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# Perivascular leukocyte clusters are essential for efficient activation of effector T cells in the skin

Yohei Natsuaki[1,2,15], Gyohei Egawa[1,15], Satoshi Nakamizo[1], Sachiko Ono[1], Sho Hanakawa[1], Takaharu Okada[3], Nobuhiro Kusuba[1], Atsushi Otsuka[1], Akihiko Kitoh[1], Tetsuya Honda[1], Saeko Nakajima[1], Soken Tsuchiya[4], Yukihiko Sugimoto[4], Ken J Ishii[5,6], Hiroko Tsutsui[7], Hideo Yagita[8], Yoichiro Iwakura[9,10], Masato Kubo[11,12], Lai guan Ng[13], Takashi Hashimoto[2], Judilyn Fuentes[14], Emma Guttman-Yassky[14], Yoshiki Miyachi[1] & Kenji Kabashima[1]

**It remains largely unclear how antigen-presenting cells (APCs) encounter effector or memory T cells efficiently in the periphery. Here we used a mouse contact hypersensitivity (CHS) model to show that upon epicutaneous antigen challenge, dendritic cells (DCs) formed clusters with effector T cells in dermal perivascular areas to promote *in situ* proliferation and activation of skin T cells in a manner dependent on antigen and the integrin LFA-1. We found that DCs accumulated in perivascular areas and that DC clustering was abrogated by depletion of macrophages. Treatment with interleukin 1α (IL-1α) induced production of the chemokine CXCL2 by dermal macrophages, and DC clustering was suppressed by blockade of either the receptor for IL-1 (IL-1R) or the receptor for CXCL2 (CXCR2). Our findings suggest that the dermal leukocyte cluster is an essential structure for elicitating acquired cutaneous immunity.**



Boundary tissues, including the skin, are continually exposed to foreign antigens, which must be monitored and possibly eliminated. Upon exposure to foreign antigens, skin dendritic cells (DCs), including epidermal Langerhans cells (LCs), capture the antigens and migrate to draining lymph nodes (LNs), where the presentation of antigen to naive T cells occurs mainly in the T cell zone. In this location, the accumulation of naive T cells in the vicinity of DCs is mediated by signaling via the chemokine receptor CCR7 (ref. 1). The T cell zone in the draining LNs facilitates the efficient encounter of antigen-bearing DCs with antigen-specific naive T cells.

In contrast to T cells in LNs, the majority of T cells in the skin, including infiltrating skin T cells and skin-resident T cells, have an effector-memory phenotype[2]. In addition, the presentation of antigen to skin T cells by antigen-presenting cells (APCs) is the crucial step in the elicitation of acquired skin immune responses, such as contact dermatitis. Therefore, we investigated how antigen presentation occurs in the skin and if it is different from antigen presentation in LNs. Published studies using mouse contact hypersensitivity (CHS) as a model of human contact dermatitis have revealed that dermal DCs (dDCs) have a pivotal role in the transport and presentation of antigen to the LNs, but epidermal LCs do not[3]. In the skin, however, it remains unclear which subset of APCs presents antigens to skin T cells and how skin T cells efficiently encounter APCs. In addition, dermal macrophages are key modulators in CHS responses[4], but the precise mechanisms by which macrophages are involved in the recognition of antigen in the skin have not yet been clarified. These unanswered questions prompted us to investigate where skin T cells recognize antigens and how skin T cells are activated in the elicitation phase of acquired cutaneous immune responses such as CHS.

When keratinocytes encounter foreign antigens, they immediately produce various proinflammatory mediators, such as interleukin 1 (IL-1) and tumor-necrosis factor, in an antigen-nonspecific manner[5,6]. Proteins of the IL-1 family are considered important modulators in CHS responses because the activation of hapten-specific T cells is impaired in mice deficient in both IL-1α and IL-1β but not in mice deficient in tumor-necrosis factor[7]. IL-1α and IL-1β are agonistic ligands of the receptor for IL-1 (IL-1R). While IL-1α is stored in keratinocytes and is secreted upon exposure to nonspecific stimuli, IL-1β is produced mainly by epidermal LCs and dermal mast cells in an inflammasome-dependent manner via activation of the cytoplasmic pattern-recognition receptor NLRP3 and of caspase-1 and caspase-11. Because IL-1α and IL-1β are crucial in the initiation of acquired

[1]Department of Dermatology, Kyoto University Graduate School of Medicine, Kyoto, Japan. [2]Department of Dermatology, Kurume University School of Medicine, Fukuoka, Japan. [3]Research Unit for Immunodynamics, RIKEN Research Center for Allergy and Immunology, Kanagawa, Japan. [4]Department of Pharmaceutical Biochemistry, Graduate School of Pharmaceutical Sciences, Kumamoto University, Kumamoto, Japan. [5]Laboratory of Adjuvant Innovation, National Institute of Biomedical Innovation, Osaka, Japan. [6]Laboratory of Vaccine Science, WPI Immunology Frontier Research Center, Osaka University, Osaka, Japan. [7]Department of Microbiology, Hyogo College of Medicine, Hyogo, Japan. [8]Department of Immunology, Juntendo University School of Medicine, Tokyo, Japan. [9]Research Institute for Biomedical Sciences, Tokyo University of Science, Chiba, Japan. [10]Medical Mycology Research Center, Chiba University, Chiba, Japan. [11]Laboratory for Cytokine Regulation, RIKEN center for Integrative Medical Science, Kanagawa, Japan. [12]Division of Molecular Pathology, Research Institute for Biomedical Science, Tokyo University of Science, Chiba, Japan. [13]Singapore Immunology Network, Agency for Science, Technology and Research, Biopolis, Singapore. [14]Department of Dermatology, Icahn School of Medicine at Mount Sinai School Medical Center, New York, New York, USA. [15]These authors contributed equally to this work. Correspondence should be addressed to K.K. (kaba@kuhp.kyoto-u.ac.jp).

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immune responses such as CHS, it is of great interest to understand how IL-1 modulates the recognition of antigen by skin T cells.

Using a mouse CHS model, here we examined how DCs and effector T cells encounter each other efficiently in the skin. We found that upon encountering antigenic stimuli, dDCs formed clusters in which effector T cells were activated and proliferated in an antigen-dependent manner. These DC–T cell clusters were initiated by skin macrophages via IL-1R signaling and were essential for the establishment of cutaneous acquired immune responses.

## RESULTS

### Formation of DC–T cell clusters at antigen-challenged sites

To explore the accumulation of cells of the immune system in the skin, we examined the clinical and histological features of the elicitation of human allergic contact dermatitis. Allergic contact dermatitis is the most common of eczematous skin diseases, affecting 15–20% of the general population worldwide[8], and is mediated by T cells. Although antigens should be spread evenly over the surface of skin, clinical manifestations commonly include discretely distributed small vesicles (**Fig. 1a**), which suggests an uneven occurrence of intense inflammation. Histological examination of allergic contact dermatitis showed spongiosis, intercellular edema in the epidermis and colocalization of perivascular infiltrates of $CD3^+$ T cells and spotty accumulation of $CD11c^+$ DCs in the dermis, especially beneath the vesicles (**Fig. 1b**). These findings led us to hypothesize that focal accumulation of T cells and DCs in the dermis might contribute to vesicle formation in early eczema.

To characterize the DC–T cell clusters in elicitation reactions, we used two-photon microscopy to obtain time-lapse images in a mouse model of CHS. We isolated T cells from the draining LNs of mice sensitized with the hapten DNFB (2,4-dinitrofluorobenzene), labeled the cells with fluorescent dye and transferred them into mice that express the common DC marker CD11c tagged with yellow fluorescent protein (YFP). In the steady state, $YFP^+$ dDCs distributed diffusely (**Fig. 1c**), representative of nondirected movement in a random fashion (**Supplementary Fig. 1**), as reported before[9]. After topical challenge with DNFB, $YFP^+$ dDCs transiently increased their velocity and formed clusters in the dermis, with the clusters becoming larger and more evident after 24 h (**Fig. 1c** and **Supplementary Movie 1**). At the same time, transferred T cells accumulated in the DC clusters and interacted with $YFP^+$ DCs for several hours (**Fig. 1d** and **Supplementary Movie 2**). Thus, we observed accumulation of DCs and T cells in the dermis in mice during CHS responses. We noted that the intercellular spaces between keratinocytes overlying the DC–T cell clusters in the dermis were enlarged (**Fig. 1e**), which replicated observations made for human allergic contact dermatitis (**Fig. 1b**).

We next sought to determine which of the two main DC populations in skin, epidermal LCs or dDCs, was essential for the elicitation of CHS. To deplete mice of all cutaneous DC subsets, we used mice with sequence expressing the diphtheria toxin receptor (DTR) under the control of the promoter of the gene encoding langerin as recipients (in such 'Langerin-DTR' mice, treatment with diphtheria toxin (DT) leads to depletion of langerin-positive cells) and mice that express a transgene encoding DTR under the control of promoter of the gene encoding CD11c as donors (in such 'CD11c-DTR' mice, treatment with DT leads to transient depletion of $CD11c^+$ DC populations). To selectively deplete mice of LCs or dDCs, we transferred bone marrow (BM) cells from C57BL/6 mice or CD11c-DTR mice into Langerin-DTR or C57BL/6 mice, respectively (**Supplementary Fig. 2a,b**). We injected DT into the chimeras to ensure depletion of each DC subset before elicitation and found that ear swelling and inflammatory histological findings were significantly attenuated in the absence of dDCs but not in the absence of LCs (**Fig. 1f** and **Supplementary Fig. 2c**). In addition, production of interferon-γ (IFN-γ) in skin T cells was substantially suppressed in mice depleted of dDCs (**Fig. 1g**). These results suggested that dDCs, not epidermal LCs, were essential for T cell activation and the elicitation of CHS responses.

### Antigen-dependent proliferation of skin effector T cells *in situ*

To evaluate the effect of DC–T cell clusters in the dermis, we determined whether T cells had acquired the ability to proliferate via the accumulation of DC–T cell clusters in the dermis. We purified $CD4^+$ or $CD8^+$ T cells from the draining LNs of DNFB-sensitized mice, labeled

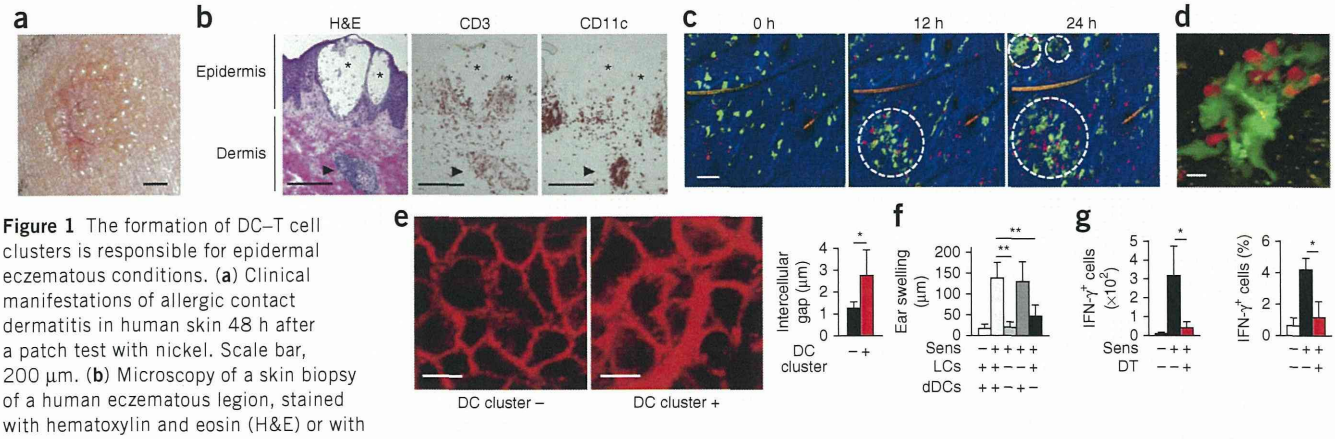


**Figure 1** The formation of DC–T cell clusters is responsible for epidermal eczematous conditions. (**a**) Clinical manifestations of allergic contact dermatitis in human skin 48 h after a patch test with nickel. Scale bar, 200 μm. (**b**) Microscopy of a skin biopsy of a human eczematous legion, stained with hematoxylin and eosin (H&E) or with antibody to CD3 (anti-CD3) or anti-CD11c. *, epidermal vesicles; arrowheads indicate dDC–T cell clusters. Scale bars, 250 μm. (**c**) Sequential images of leukocyte clusters in the elicitation phase of CHS. White outlined areas indicate dermal accumulation of DCs (green) and T cells (red). Scale bar, 100 μm. (**d**) Enlargement of DC–T cell cluster in **c**. Scale bar,10 μm. (**e**) Intercellular edema of the epidermis overlying a DC–T cell cluster in the dermis, with keratinocytes (red) visualized with isolectin B4 (left), and distance between adjacent keratinocytes above (+) or not above (–) a DC–T cell cluster ($n = 20$ images per condition) (right). Scale bars, 10 μm. (**f**) Ear swelling 24 h after CHS with (+) or without (–) sensitization (Sens) and with (–) or without (+) subset-specific depletion of DCs ($n = 5$ mice per group). (**g**) Quantification (left) and frequency (right) of IFN-γ-producing T cells in the ear 18 h after CHS with or without sensitization (as in **f**) and with (DT +) or without (DT –) depletion of dDCs ($n = 5$ mice per group). $^*P < 0.05$ and $^{**}P < 0.001$ (unpaired Student's *t*-test). Data are representative of five independent experiments (**a**–**d**) or three experiments (**f**,**g**) or are pooled from three experiments (**e**; error bars (**e**–**g**), s.d.).

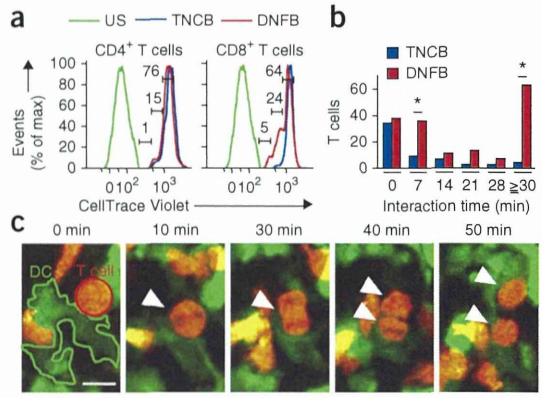


**Figure 2** Antigen-dependent T cell proliferation in DC–T cell clusters. (**a**) Proliferation $CD4^+$ T cells (left) or $CD8^+$ T cells (right) in the skin of recipient mice 24 h after transfer of CellTrace Violet–labeled cells from donor mice left unsensitized (US) or sensitized with DNFB or TNCB, assessed as dilution of tracer in the challenged sites. Numbers adjacent to bracketed lines indicate percent cells that had proliferated. (**b**) Conjugation time of dDCs with T cells sensitized with DNFB ($n = 160$ T cells) or TNCB ($n = 60$ T cells), assessed at 24 h after challenge with DNFB. $^*P < 0.05$ (unpaired Student's *t*-test). (**c**) Sequential images of dividing T cells (red) in DC–T cell clusters. Green, dDCs; arrowheads indicate a dividing T cell. Data are representative of three experiments.

the cells with a division-tracking dye and transferred the cells into naive mice. Twenty-four hours after the application of DNFB to the recipient mice, we collected the skin to evaluate T cell proliferation by dilution of fluorescence intensity. Most of the infiltrating T cells (>90%) were $CD44^+CD62L^-$ effector T cells (**Supplementary Fig. 2d**). Among the infiltrating T cells, $CD8^+$ T cells proliferated actively, whereas the $CD4^+$ T cells showed low proliferative potency (**Fig. 2a**). This T cell proliferation was antigen dependent because T cells sensitized with the hapten TNCB (2,4,6-trinitrochlorobenzene) exhibited low proliferative activity in response to the application of DNFB (**Fig. 2a**). In line with that finding, the DC–T cell conjugation time was prolonged in the presence of the cognate antigen DNFB (**Fig. 2b**), and the T cells interacting with DCs within DC–T cell clusters proliferated (**Fig. 2c** and **Supplementary Movie 3**). These findings indicated that skin effector T cells conjugated with DCs and proliferated *in situ* in an antigen-dependent manner.

### LFA-1-dependent activation of $CD8^+$ T cells in DC–T cell clusters

Sustained interaction between DCs and naive T cells, known as the 'immunological synapse', is maintained by cell adhesion molecules[10]. In particular, the integrin LFA-1 (CD58) on T cells binds to cell-surface glycoproteins, such as the intercellular adhesion molecule ICAM-1, on APCs, which is essential for the proliferation and activation of naive T cells during antigen recognition in the LNs. To determine whether LFA-1–ICAM-1 interactions are required for the activation of effector T cells in DC–T cell clusters in the skin, we elicited a CHS response in mouse ear skin with DNFB, then injected KBA, a neutralizing antibody to LFA-1, intravenously 14 h later. Such administration of KBA reduced the accumulation of T cells in the dermis (**Fig. 3a**). The velocity of T cells in the cluster was 0.65 ± 0.29 μm/min (mean ± s.d.) at 14 h after the DNFB challenge and increased up to threefold (1.64 ± 1.54 μm/min) at 8 h after treatment with KBA, while it was not affected by treatment with the isotype-matched control antibody immunoglobulin G (IgG) (**Fig. 3b**). At the outside of clusters, T cells smoothly migrated at the mean velocity of 2.95 ± 1.19 μm/min, consistent with published results[11], and this was not affected by treatment with the control antibody IgG (data not shown). Treatment with KBA also significantly attenuated ear swelling (**Fig. 3c**) as well as IFN-γ production by skin $CD8^+$ T cells (**Fig. 3d,e**). These results suggested that the DC–effector T cell conjugates were integrin dependent, similar to the DC–naive T cell interactions in draining LNs.

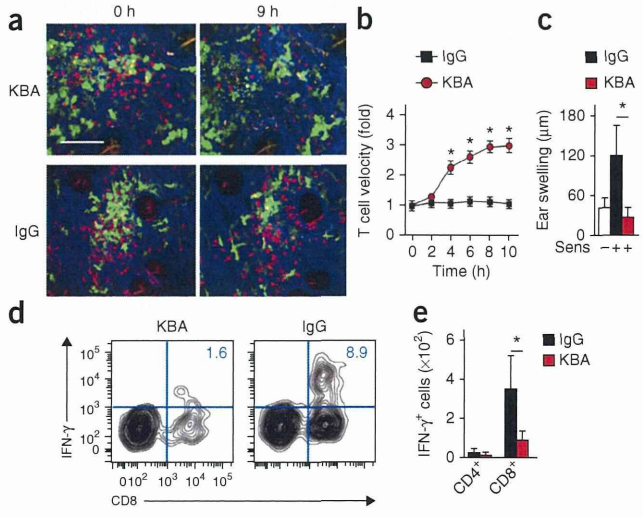


**Figure 3** LFA-1 is essential for the persistence of DC–T cell clustering and for T cell activation in the skin. (**a**) Clusters of DCs (green) and T cells (red) in the DNFB-challenged site before (0 h) and 9 h after treatment with KBA (LFA-1-neutralizing antibody) or IgG (isotype-matched control antibody). Scale bar, 100 μm. (**b**) T cell velocity in DNFB-challenged sites at various times (horizontal axis) after treatment with KBA or IgG ($n = 30$ T cells per group), presented relative to velocity at time 0, set as 1. (**c**) Ear swelling 24 h after treatment with KBA or IgG in mice ($n = 5$ per group) left unsensitized (Sens –) or challenged with DNFB (Sens +). (**d,e**) IFN-γ production by $CD8^+$ T cells (**d**) and quantification of IFN-γ-producing cells in the $CD4^+$ or $CD8^+$ population (**e**) in skin from mice ($n = 5$ per group) challenged with DNFB, then treated with KBA or IgG 12 h later, assessed 6 h after antibody treatment. Numbers in top right quadrants (**d**) indicate percent IFN-γ$^+$$CD8^+$ T cells. $^*P < 0.05$ (unpaired Student's *t*-test). Data are representative of three experiments (error bars (**b**,**c**,**e**), s.d.).

### dDC clustering requires skin macrophages

We next examined the factors that initiated the accumulation of DC–T cell clusters. dDC clusters also formed in response to the initial application of hapten (sensitization phase), but their number decreased significantly 48 h after sensitization, while DC clusters persisted for 48 h in the elicitation phase (**Fig. 4a** and **Supplementary Fig. 3a**). These DC clusters were abrogated 7 d after application of DNFB (data not shown). These observations suggested that the accumulation of DC–T cell clusters was initiated by DC clustering, which then induced the accumulation, proliferation and activation of T cells, a process that depended on the presence of antigen-specific effector T cells *in situ*. DC clusters were also induced by solvents (such as acetone) or adjuvants (such as dibutylphthalic acid) and by pathogenic inoculation with *Mycobacterium bovis* bacillus Calmette-Guérin (**Supplementary Fig. 3b,c**). In addition, we observed DC clusters not only in the ear skin but also in other regions, such as the back skin and the footpad (**Supplementary Fig. 3d**). These results suggested that the formation of DC clusters was not an ear-specific event but was a general mechanism during skin inflammation.

The abundance of DC clusters in response to the application of DNFB was not altered in mice that lack T cells and B cells (recombinase

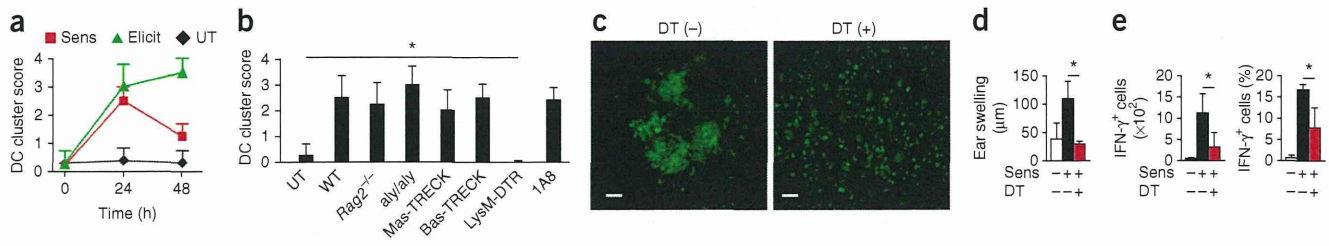


**Figure 4** Macrophages are essential for DC cluster formation. (**a**) Score of DC cluster abundance in mice ($n = 4$ per group) left untreated (UT) or 24 h and 48 h after application of DNFB in the sensitization or elicitation phase of CHS; scores were assigned according to the size and number of clusters. (**b**) Score of DC cluster abundance (as in **a**) in untreated wild-type mice (UT), in DNFB-treated wild-type (C57BL/6) mice (WT), RAG-2-deficient mice (*Rag2*$^{-/-}$), aly/aly mice (aly/aly), DT-treated Mas-TRECK or Bas-TRECK mice or DT-treated C57BL/6 recipients of LysM-DTR BM cells, and in wild-type mice treated with 1A8 (anti-Ly6G) ($n = 4$ mice per group). (**c**) DC clusters in C57BL/6 mice chimeras given LysM-DTR BM with (right) or without (left) treatment of recipients with DT. Scale bars, 100 μm. (**d**) Ear swelling in C57BL/6 chimeras ($n = 5$ per group) given LysM-DTR BM with or without treatment with DT, assessed 24 h after no DNFB (Sense –) or application of DNFB to the recipients. (**e**) Quantification (left) and frequency (right) of IFN-γ-producing CD8$^+$ T cells in mice as in **d** ($n = 5$ per group). *$P < 0.05$ (unpaired Student's *t*-test). Data are representative of three (**a**,**c**,**e**), two (**b**) or four (**d**) experiments (error bars (**b**,**d**,**e**), s.d.).

RAG-2–deficient mice), in mice deficient in lymphoid tissue–inducer cells (alymphoblastic (aly/aly) mice)[12] or in mice depleted of mast cells or basophils (Mas-TRECK or Bas-TRECK mice treated with DT)[13,14] (**Fig. 4b**). In contrast, DC clustering was abrogated in C57BL/6 mice given transfer of BM from LysM-DTR mice (with sequence encoding a DTR cassette inserted into the gene encoding lysozyme M) followed by treatment of the recipients with DT to ensure depletion of both macrophages and neutrophils (**Fig. 4b**,**c**). Depletion of neutrophils alone, by administration of antibody 1A8 to Ly6G, did not interfere with the formation of DC clusters (**Fig. 4b**), which suggested that macrophages were required during the formation of DC clusters, but neutrophils were not. Of note, the formation of DC clusters was not attenuated by treatment with the LFA-1-neutralizing antibody KBA (**Supplementary Fig. 3e**,**f**), which suggested that macrophage-DC interactions were LFA-1 independent. Consistent with the formation of DC clusters, elicitation of the CHS response (**Fig. 4d**) and IFN-γ production by skin T cells (**Fig. 4e**) were significantly suppressed in chimeras given LysM-DTR BM and treated with DT. Thus, skin macrophages were required for the formation of DC clusters, which was necessary for T cell activation and the elicitation of CHS.

## Perivascular DCs clustering requires macrophages

To examine the migratory kinetics of dermal macrophages and DCs *in vivo*, we visualized them by two-photon microscopy. *In vivo* labeling of blood vessels with dextran conjugated to the hydrophobic red fluorescent dye TRITC (tetramethylrhodamine isothiocyanate) revealed that dDCs distributed diffusely in the steady state (**Fig. 5a**, left). After application of DNFB to the ears of mice previously sensitized with DNFB, dDCs accumulated mainly around post-capillary venules (**Fig. 5a**, right, and **b**). Time-lapse imaging revealed that some dDCs showed directional migration toward TRITC$^+$ cells that were labeled red by incorporation of extravasated TRITC-dextran (**Fig. 5c** and **Supplementary Movie 4**). Most of the TRITC$^+$ cells were F4/80$^+$CD11b$^+$ macrophages (**Supplementary Fig. 4a**). These observations prompted us to investigate the role of macrophages in DC accumulation. We used a chemotaxis assay to determine whether macrophages attracted the DCs. We isolated dDCs and dermal macrophages from dermal skin cell suspensions and incubated them for 12 h in a Transwell assay. dDCs placed in the upper wells migrated efficiently to lower wells that contained dermal macrophages (**Fig. 5d**). However, we did not observe such dDC migration when macrophages were absent from the lower wells (**Fig. 5d**). Thus, dermal macrophages were able to attract dDCs *in vitro*, which may have led to the accumulation of dDCs around post-capillary venules.

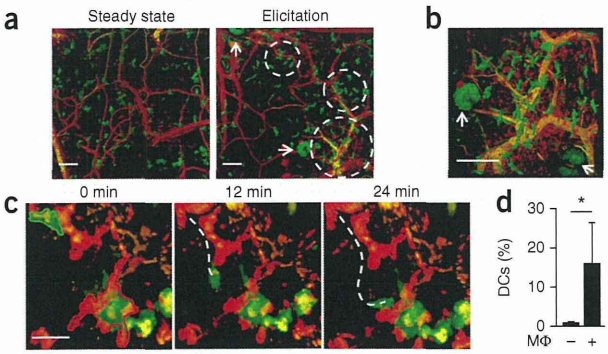


**Figure 5** Macrophages mediate the perivascular formation of DC clusters. (**a**) Distribution of dDCs (green) in the steady state (left) and in the elicitation phase of CHS (right). White outlined areas indicate DC clusters; arrows indicate sebaceous glands visualized with BODIPY (green); yellow and red, blood vessels; red, macrophages. Scale bars, 100 μm. (**b**) Enlargement of a perivascular DC cluster. Arrows indicate sebaceous glands of hair follicles. Scale bar, 100 μm.(**c**) Sequential images of dDCs (green) and macrophages (red) in the elicitation phase of CHS. White dashed line represents the track of a DC. Scale bar, 30 μm. (**d**) Chemotaxis of dDCs in the presence (+) or absence (–) of macrophages (MΦ) prepared from ear skin, presented as the frequency of dDCs that transmigrated into the lower chamber of a Transwell (relative to input dDCs). *$P < 0.05$ (unpaired Student's *t*-test). Data are representative of three experiments (error bars (**d**), s.d.).

## DC cluster formation requires IL-1α upon antigen challenge

We attempted to explore the mechanism underlying the formation of DC clusters. We observed that DC accumulation occurred during the first application of hapten (**Fig. 4a**), which suggested that an antigen-nonspecific mechanism, such as production of the proinflammatory mediator IL-1, may initiate DC clustering. DNFB-induced accumulation of DCs was not suppressed in mice deficient in NLRP3 or deficient in caspase-1 and caspase-11 more than their wild-type counterparts, but it was significantly lower in IL-1R1-deficient mice (which lack the receptor for IL-1α and IL-1β and for the IL-1 receptor antagonist (IL-1ra)) than in their wild-type counterparts, as well as after the subcutaneous administration of IL-1ra than before treatment with the antagonist (**Fig. 6a**,**b**). Consistent with those observations, the elicitation of CHS and IFN-γ production by skin T cells were significantly attenuated in mice that lacked both IL-1α and IL-1β (**Fig. 6c**,**d**). In addition, the formation of dDC clusters was suppressed significantly by the subcutaneous injection of a neutralizing antibody to IL-1α but

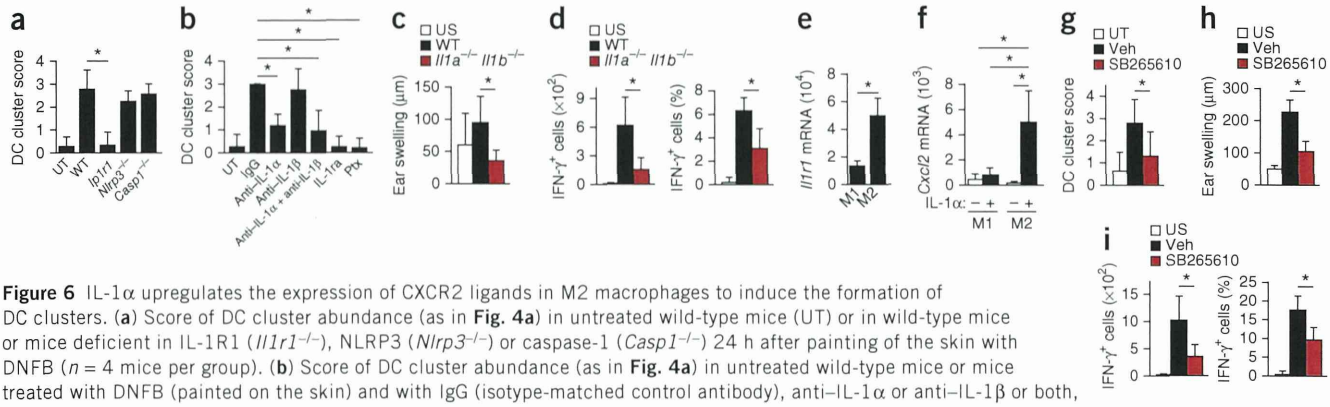


**Figure 6** IL-1α upregulates the expression of CXCR2 ligands in M2 macrophages to induce the formation of DC clusters. (**a**) Score of DC cluster abundance (as in **Fig. 4a**) in untreated wild-type mice (UT) or in wild-type mice or mice deficient in IL-1R1 (*Il1r1*$^{-/-}$), NLRP3 (*Nlrp3*$^{-/-}$) or caspase-1 (*Casp1*$^{-/-}$) 24 h after painting of the skin with DNFB ($n = 4$ mice per group). (**b**) Score of DC cluster abundance (as in **Fig. 4a**) in untreated wild-type mice or mice treated with DNFB (painted on the skin) and with IgG (isotype-matched control antibody), anti–IL-1α or anti–IL-1β or both, recombinant IL-1ra or pertussis toxin (Ptx), assessed 24 h after treatment with hapten ($n = 4$ mice per group). (**c**,**d**) Ear swelling 24 h after application of DNFB (**c**) and quantification (**d**, left) and frequency (**d**, right) of IFN-γ-producing CD8$^+$ T cells in the ear 18 h after application of DNFB (**d**) in unsensitized wild-type mice (US) or in mice lacking both IL-1α and IL-1β (*Il1a*$^{-/-}$*Il1b*$^{-/-}$) and wild-type mice given adoptive transfer of DNFB-sensitized T cells ($n = 5$ mice per group). (**e**) Quantitative RT-PCR analysis of *Il1r1* mRNA in M1 or M2 macrophages ($n = 4$ mice per group). (**f**) Quantitative RT-PCR analysis of *Cxcl2* mRNA in M1 or M2 macrophages cultured with (+) or without (–) IL-1α. (**g**) Score of DC cluster abundance (as in **Fig. 4a**) in untreated wild-type mice (UT) or in mice treated with DNFB (painted on the skin) in the presence (SB265610) or absence (vehicle (Veh)) of a CXCR2 inhibitor, assessed 24 h after treatment with DNFB ($n = 4$ mice per group). (**h**,**i**) Ear swelling 24 h after application of DNFB (**h**) and quantification (**i**, right) and frequency (**i**, left) of IFN-γ-producing CD8$^+$ T cells 18 h after application of DNFB (**i**) in unsensitized wild-type mice (US) or in mice treated with DNFB in the presence or absence of the CXCR2 inhibitor SB265610 ($n = 5$ mice per group). *$P < 0.05$ (unpaired Student's *t*-test). Data are representative of two (**a**,**c**,**d**) or three (**b**,**e**–**i**) experiments (error bars, s.d.).

was suppressed only marginally by a neutralizing antibody to IL-1β (**Fig. 6b**). Because keratinocytes are known to produce IL-1α upon application of a hapten[15], our results suggested a major role for IL-1α in mediating the formation of DC clustering.

### M2 macrophages produce chemokine CXCL2 to attract dDCs

To further characterize how macrophages attract dDCs, we examined expression of the gene encoding IL-1Rα (*Il1r1*) in BM-derived classically activated (M1) and alternatively activated (M2) macrophages, classified as such on the basis of differences in the expression of *Tnf*, *Nos2*, *Il12a*, *Arg1*, *Retnla* and *Chi313* mRNA[16] (**Supplementary Fig. 4b**). We found that M2 macrophages had higher expression of *Il1r1* mRNA than did M1 macrophages (**Fig. 6e**). We also found that subcutaneous injection of pertussis toxin, an inhibitor specific for inhibitory regulative G protein, almost completely abrogated the formation of DC clusters in response to hapten stimuli (**Fig. 6b**), which suggested that signaling through chemokines coupled to the inhibitory regulative G protein was required for the formation of DC clusters.

We next used microarray analysis to examine the effect of IL-1α on the expression of chemokine-encoding genes in M1 and M2 macrophages. Treatment with IL-1α did not enhance such expression in M1 macrophages, whereas it increased the expression of *Ccl5*, *Ccl17*, *Ccl22* and *Cxcl2* mRNA in M2 macrophages (**Supplementary Table 1**). Among those, *Cxcl2* mRNA expression was enhanced most prominently by treatment with IL-1α, a result we confirmed by real-time PCR analysis (**Fig. 6f**). Consistently, *Cxcl2* mRNA expression was much higher in DNFB-painted skin than in untreated skin (**Supplementary Fig. 5a**) and was not affected by neutrophil depletion with the 1A8 antibody to Ly6G (**Supplementary Fig. 5b**,**c**). In addition, IL-1α-treated dermal macrophages produced *Cxcl2* mRNA *in vitro* (**Supplementary Fig. 5d**). These results suggested that dermal macrophages, but not neutrophils, were the main source of CXCL2 during CHS. We also detected high expression of *Cxcr2* mRNA (which encodes the receptor for CXCL2) in DCs (**Supplementary Fig. 5e**); this prompted us to examine the role of CXCR2 in dDCs. The formation of DC clusters in response to DNFB was substantially reduced by intraperitoneal administration of the CXCR2 inhibitor SB265610 (ref. 17) (**Fig. 6g**). In addition, treatment with SB265610 during the elicitation of CHS with DNFB inhibited ear swelling (**Fig. 6h**) and IFN-γ production by skin T cells (**Fig. 6i**).

Together our results indicated that in the absence of effector T cells specific for a cognate antigen (i.e., in the sensitization phase of CHS), DC clustering was a transient event, and hapten-carrying DCs migrated into draining LNs to establish sensitization. On the other hand, in the presence of the antigen and antigen-specific effector or memory T cells, DC clustering was followed by accumulation of T cells (i.e., in the elicitation phase of CHS) (**Supplementary Fig. 6**). Thus, dermal macrophages were essential for initiating the formation of DC clusters through the production of CXCL2, and DC clustering had a role in the efficient activation of skin T cells.

## DISCUSSION

Although the mechanistic events in the sensitization phase in cutaneous immunity have been studied thoroughly over 20 years[18,19], the types of immunological events that occur during the elicitation phases in the skin has remained unclear. Here we have described the antigen-dependent induction of DC–T cell clusters in the skin in a mouse model of CHS and showed that DC–effector T cell interactions in these clusters were required for the induction of efficient antigen-specific immune responses in the skin. We found that dDCs, but not epidermal LCs, were essential for the presentation of antigen to skin effector T cells and that they exhibited sustained association with effector T cells in an antigen- and LFA-1-dependent manner. IL-1α, not the inflammasome, initiated the formation of these perivascular DC clusters.

Epidermal contact with antigens triggers the release of IL-1 in the skin[15]. Published studies have shown that the epidermal keratinocytes constitute a major reservoir of IL-1α[6] and that mechanical stress applied to keratinocytes permits the release of large amounts of IL-1α even in the absence of cell death[20]. The cellular source of IL-1α in this process remains unclear. We found that IL-1α activated macrophages that subsequently attracted dDCs, mainly to areas around post-capillary venules, where effector T cells are known to transmigrate from the blood into the skin[21]. In the presence of the antigen and antigen-specific

effector T cells, DC clustering was followed by T cell accumulation. Therefore, we propose that these perivascular dDC clusters may provide antigen-presentation sites for efficient activation of effector T cells. This is suggested by the observations that CHS responses and intracutaneous T cell activation were attenuated substantially in the absence of these clusters, in conditions of macrophage depletion or inhibition of integrin function, IL-1R signaling[22,23] or CXCR2 signaling[24].

In contrast to antigen presentation in the skin, antigen presentation in other peripheral barrier tissues is relatively well understood. In submucosal areas, specific sentinel lymphoid structures (mucosa-associated lymphoid tissue (MALT)) serve as peripheral antigen-presentation sites[25], and lymphoid follicles are present in non-inflammatory bronchi (bronchus-associated lymphoid tissue (BALT)). These structures serve as antigen-presentation sites in nonlymphoid peripheral organs. By analogy, the concept of skin-associated lymphoid tissue (SALT) was proposed in the early 1980s, on the basis of findings that cells in the skin are able to capture, process and present antigens[26,27]. However, the role of cellular skin components as antigen-presentation sites has remained uncertain. Here we have identified an inducible structure formed by dermal macrophages, dDCs and effector T cells, which seemed to accumulate sequentially. Because formation of this structure was essential for efficient activation of effector T cells, these inducible leukocyte clusters may function as SALTs. Unlike leukocyte clusters in MALT, these leukocyte clusters were not found in the steady state but were induced during the development of an adaptive immune response. Therefore, these clusters might be better called 'inducible SALTs', similar to inducible BALTs in the lung[28]. In contrast to the cells present in inducible BALT, we did not identify naive T cells or B cells in SALT (data not shown), which suggested that the leukocyte clusters in the skin may be specialized for the activation of effector T cells but not for the activation of naive T cells. Our findings suggest that approaches for the selective inhibition of this structure may have novel therapeutic benefit in inflammatory disorders of the skin.

## METHODS

Methods and any associated references are available in the online version of the paper.

**Accession codes.** GEO: microarray data, GSE53680.

*Note: Any Supplementary Information and Source Data files are available in the online version of the paper.*

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AUTHOR CONTRIBUTIONS

Y.N., G.E. and K.K. designed this study and wrote the manuscript; Y.N., G.E., S. Nakamizo, S.O., S.H., N.K., A.O., A.K., T. Honda and S. Nakajima performed the experiments and analyzed data; S.T. and Y.S. did experiments related to microarray analysis; K.J.I., H.T., H.Y., Y.I., M.K. and L.g.N. developed experimental reagents and gene-targeted mice; J.F. and E.G.-Y. did experiments related to immunohistochemistry of human samples; T.O., T. Hashimoto, Y.M. and K.K. directed the project and edited the manuscript; and all authors reviewed and discussed the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.



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## ONLINE METHODS

**Mice.** 8- to 12-week-old female C57BL/6 mice were used in this study. C57BL/6N mice were from SLC. Langerin-eGFP-DTR mice[29], CD11c-DTR mice[30], CD11c-YFP mice (that express CD11c tagged with YFP)[31], LysM-DTR mice[32], RAG-2-deficient mice[33], Mas-TRECK mice[13,14], Bas-TRECK mice[13,14], ALY/NscJcl- aly/aly mice[12], IL-1α/β-deficient mice[34], IL-1R1-deficient mice[35], NLRP3-deficient mice[36] and caspase-1/11-deficient mice[37] have been described. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Kyoto University Graduate School of Medicine.

**Human subjects.** Biopsy samples of human skin were obtained from a nickel-reactive patch after 48 h after placement of nickel patch tests in patients with previously proven allergic contact dermatitis. A biopsy of petrolatum-occluded skin was also obtained as a control. Informed consent was obtained under protocols approved by the Institutional Review Board at the Icahn School of Medicine at Mount Sinai School Medical Center, and the Rockefeller University in New York.

**Induction of CHS responses.** Mice were sensitized on shaved abdominal skin with 25 μl 0.5% (wt/vol) DNFB (1-fluoro-2,4-dinitrofluorobenzene ; Nacalai Tesque) dissolved in acetone and olive oil (at a ratio of 4:1). Five days later, the ears were challenged with 20 μl 0.3% DNFB. For adoptive transfer, T cells were magnetically sorted, with an autoMACS (Miltenyi Biotec), from the draining LNs of sensitized mice and then were transferred intravenously ($1 \times 10^7$ cells) into naive mice.

**Depletion of cutaneous DC subsets, macrophages and neutrophils.** For depletion of all cutaneous DC subsets (including LCs), 6-week-old Langerin-DTR mice were irradiated (two doses of 550 rads given 3 h apart) and were given transfer of $1 \times 10^7$ BM cells from CD11c-DTR mice. Eight weeks later, 2 μg DT (Sigma-Aldrich) was injected intraperitoneally. For selective depletion of LCs, irradiated Langerin-DTR mice were given transfer of BM cells from C57BL/6 mice, and 1 μg DT was injected. For selective depletion of dDCs, irradiated C57BL/6 mice were given transfer of BM cells from CD11c-DTR mice, and 2 μg DT was injected. For depletion of macrophages, irradiated C57BL/6 mice were given transfer of BM cells from LysM-DTR mice and 800 ng DT was injected. For depletion of neutrophils, anti-Ly6G (1A8; BioXCell) was administered to mice intravenously at a dose of 0.5 mg per mouse 24 h before experiments.

**Time-lapse imaging of cutaneous DCs, macrophages and T cells.** Cutaneous DCs were observed in CD11c-YFP mice. For labeling of cutaneous macrophages *in vivo*, 5 mg TRITC-dextran (Sigma-Aldrich) was injected intravenously and mice were allowed to 'rest' for 24 h. At that time, cutaneous macrophages became fluorescent because they had incorporated extravasated dextran. For labeling of skin-infiltrating T cells, T cells from DNFB-sensitized mice were labeled with CellTracker Orange (CMTMR (5-(and-6)-(((4-chloromethyl)benzoyl) amino)tetramethylrhodamine); Invitrogen) and were adoptively transferred into recipient mice. Keratinocytes and sebaceous glands were visualized by subcutaneous injection of isolectin B4 (Invitrogen) and BODIPY (Molecular Probes), respectively. Mice were positioned on a heating plate on the stage of a two-photon IX-81 microscope (Olympus) and their ear lobes were fixed beneath a cover slip with a single drop of immersion oil. Stacks of ten images, spaced 3 μm apart, were acquired at intervals of 1–7 min for up to 24 h. For calculation of T cell and DC velocities, movies were processed and analyzed with Imaris 7.2.1 software (Bitplane).

**Histology and immunohistochemistry.** For histological examination, tissues were fixed with 10% formalin in phosphate-buffered saline, then were embedded in paraffin. Sections with a thickness of 5 μm were prepared and then were stained with hematoxylin and eosin. For whole-mount staining, the ears were split into dorsal and ventral halves and were incubated for 30 min at 37 °C with 0.5 M ammonium thiocyanate. Then the dermal sheets were separated and fixed in acetone for 10 min at −20 °C. After treatment with Image-iT FX Signal Enhancer (Invitrogen), the sheets were incubated with antibody to mouse MHC class II (M5/114.15.2; eBioscience) followed by incubation with antibody to rat IgG conjugated to Alexa Fluor 488 (A-11006; Invitrogen) or Alexa Fluor 594 (A-11007; Invitrogen). The slides were mounted with a ProLong Antifade kit with the DNA-binding dye DAPI (4′6-diamidino-2-phenylindole; Molecular Probes) and were observed with a fluorescent microscope (BZ-900; KEYENCE). The number and size of DC clusters were evaluated in ten fields of 1 $mm^2$ per ear and were assigned scores according to the criteria in **Supplementary Figure 5a**.

**Cell isolation and flow cytometry.** For the isolation of skin lymphocytes, the split ears were incubated for 1 h at 37 °C in digestion buffer (RPMI medium supplemented with 2% FCS, 0.33 mg/ml of Liberase TL (Roche) and 0.05% DNase I (Sigma-Aldrich)). After that incubation, the tissues were disrupted by passage through a 70-μm cell strainer and stained with the appropriate antibodies (identified below). For analysis of intracellular cytokine production, cell suspensions were obtained in the presence of 10 μg/ml of brefeldin A (Sigma-Aldrich) and were fixed with Cytofix Buffer and permeabilized with Perm/Wash Buffer according to the manufacturer's protocol (BD Biosciences). Cells were stained with the following: antibody to mouse CD4 (GK1.5), anti-CD8 (53-6.7), anti-CD11b (M1/70), anti-CD11c (N418), anti-B220 (RA3-6B2), antibody to MHC class II (M5/114.15.2), anti-F4/80 (BM8), anti-IFN-γ (XMG1.2), anti-Gr1 (RB6-8c5) and 7-amino-actinomycin D (all from eBioscience); anti-mouse CD45 (30-F11) and anti-TCR-β (H57-597; both from BioLegend); and anti-CD16-CD32 (2.4G2; BD Biosciences). Flow cytometry was done with an LSR Fortessa (BD Biosciences) and data were analyzed with FlowJo software (TreeStarA).

**Chemotaxis assays.** Chemotaxis was assessed as described with some modifications[38]. The dermis of the ear skin was minced and then was digested for 30 min at 37 °C with 2 mg/ml collagenase type II (Worthington Biochemical) containing 1 mg/ml hyaluronidase (Sigma-Aldrich) and 100 μg/ml DNase I (Sigma-Aldrich). DDCs and macrophages were isolated with an autoMACS. Alternatively, BM-derived DCs and macrophages were prepared. $1 \times 10^6$ DCs were added to a Transwell insert with a pore size of 5 μm (Corning), and $5 \times 10^5$ macrophages were added to the lower wells, and the cells were incubated for 12 h at 37 °C. A known number of fluorescent reference beads (FlowCount fluorospheres; Beckman Coulter) were added to each sample to allow accurate quantification of cells that had migrated to the lower wells by flow cytometry.

**Cell proliferation assay.** Mice were sensitized with 25 μl 0.5% DNFB or 7% trinitrochlorobenzene (Chemical Industry). Five days later, T cells were magnetically separated from the draining LNs of each group of mice and were labeled with CellTrace Violet according to the manufacturer's protocol (Invitrogen). $1 \times 10^6$ T cells were adoptively transferred into naive mice, and the ears of the recipient mice were challenged with 20 μl of 0.5% DNFB. 24 h later, ears were collected and analyzed by flow cytometry.

***In vitro* differentiation of DCs and M1 and M2 macrophages from BM cells.** BM cells from the tibias and fibulas were plated at a density of $5 \times 10^6$ cells per 10-cm dish on day 0. For DC differentiation, cells were cultured at 37 °C in 5% $CO_2$ in cRPMI medium (RPMI medium supplemented with 1% L-glutamine, 1% HEPES, 0.1% 2-mercaptoethanol and 10% FBS) containing 10 ng/ml granulocyte-macrophage colony-stimulating factor (Peprotech). For macrophage differentiation, BM cells were cultured in cRPMI medium containing 10 ng/ml macrophage colony-stimulating factor (Peprotech). The medium was replaced on days 3 and 6 and cells were harvested on day 9. For the induction of M1 macrophages or M2 macrophages, cells were stimulated for 48 h with IFN-γ (10 ng/ml; R&D Systems) or with IL-4 (20 ng/ml; R&D Systems), respectively.

***In vitro* IL-1α-stimulation assay of dermal macrophages.** Dermal macrophages were separated from mice deficient in IL-1α and IL-1β[34] to avoid preactivation during cell preparations. Split ears were treated for 30 min at 37 °C with 0.25% trypsin and EDTA for removal of the epidermis, then were minced and then incubated with collagenase as described above. $CD11b^+$ cells were separated by magnetic-activated cell sorting, and $2 \times 10^5$ cells per well in 96-well plates were incubated for 24 h with or without 10 ng/ml IL-1α (R&D Systems).





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**Blocking assay.** For the LFA-1-blocking assay, mice were given intravenous injection of 100 μg KBA (neutralizing antibody to LFA-1; a gift from H. Yagita) 12–14 h after challenge with 20 μl 0.5% DNFB. For blockade of IL-1R, mice were given subcutaneous injection of 10 μg recombinant mouse IL-1ra (PROSPEC) 5 h before challenge. For blockade of CXCR2, mice were given intraperitoneal treatment with 50 μg CXCR2 inhibitor[17] (SB265610; Tocris Bioscience) 6 h before and at the time of painting of the skin with hapten.

**Quantitative PCR analysis.** Total RNA was isolated with an RNeasy Mini kit (Qiagen, Hilden, Germany). cDNA was synthesized with a PrimeScript RT reagent kit and random hexamers according to the manufacturer's protocol (TaKaRa). A LightCycler 480 and LightCycler SYBR Green I Master mix were used according to the manufacturer's protocol (Roche) for quantitative PCR (primer sequences, **Supplementary Table 2**). The expression of each gene was normalized to that of the control gene *Gapdh*.

**Microarray analysis.** Total RNA was isolated with an RNeasy Mini Kit according to the manufacturer's protocol (Qiagen). An amplified sense-strand DNA product was synthesized with the Ambion WT Expression Kit (Life Technologies), was fragmented and labeled by the WT Terminal Labeling and Controls Kit (Affymetrix) and was hybridized to a Mouse Gene 1.0 ST Array (Affymetrix). We used the robust multiarray average algorithm for log transformation ($\log_2$) and normalization of the GeneChip data.

**General experimental design and statistical analysis.** For animal experiments, a sample size of three to five mice per group was used on the basis of past experience in generating statistical significance. Mice were randomly assigned to study groups and no specific randomization or blinding protocol was used. Sample or mouse identity was not masked for any of these studies. Prism software (GraphPad) was used for statistical analyses. Normal distribution was assumed a priori for all samples. Unless indicated otherwise, an unpaired parametric *t*-test was used for comparison of data sets. In cases in which the data-point distribution was not Gaussian, a nonparametric *t*-test was also applied. *P* values of less than 0.05 were considered significant.


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SHORT COMMUNICATION

# DIHS/DRESS with Remarkable Eosinophilic Pneumonia Caused by Zonisamide

**Rintaro Shibuya[1], Hideaki Tanizaki[1], Saeko Nakajima[1], Itsuko Koyanagi[2], Tatsuki R. Kataoka[2], Yoshiki Miyachi[1] and Kenji Kabashima[1]***

*[1]Department of Dermatology, Kyoto University Graduate School of Medicine, 54 Shogoin, Kawahara-cho, Sakyo-ku, Kyoto 606-8507, and [2]Department of Diagnostic Pathology, Kyoto University Hospital, Kyoto, Japan. *E-mail: kaba@kuhp.kyoto-u.ac.jp*

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Drug-induced hypersensitivity syndrome (DIHS), also known as drug reaction with eosinophilia and systemic symptoms (DRESS), is a rare and severe skin disease associated with systemic findings, such as fever, eosinophilia, lymphadenopathy, and internal organ involvement that typically develops 2 to 6 weeks after the drug intake, following a prolonged course with frequent flare-ups and relapses over weeks or even months after discontinuing the drug. This syndrome has been reported to be associated with the intake of anticonvulsants, sulfonamides and allopurinol (1). Several mechanisms are involved in its pathophysiology, such as drug toxicity, immunological imbalance and reactivation of the herpes virus family members. Recently, a certain type of human leucocyte antigen (HLA) was identified as a predisposing factor in DIHS/DRESS. Here, we describe a case of DIHS/DRESS with remarkable eosinophilic pneumonia caused by zonisamide.

## CASE REPORT

A 46-year-old woman was referred to our hospital with a spiking fever, dry cough and skin rash. She had a past medical history of subarachnoid haemorrhage due to hereditary haemorrhagic telangiectasia (HHT) and had commenced zonisamide 8 months before. She had been administered with zonisamide for 2 months, and followed by a washout period of 4 months. On the 41st day of the second course of treatment, she developed fever with chills, dry cough, and progressive non-pruritic maculopapular eruption with mucosal involvement (Fig. 1 a, b).

She was hospitalised for further evaluation. Blood tests revealed white blood cell count 8,800/mm$^3$ (11% eosinophils, 49% neutrophils and 27% lymphocytes) and platelet count 227,000/mm$^3$. Liver function test results were elevated: asparate aminotransferase (AST) 67 U/l, alanine aminotransferase (ALT) 133 U/l; alkaline phosphatase (ALP) 220 U/l. Serologic tests for viral infections, including hepatitis B, hepatitis C, Epstein-Barr virus (EBV), cytomegalovirus, and human herpes virus 6 (HHV6) were negative. A chest radiograph did not reveal a pulmonary infiltration. A skin biopsy of the erythematous eruption on her abdomen exhibited spongiosis and vacuolar degeneration of epidermal basal keratinocytes. Lymphocytic perivascular infiltration was present in the dermis (Fig. 1 c, d). Immunohistochemical staining showed that lymphocytes were CD8$^+$ lymphocytes with sparse Foxp3 positive lymphocytes in the upper dermis (Fig. 1 e, f).

Based upon the patient's clinical, laboratory, and pathological findings, DIHS/DRESS was suspected. Zonisamide was discontinued immediately and topical steroids were initiated. On the 11th day of hospitalisation, however, her dry cough was exacerbated. Laboratory tests revealed leucocytosis (11,800/μl) with eosinophilia (3,800/μl) and atypical lymphocytosis. HHV6 IgG titre was increased from × 20 (at day 4) to × 120 (at day 17), confirming a reactivation of HHV6. Drug-induced lymphocyte stimulation test (DLST) for zonisamide was positive (a stimulation index of 338.2 %). Chest computed tomography (CT) showed multiple bilateral nodular lesions with surrounding ground-glass-opacity halo (Fig. 1g). Eosinophilic pneumonia (EP) due to zonisamide was suspected and systemic steroid therapy (0.5 mg/kg/day of prednisolone) was commenced. Peripheral eosinophils decreased and her pulmonary lesions improved after one week. Oral prednisolone was tapered and she was discharged on the 35th day of hospitalisation.

## DISCUSSION

The diagnosis of DIHS/DRESS is based upon clinical and laboratory findings. The European Registry of Severe Cutaneous Adverse Reaction (RegiSCAR) study group has devised a scoring system (2). Our case was scored as "7" which is classified as a "definite" case (Table SI[1]). Although, bronchoscopy was not successfully performed due to cardiopulmonary arrest during the procedure, the findings of high eosinophil counts and pneumonia developing 2 weeks after the onset of DRESS, along with the dramatic clinical response to glucocorticoids, strongly suggest that EP was a part of DRESS/DIHS rather than a separate adverse effect of zonisamide. Although pulmonary involvement is rarely reported, it may lead to life threatening adult respiratory distress syndrome (3, 4).

The pathogenesis of DIHS/DRESS remains unclarified, but reactivation of the herpes virus family has been reported at the onset of DIHS/DRESS (5). Tumour necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) secreted by the anti-EBV CD8$^+$ T lymphocytes in cutaneous and visceral lesions may contribute to the development of DIHS/DRESS at the early stage (6). One study has demonstrated a switch in the predominant drug-specific proliferating T-cell population in the course of DIHS/DRESS; CD8$^+$ lymphocytes were predominant initially, whereas CD4$^+$ lymphocytes and regulatory T (Treg) cells (CD4$^+$CD25$^+$Foxp3$^+$) proliferated at the recovery stage (7). In our case, immunohistochemical staining showed abundant CD8$^+$ lymphocytes and few Treg cells infiltration in the upper dermis, reflecting the acute phase of DIHS/DRESS.

[1]http://www.medicaljournals.se/acta/content/?doi=10.2340/00015555-1863

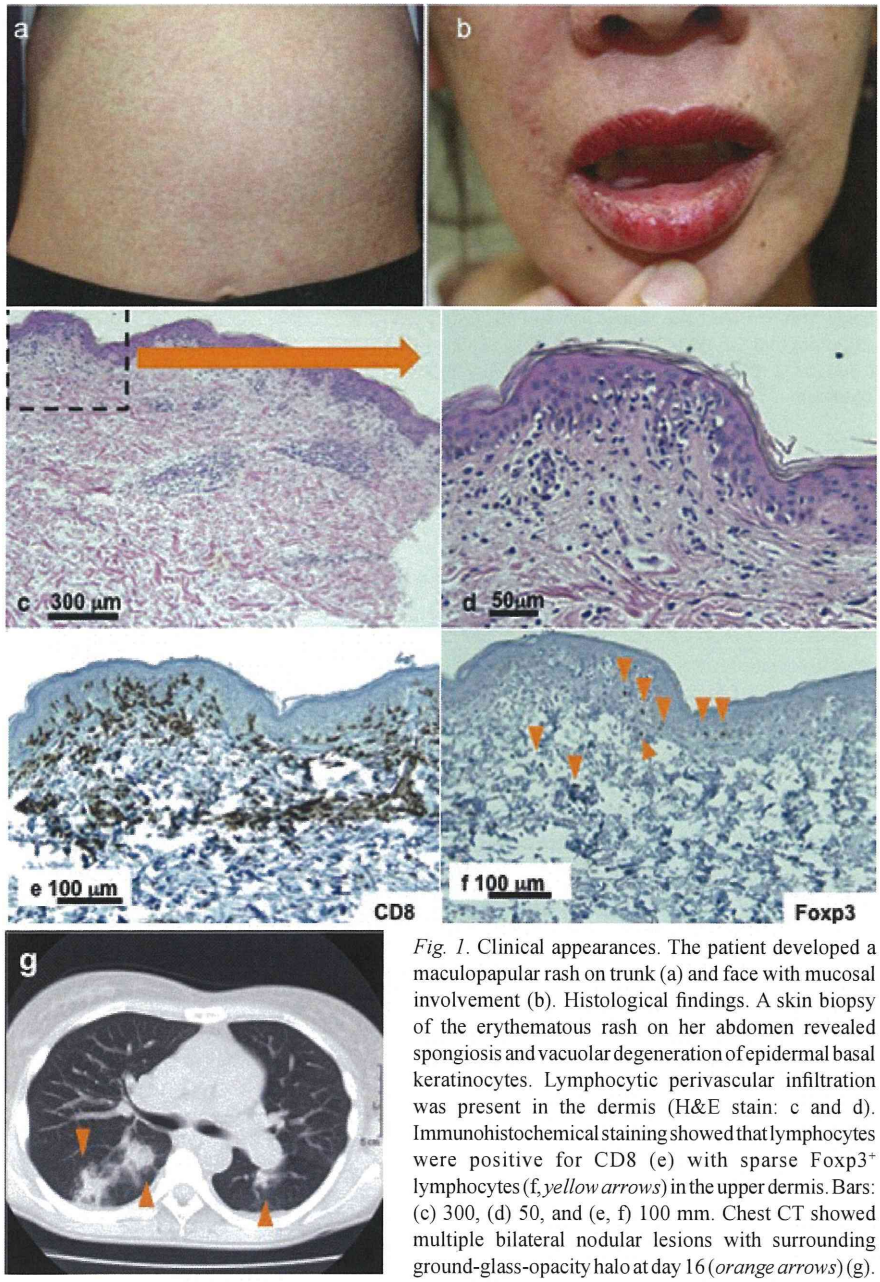


*Fig. 1.* Clinical appearances. The patient developed a maculopapular rash on trunk (a) and face with mucosal involvement (b). Histological findings. A skin biopsy of the erythematous rash on her abdomen revealed spongiosis and vacuolar degeneration of epidermal basal keratinocytes. Lymphocytic perivascular infiltration was present in the dermis (H&E stain: c and d). Immunohistochemical staining showed that lymphocytes were positive for CD8 (e) with sparse Foxp3$^+$ lymphocytes (f, *yellow arrows*) in the upper dermis. Bars: (c) 300, (d) 50, and (e, f) 100 mm. Chest CT showed multiple bilateral nodular lesions with surrounding ground-glass-opacity halo at day 16 (*orange arrows*) (g).

At present, we do not have clear evidence how EP was induced by zonisamide in DRESS/DIHS. Pulmonary involvement in DIHS/DRESS is known to be induced by a certain drug (3, 4). We consider that pulmonary involvement in DIHS/DRESS is not related to specific drugs only but may be related to the patient's underlying condition, such as HHT. Antigen-presenting cells (e.g. alveolar macrophages) are known to ingest drugs and present them to T helper cells to release interleukin-5, resulting in eosinophil proliferation (8). Therefore, in patients with HHT, blood vessels tend to be fragile and prone to bleeding (9), which may cause the accumulation of zonisamide into the lung and stimulate macrophages to initiate the immunologic cascade. Clinicians should be aware of the possible involvement of the lung in the course of DIHS/DRESS.

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