletion of Tregs by a neutralizing antibody was technically difficult. To solve this problem, Foxp3 reporter mice expressing human CD2 and CD52 chimeric protein have been generated and designated as Foxp3^{hCD2/hCD52} mice (Tomura *et al.*, 2010). In the mice, because Foxp3⁺ cells coexpress hCD2/hCD52 on the cellular surface, Foxp3⁺ Tregs are depleted with neutralizing anti-hCD52 antibody. When Tregs were depleted during the sensitization phase, it caused higher effector CD4⁺ and CD8⁺ T-cell induction and led to enhanced and prolonged ear swelling, indicating that endogenous Tregs contribute significantly to the control of sensitization (Honda *et al.*, 2010b). In addition, Vocanson *et al.* (Vocanson *et al.*, 2006) have reported that, by depleting CD4⁺ T cells before sensitization, CHS developed in mice with the weak hapten. This did not normally induce CHS in mice, and it therefore suggests the suppressive role of endogenous Tregs in sensitization.

When Tregs were depleted during the elicitation phase, it also led to an enhanced and prolonged ear-swelling response, indicating the importance of Tregs in the termination of inflammation (Tomura *et al.*, 2010). In fact, the

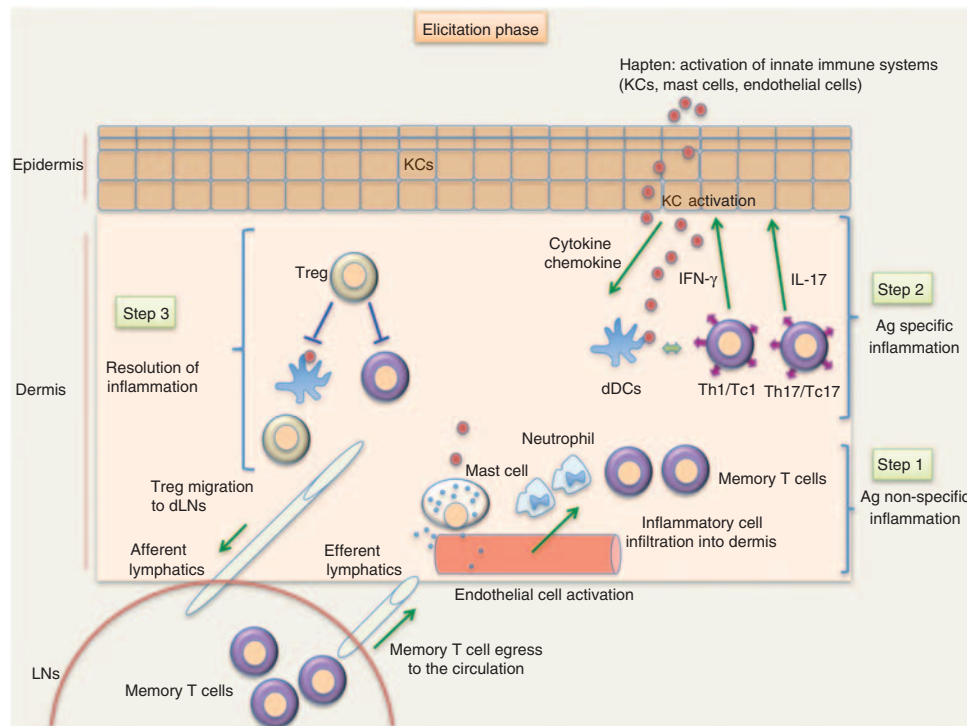


Figure 3. A schematic view of the elicitation phase. Step 1: Upon reexposure to haptens, keratinocytes (KCs) and mast cells are activated and produce various chemical mediators, which activate endothelial cells and cause inflammatory cell infiltration, including antigen-specific T cells. Step 2: Infiltrated antigen-specific effector T cells are activated and produce proinflammatory cytokines and chemokines, which activate KCs and cause further inflammatory cell infiltration. Step 3: 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. Ag, antigen; dDC, dermal DC; dLNs, draining lymph nodes; LC, Langerhans cell; LN, lymph node; Tc, T cytotoxic; Th, T helper; Treg, regulatory T cell.

number of Tregs in skin significantly increased during the process of skin inflammation, suggesting that Tregs have a suppressive role at inflammatory sites. It was reported that CCR4 is a critical receptor for Treg migration into skin under homeostatic conditions (Dudda *et al.*, 2008). A recent report, however, indicates that loss of CCR4 does not necessarily inhibit Treg migration in the elicitation site, because CCR4-deficient mice exhibited a similar or even elevated number of Tregs in the skin after elicitation (Lehtimäki *et al.*, 2010). In the context of CHS, other chemokine receptors, such as CCR10, may compensate for the function of CCR4.

Recirculation of Tregs from skin to the dLNs

Although a number of studies have been conducted to dissect the T cell-infiltration mechanisms to skin, it remained unclear how and to where T cells migrate after the infiltration. Using a new cell-labeling system with the photo-convertible protein Kaede, we succeeded in tracking the migration of T cells after their infiltration into the skin. When Kaede protein is irradiated with violet light, its fluorescence turns from green to red, which enables us to label the cells for tracking their mobilization. Using this system, it was reported that skin-infiltrated T cells moved from skin to dLNs in both the steady and inflammatory conditions (Tomura *et al.*, 2010). In addition, Tregs migrated rather selectively in the inflammatory condition (elicitation phase) than in the steady condition. Moreover, Tregs that circulated to dLNs had a potential to

remigrate into the skin. Although the mechanism of how Tregs migrate from skin to dLNs remains unclear, skin Tregs may use CCR7-dependent mechanisms for their entry into lymph vessels (Kabashima *et al.*, unpublished observation). Interestingly, the skin-derived Tregs exhibited an activated phenotype with high expression of cytotoxic T-lymphocyte antigen-4 (CTLA-4) and IL-10, and they had much more potent suppressive activities compared with resident Tregs in the dLNs, suggesting that skin Tregs exert their potent suppressive activity not only in skin but also in dLNs by circulating in the body.

Importantly, using the above cell-labeling systems, we observed that other skin-infiltrated cells, such as non-Treg CD4⁺ T cells and CD8⁺ T cells, migrated from the skin to dLNs (Kabashima *et al.*, unpublished observation). The function analyses of those skin-derived cells, which will further clarify the complex regulatory mechanisms of CHS, are now ongoing.

In addition to Tregs, the involvement of regulatory B cells (Bregs) has also been proposed as another regulatory mechanism in CHS. Bregs are identified as a CD1b^{high}CD5⁺ B-cell population (Bouaziz *et al.*, 2008). They represent 1–2% of spleen B220⁺ cells and produce abundant amounts of IL-10 (Yanaba *et al.*, 2008). CD19-deficient mice exhibited augmented CHS responses, and the transfer of Bregs into CD19-deficient mice normalized the extent of inflammation in CHS (Watanabe *et al.*, 2007b).

Table 3. 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-1 β , IL-18, and PGE ₂	Recruitment of inflammatory cells through chemokine (CXCL1, CXCL2, CCL17, CCL27, CXCL9, and 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, and CXCL1
	Neutrophils		Production of pro-inflammatory cytokines (TNF- α), 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 hours after hapten 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 hours after hapten application	Possible Ag presentation in dLNs and skin
	Langerin [−] dDCs (approximately 90% of dDCs)	Ag presentation in dLNs to promote effector T-cell differentiation and proliferation	Possible Ag presentation in dLNs and skin
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-cell 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
B cells	B-1 cells	IgM production	Activation of mast cells through IgM/C5a and promotion of inflammatory cell infiltration
	Breg		Regulation of inflammation via IL-10

Abbreviations: Ag, antigen; Breg, regulatory B cell; CHS, contact hypersensitivity; DC, dendritic cell; dLNs, draining lymph nodes; KC, keratinocyte; Tc, T cytotoxic; Th, T helper; TNF, tumor necrosis factor; Treg, regulatory T cell.

CONCLUSION AND DISCUSSION

Taken together, the broad view of CHS development during the sensitization and elicitation phases is summarized in Figures 2 and 3. By integrating the newly identified cell subsets and by introducing innate immunity into CHS, our understanding of the mechanisms of CHS has significantly advanced compared with a decade ago. At the same time, however, some key questions remain unanswered, such as the role of LCs in the sensitization phase and the identification of the responsible DC subsets in the elicitation phase. These questions should be examined in future research.

The heterogeneity of the mechanism of CHS may be another important point to be addressed. Although multiple T-cell subsets or cytokines have been reported to be involved in the development of CHS, their relative importance appears to depend upon the experimental model used. This is probably attributable to the fact that CHS is created by the cooperation of multiple factors, such as the microenvironment in which the antigen is presented, the type of antigen itself, and the subject's genetic background. Systemic analysis of these factors from the viewpoints of innate

immunity may also provide important clues and enhance our understanding (Table 3).

Therefore, 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. Future studies focusing on this topic will develop novel therapeutic approaches to control cutaneous inflammatory diseases.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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