Perivascular leukocyte cluster: an essential structure for efficient effector T cell activation in the skin

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It remains largely unclear how antigen-presenting cells encounter effector/memory T cells efficiently in the periphery. Here we used a murine contact hypersensitivity model to analyze antigen presentation in the skin. 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 effector T cells in antigen- and integrin LFA-1-dependent manners. Intriguingly, DCs were attracted toward perivascular area and DC clustering was abrogated by macrophage-depletion. IL-1α treatment induced CXCL2 production from dermal macrophages, and DC clustering was suppressed by blockade of either IL-1R or CXCR2. These findings suggest that dermal leukocyte cluster is an essential structure for elicitation of the acquired cutaneous immunity.

Boundary tissues, including the skin, are continually exposed to foreign antigens that must be monitored and characterized for possible elimination. Upon exposure to skin, skin dendritic cells (DCs), including epidermal Langerhans cells (LCs), capture the antigens and migrate to draining lymph nodes (LNs). In the LNs, antigen presentation to naïve T cells occurs mainly in the T cell zone, where naïve T cells accumulate in the vicinity of DCs via CCR7-signaling[1]. This structure facilitates the efficient encounter of antigen-bearing DCs with antigen-specific naïve T cells.

On the other hand, antigen presentation within the skin is the crucial step in elicitation of acquired skin immune responses. Since the majority of skin-infiltrating T cells are effector memory-phenotype and either naïve T cells or B cells are almost absent in the skin[2], the antigen-presentation mechanism in the skin should be substantially different from that in LNs. Using a murine contact hypersensitivity (CHS) model, previous studies have revealed that dermal DCs (dDCs), but not epidermal LCs, play a pivotal role in the antigen-carrying and presentation process in LNs[3]. In the skin, however, it remains unclear which subset of antigen-presenting cells (APCs) presents antigens to peripheral effector/memory T cells, and how skin-infiltrating effector/memory T cells efficiently encounter APCs. On the other hand, dermal macrophage is one of a key modulator in CHS response[4], but the precise mechanism behind how macrophages are involved in an antigen recognition process in the skin has not yet been clarified. These unsolved puzzles prompted us to focus on the peripheral antigen-recognition mechanism by skin-infiltrating effector/memory T cells.

When keratinocytes encounter foreign antigens, they immediately produce various pro-inflammatory mediators such as interleukin (IL)-1 and tumor necrosis factor (TNF)-α in an antigen-nonspecific manner[5, 6]. In particular, the IL-1 family proteins have been considered to be important modulators in CHS responses, because hapten-specific T cell activation was shown to be impaired in IL-1α/β-deficient mice but not in TNF-α-deficient mice[7]. The agonistic ligands of the IL-1 receptor (IL-1R) are IL-1α and IL-1β. While IL-1α is stored in keratinocytes and secreted upon exposure to nonspecific stimuli, IL-1β is produced mainly by epidermal LCs and dermal mast cells in an inflammasome-dependent manner via NALP3 and caspase 1/11 activation. Since these pro-inflammatory mediators are crucial even in the initiation of acquired immune responses, it is of great interest how IL-1 modulates antigen recognition by skin-infiltrating T cells.

Using a murine CHS model, here we examined how DCs and effector T cells encounter each other efficiently in the skin. We found that dDCs formed clusters upon antigen stimuli,

wherein effector T cells proliferated and activated 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

DC–T cell clusters are formed at antigen-challenged sites

To explore immune cell accumulation in the skin, we examined the clinical and histological features of elicitation of human allergic contact dermatitis. Allergic contact dermatitis is the most common eczematous skin diseases, affecting 15–20% of the general population worldwide[8], and is mediated by T cells. Although antigens may be applied relatively evenly over the surface of skin, clinical manifestations commonly include discretely distributed small vesicles (**Fig. 1a**), suggesting an uneven occurrence of intense inflammation. Histological examination of allergic contact dermatitis has shown spongiosis, intercellular edema in the epidermis, and co-localization 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 accumulations of T cells and DCs in the dermis may contribute to vesicle formation in early eczema.

To characterize the DC–T cell clusters in elicitation reactions, we obtained time-lapse images in a murine model of CHS using two-photon microscopy. T cells were isolated from the draining LNs of 2, 4-dinitrofluorobenzene (DNFB)-sensitized mice, labeled and transferred into CD11c-yellow fluorescent protein (YFP) mice. In the steady state, YFP^+ dDCs distributed diffusely (**Fig. 1c**), representing nondirected movement, as reported previously (**Supplementary Fig. 1**). After topical challenge with DNFB, YFP^+ dDCs transiently increased their velocities and formed clusters in the dermis, becoming larger and more evident over 24 h (**Fig. 1c circles 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, the accumulation of DCs and T cells in the dermis is provoked in mice during CHS responses. Intriguingly, the intercellular spaces between keratinocytes overlying the DC–T cell clusters in the dermis were enlarged (**Fig. 1e**), replicating what is seen in human allergic contact dermatitis. We then sought to determine which of the two major DC populations in skin, epidermal LCs or dDCs, were essential for the elicitation of CHS, using bone marrow (BM) chimeric CD11c-diphtheria toxin receptor (DTR) and Langerin-DTR mice (**Supplementary Fig. 2**). We 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. 3**). In addition, interferon (IFN)-γ-production by skin-infiltrating T cells was strongly suppressed in dDC-depleted mice (**Fig. 1g**).These results suggest that dDCs, and not epidermal LCs, are essential for the elicitation of CHS responses.

Skin-infiltrating effector T cells proliferate *in situ* in an antigen-dependent manner

To evaluate the impact of DC–T cell clusters in the dermis, we determined whether T cells had acquired the ability to proliferate via DC–T cell accumulation in the dermis. $CD4^+$ or $CD8^+$ T cells purified from the draining LNs of DNFB-sensitized mice were labeled with CellTrace™ Violet and transferred into naïve mice. Twenty-four hours after DNFB application, we collected the skin to evaluate T cell proliferation by dilution of fluorescent intensity, and we found that the majority of infiltrating T cells were $CD44^+$ $CD62L^-$ effector memory phenotype (**Supplementary Fig. 4**) and that 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, since 2,4,6-trinitrochlorobenzene (TNCB)-sensitized T cells exhibited low proliferative activities in response to DNFB application (**Fig. 2a**). In line with this finding, the DC–T cell conjugation time was prolonged in the presence of cognate antigens (**Fig. 2b**), and the T cells interacting with DCs divided within DC–T cell clusters (**Fig. 2c, and Supplementary Movie 3**).

$CD8^+$ T cell activation in DC–T cell clusters is LFA-1 dependent

A sustained interaction between DCs and naïve T cells, which has been named as immunological synapse, is maintained with cell adhesion molecules[9]. Particularly, lymphocyte function-associated antigen-1 (LFA-1), an integrin on T cells bound to intercellular adhesion molecule-1 (ICAM-1) on the APCs, is essential for T cell proliferation and activation in an antigen-recognition process in LNs. To examine whether LFA-1-ICAM-1 interaction is required for effector T cell activation in DC–T cell clusters in the skin, an anti-LFA-1 neutralizing antibody, KBA, was intravenously injected 14 h after elicitation. Intriguingly, accumulated T cells were dispersed by KBA treatment (**Fig. 3a**). The velocity of T cells in the cluster was 0.65 ± 0.29 μm/min at the beginning (14 h after DNFB challenge) and increased up to 3-fold (1.64± 1.54 μm/min) at 8 h after KBA-treatment, while it was not affected by isotype-matched control IgG treatment (**Fig. 3b**). At the outside of the cluster, T

cells smoothly migrated at the mean velocity of 2.95 ± 1.19 μm/min, which was in consistent with our previous report[10]. And again, it was not affected by control-IgG treatment (data not shown). KBA treatment also attenuated ear swelling significantly (**Fig. 3c**), as was IFN-γ production by skin-infiltrating $CD8^+$ T cells (**Fig. 3d and e**). These findings suggest that DC–effector T cell conjugations are integrin-dependent, as seen in the DC–naïve T cell interactions in draining LNs.

Skin macrophages are required for dDC clustering

We next examined the initiation factors of DC–T cell accumulation. Intriguingly, dDC clusters were also formed in response to the initial application of hapten (sensitization phase). But their number was significantly decreased 48 h after sensitization, while DC clusters persisted for 48 h in the elicitation phase (**Fig. 4a and Supplementary Fig. 5a**). These DC clusters were abrogated 7 days after DNFB application (data not shown). These observations suggest that DC–T cell accumulation is initiated by DC clustering, which then induces the accumulation, proliferation, and activation of T cells, a process that depends 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 *Mycobacterium bovis BCG*-inoculation (**Supplementary Fig. 5b and c**). In addition, DC cluster formation was observed not only in the ear but also in other regions such as back skin and footpad (**Supplementary Fig. 5d**). These results suggest that DC cluster formation is not an ear-specific event, but a fundamental mechanism to elicit skin inflammation.

The initial DC clusters were not attenuated in recombination activating gene 2 (RAG2)-deficient mice in which T and B cells were absent, in lymphoid tissue inducer cell-deficient *aly/aly* mice [11], or in mast cell or basophil-depleted conditions using MasTRECK or BasTRECK mice[12, 13] (**Fig. 4b**). In contrast, DC clusters were abrogated in LysM-DTR BM chimeric mice, in which both macrophages and neutrophils were depleted by treatment with diphtheria toxin (DT) (**Fig. 4b and c**). Since the depletion of neutrophils alone by administration of anti-Ly6G antibody (1A8) did not interfere with DC cluster formation (**Fig. 4b**), it seems that macrophages, but not neutrophils, play roles during the formation of DC clusters. Of note, DC cluster formation was not attenuated by anti-LFA-1 neutralizing KBA antibody-treatment (**Supplementary Fig. 6**), suggesting that macrophage-DC interaction was LFA-1 independent. Consistent with the DC cluster formation, the elicitation of the CHS response (**Fig. 4d**) and IFN-γ production by skin-infiltrating T cells (**Fig. 4e**)

were significantly suppressed in LysM-DTR BM chimeric mice treated with DT.

Macrophages are essential for perivascular DCs clustering upon antigen-challenge

To examine the kinetics of dermal macrophage and DCs *in vivo*, we visualized them by two-photon microscopy. *In vivo* labeling of blood vessels with tetramethylrhodamine isothiocyanate (TRITC)-conjugated dextran revealed that dDCs distributed diffusely in the steady state (**Fig. 5a, left**). After hapten-application to the ear of previously sensitized mice, dDCs accumulated mainly around post-capillary venules (**Fig. 5a, right, and 5b**). Interestingly, time-lapse imaging revealed that some of dDCs showed directional migration toward TRITC-positive cells that were labeled red by incorporating extravasated TRITC-dextran (**Fig. 5c and Supplementary Movie 4**). The majority of TRITC-positive cells were F4/80$^+$ CD11b$^+$ macrophages (**Supplementary Fig. 7**). These observations prompted us to examine the role of macrophages in DC accumulation. We used a chemotaxis assay to determine whether macrophages attracted the DCs. As expected, dDCs were efficiently attracted by skin-derived macrophages (**Fig. 5d**).

IL-1α signaling is essential for DC cluster formation upon antigen challenge

We attempted to explore the underlying mechanism of DC cluster formation. Since DC accumulation occurred during the first application of hapten, an antigen-nonspecific mechanism, such as production of pro-inflammatory mediator IL-1, may initiate DC clustering. Intriguingly, DC accumulation in response to hapten was not suppressed in NALP3- or caspase-1/11-deficient mice, but they were suppressed significantly in IL-1R type1 (IL-1R1)-deficient mice or after the subcutaneous administration of an IL-1R antagonist (**Fig. 6a and b**). Consistent with these observations, the elicitation of CHS and IFN-γ production by skin-infiltrating T cells were significantly attenuated in IL-1α/β-deficient mice (**Fig. 6c and d**). In addition, the formation of dDC clusters was suppressed significantly by the subcutaneous injection of an anti-IL-1α neutralizing antibody, but only marginally by an anti-IL-1β neutralizing antibody (**Fig. 6b**). Given that IL-1α production by keratinocytes upon hapten application is a well-known phenomenon[14], our results support a major role for IL-1α in mediating the formation of DC clustering.

M2-phenotype macrophages produce CXCL2 to attract dDCs

To further characterize how macrophages attract dDCs, we examined *Il1r1* expression in

BM-derived M1- and M2-phenotype macrophages based on the differential mRNA expression of TNF-α, Nos2, IL-12a, Arg-1, Retnla, and Chi313 (**Supplementary Fig. 8**) [15]. We found that M2-phenotype macrophages expressed higher levels of *Il1r1*compared to M1-phenotype macrophages (**Fig. 6e**). We also found that a subcutaneous injection of pertussis toxin, a Gi-specific inhibitor, completely attenuated DC cluster formation in response to hapten-stimuli (**Fig. 6b**). We therefore focused on the expression levels of Gi-coupled chemokine signaling in the following studies.

We employed microarray analysis to examine chemokine expression levels in M1- and M2-phenotype macrophages with or without IL-1α stimulation. No chemokine expression was enhanced in M1-phenotype macrophages with IL-1α treatment, whereas CCL5, CCL17, CCL22, and CXCL2 mRNA expression levels were increased in M2-phenotype macrophages (**Supplementary Table 1**). Among them, CXCL2 expression was enhanced most prominently by treatment with IL-1α, which was also validated by real-time polymerase chain reaction (PCR) analysis (**Fig. 6f**). Consistently, CXCL2 expression was significantly increased in DNFB-painted skin (**Supplementary Fig. 9a**) and was not affected by neutrophil-depletion with 1A8 (**Supplementary Fig. 9b and c**). In addition, IL-1α-treated dermal macrophages produced CXCL2 *in vitro* (**Supplementary Fig. 9d**). These results suggest that dermal macrophages, but not neutrophils, are the major sources of CXCL2 during CHS. We detected mRNA of CXCR2, the receptor for CXCL2, at high levels in DCs (**Supplementary Fig. 9e**). These findings prompted us to examine the role of CXCR2 on dDCs. The formation of DC clusters in response to hapten stimuli was attenuated substantially via intraperitoneal administration of a CXCR2 inhibitor SB265610 [16] (**Fig. 6g**). In addition, SB265610-treatment during the elicitation of CHS inhibited the ear swelling (**Fig. 6h**) and IFN-γ production by skin infiltrating T cells (**Fig. 6i**). Taken together, these findings suggest that dermal macrophages are essential for initiating DC cluster formation through the production of CXCL2, and that DC clustering plays an important role for efficient activation of skin-infiltrating T cells.

DISCUSSION

Here we have identified leukocyte clusters in the skin induced by antigen challenge using the murine CHS model. This is the first study that clearly analyses how DCs and effector/memory T cells encounter one another and then interact to induce efficient

antigen-specific immune responses in the skin. We demonstrated that dDCs, but not epidermal LCs, are essential for antigen presentation to skin-infiltrating effector T cells. In addition, dDCs exhibit sustained association with effector T cells in an antigen- and LFA-1-dependent manner. Intriguingly, IL-1α and not the inflammasome, initiates the formation of perivascular DC clusters.

Although the mechanistic events in the sensitization phase in cutaneous immunity have been studied thoroughly over 20 years[17, 18], one of the unresolved important questions in cutaneous immunity is what types of immunological events occur during the elicitation phases. Our present study illustrates the antigen recognition process in the skin (**Fig. 7**). Epidermal contact with antigens triggers release of IL-1 in the skin[14]. Previous studies have shown that the epidermal keratinocytes constitute a major reservoir of IL-1α[6] and mechanical stress to keratinocytes permits release of large amounts of IL-1α even in the absence of cell death[19]. The precise source and production mechanism of IL-1α in this process need to be determined in future studies. IL-1 signal then seems to activate macrophages that subsequently attract dDCs, mainly to areas around post-capillary venules where effector/memory T cells in the blood transmigrate into the skin[20]. In the absence of antigen-specific effector/memory T cells to a cognate antigen (i.e. in the sensitization phase of CHS; **Fig. 7a**), DC clustering is a transient event, and hapten-carrying DCs migrate into draining LNs to establish sensitization. On the other hand, DC clustering is followed by T cell accumulation in the presence of the antigen and antigen-specific effector/memory T cells (i.e. in the elicitation phase of CHS; **Fig. 7b**). We propose that these perivascular dDC clusters may provide antigen-presentation sites for efficient effector T cell activation. In fact, CHS responses and intracutaneous T cell activation were attenuated significantly in the absence of the leukocyte clusters by depleting macrophages, or inhibiting integrin functions (**Fig. 3**), IL-1R signaling[21, 22], or CXCR2[23].

In contrast to the skin, antigen presentations in other peripheral organs are rather well clarified. For example, in submucosal areas, specific sentinel lymphoid tissues, called mucosa-associated lymphoid tissue (MALT), serve as peripheral antigen presentation sites[24], and lymphoid follicles are present in the normal bronchi (bronchus-associated lymphoid tissue; BALT). These tissues serve as antigen presentation sites in non-lymphoid peripheral organs. By analogy, the concept of skin-associated lymphoid tissue (SALT) was proposed in the early 1980's, based on findings that cells in the skin are capable of capturing, processing, and presenting antigens[25, 26]. However, the roles played by the various skin components as

antigen presentation sites remain uncertain. In this study, we have identified the inducible structure formed by dermal macrophages, dDCs and effector T cells sequentially. Since formation of this structure is essential for efficient effector T cell activation, these inducible leukocyte clusters may function as SALT. Unlike MALT, these leukocyte clusters are not found in the steady state thus far but inducible during the development of acquired immune response. Therefore, this cluster may be better named as inducible SALT (iSALT), like inducible BALT (iBALT) in the lung[27]. In contrast to iBALT, naïve T cells or B cells are absent therein thus far (data not shown), suggesting that the leukocyte clusters in the skin may be specialized for effector T cell activation but not for naïve T cell activation. Our findings suggest that approaches to the selective inhibition of this structure may have novel therapeutic benefit in inflammatory disorders of the skin.

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METHODS

Mice

Female 8- to 12-week-old C57BL/6-background mice were used in this study. C57BL/6N mice were purchased from SLC (Shizuoka, Japan). Langerin-eGFP-DTR[28], CD11c-DTR[29], CD11c-YFP[30], LysM-DTR[31], Rag2-deficient[32], MasTRECK[12, 13], BasTRECK [12, 13], ALY/NscJcl-*aly/aly*[11], IL-1α/β-deficient[33], IL-1R1-deficient[34], NLRP3-deficient[35], and caspase-1/11-deficient mice[36] were described previously. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Kyoto University Graduate School of Medicine.

Human Subjects

Human skin biopsy samples were obtained from a nickel-reactive patch after 72 h from placement of nickel patch tests in patients with a previously proven allergic contact dermatitis. A biopsy of petrolatum-occluded skin was also obtained as a control. Informed consent was obtained under IRB approved protocols at the Icahn School of Medicine at Mount Sinai School Medical Center, and the Rockefeller University in New York.

Induction of contact hypersensitivity (CHS) response

Mice were sensitized on shaved abdominal skin with 25 μl 0.5% (w/v) 1-fluoro-2,4-dinitrofluorobenzene (DNFB; Nacalai Tesque, Kyoto, Japan) dissolved in acetone/olive oil (4/1). Five days later, the ears were challenged with 20 μl 0.3% DNFB. For adoptive transfer, T cells were magnetically sorted using auto MACS (Miltenyi Biotec, Bergisch Gladbach, Germany) from the draining LNs of sensitized mice and then transferred 1x 10^7 cells intravenously into naïve mice.

Depletion of cutaneous DC subsets, macrophages, and neutrophils

To deplete all cutaneous DC subsets (including LCs), 6-week-old Langerin-DTR mice were irradiated (two doses of 550 Rad given 3 h apart) and were transferred with 1 x 10^7 BM cells from CD11c-DTR mice. Eight weeks later, 2 μg diphtheria toxin (DT; Sigma-Aldrich, St. Louis, MO) was intraperitoneally injected. To selectively deplete LCs, irradiated Langerin-DTR mice were transferred with BM cells from C57BL/6 mice, and 1 μg DT was injected. To selectively deplete dermal DCs, irradiated C57BL/6 mice were transferred with BM cells from CD11c-DTR mice, and 2 μg DT was injected. For macrophage depletion,

irradiated C57BL/6 mice were transferred with BM cells from LysM-DTR mice and 800 ng DT was injected. For neutrophil depletion, 0.5 mg/body anti-Ly6G antibody (1A8, BioXCell, Shiga, Japan) were intravenously administered to mice 24 h before experiment.

Time-lapse imaging of cutaneous DCs, macrophages, and T cells

Cutaneous DCs were observed using CD11c-YFP mice. To label cutaneous macrophages *in vivo*, 5 mg TRITC-dextran (Sigma-Aldrich) was intravenously injected and mice were left for 24 h. At that time, cutaneous macrophages become fluorescent because they incorporated extravasated dextran. To label skin-infiltrating T cells, T cells from DNFB-sensitized mice were labeled with CellTracker Orange CMTMR (Invitrogen, Carlsbad, CA) and adoptively transferred. Keratinocytes and sebaceous glands were visualized with the subcutaneous injection of isolectin B4 (Invitrogen) and BODIPY (Molecular Probes, Carlsbad, CA), respectively. Mice were positioned on the heating plate on the stage of a two-photon microscope IX-81 (Olympus, Tokyo, Japan) and their ear lobes were fixed beneath a cover slip with a single drop of immersion oil. Stacks of 10 images, spaced 3 μm apart, were acquired at 1 to 7 min intervals for up to 24 h. To calculate T cell and DC velocities, movies from 3 independent mice were processed and analyzed using Imaris7.2.1 (Bitplane, South Windsor, CT) for each experiment.

Histology and immunohistochemistry

For histological examination, tissues were fixed with 10% formalin in phosphate buffer saline, and then embedded in paraffin. Sections with a thickness of 5 μm were prepared and subjected to staining with hematoxylin and eosin. For whole-mount staining, the ears were split into dorsal and ventral halves, and incubated with 0.5 M ammonium thiocyanate for 30 min at 37°C [37]. 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 anti-mouse MHC class II antibody (eBioscience, San Diego, CA) followed by incubation with secondary antibody conjugated to Alexa 488 or 594 (Invitrogen). The slides were mounted using a ProLong Antifade kit with DAPI (Molecular Probes) and observed under a fluorescent microscope (BZ-900, KEYENCE, Osaka, Japan). The number/size of DC clusters were evaluated in 10 fields of 1mm^2/ ear and were scored according to the criteria shown in Supplementary Fig. 5a.

Antibodies and flow cytometry

Anti-mouse CD4, CD8, CD11b, CD11c, B220, MHC class II, F4/80, IFN-γ, Gr1 antibodies, and 7-amino-actinomycin D (7AAD) were purchased from eBioscience. Anti-mouse CD45 antibody (BioLegend, San Diego, CA), anti-TCR-β antibody (BioLegend), and anti-CD16/CD32 antibody (BD Biosciences) were purchased. Flow cytometry was performed using LSRFortessa (BD Biosciences) and analyzed with FlowJo (TreeStar, San Carlos, CA).

Chemotaxis assay

Chemotaxis was performed as described previously with some modifications [37]. In brief, ear splits were minced and digested with 2 mg/ml collagenase type II (Worthington Biochemical, NY) containing 1 mg/ml hyaluronidase (Sigma-Aldrich) and 100 μg/ml DNase I (Sigma-Aldrich) for 30 min at 37°C. DDCs and macrophages were isolated using auto-MACS. Alternatively, BM-derived DCs and macrophages were prepared. 1 x 10^6 DCs were added to the 5 μm pore-size transwell insert (Corning, Cambridge, MA) and 5 x 10^5 macrophages were added into the lower wells, and the cells were incubated at 37°C for 12 h. A known number of fluorescent reference beads (FlowCount fluorospheres, Beckman Coulter, Fullerton, CA) were added to each sample to allow accurate quantification of migrated cells in the lower wells by flow cytometry.

Cell proliferation assay with CellTrace™ Violet

Mice were sensitized with 25 μl 0.5% DNFB or 7% trinitrochlorobenzene (Chemical Industry, Tokyo, Japan). Five days later, T cells were magnetically separated from the draining LNs of each group, and labeled with CellTrace™ Violet (Invitrogen) as per the manufacturer's protocol. Ten million T cells were adoptively transferred to naïve mice, and the ears were challenged with 20 μl of 0.5% DNFB. Twenty-four hours later, ears were collected and analyzed by flow cytometry.

In vitro differentiation of DCs, M1 and M2-phenotype macrophages from BM cells

BM cells from the tibias and fibulas were plated 5x10^6 cells/ 10cm dishes on day 0. For DC differentiation, cells were cultured at 37°C in 5% CO_2 in cRPMI medium (RPMI supplemented with 1% L-glutamine, 1% Hepes, 0.1% 2ME and 10% fetal bovine serum) containing 10 ng/mL GM-CSF (Peprotech, Rocky Hill, NJ). For macrophages differentiation, BM cells were cultured in cRPMI containing 10 ng/mL M-CSF (Peprotech).

Medium was replaced on days 3 and 6 and cells were harvested on day 9. To induce M1 or M2 phenotypes, cells were stimulated for 48 h with IFN-γ (10 ng/mL; R&D Systems, Minneapolis, MN) 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 IL-1α/β-deficient mice[33] to avoid pre-activation during cell preparations. Ear splits were treated with 0.25% trypsin/EDTA for 30 min at 37°C to remove epidermis and then minced and incubated with collagenase as previously described. $CD11b^+$ cells were separated using MACS and $2x10^5$ cells/well were incubated with or without 10 ng/ml IL-1α (R&D systems) in 96-well plate for 24 h.

Blocking assay

For LFA-1 blocking assay, mice were intravenously injected with 100 μg anti-LFA-1 neutralizing antibody, KBA, 12-14 h after challenge with 20 μl 0.5% DNFB. For IL-1R blocking, mice were subcutaneously injected with 10 μg IL-1R antagonist (PROSPEC, East Brunswick, NJ) 5 h before challenge. For blocking of CXCR2, mice were intraperitoneally treated with 50 μg CXCR2 inhibitor SB265610[16] (Tocris Bioscience, Bristol, UK) 6 h before and at hapten painting.

Quantitative PCR analysis

Total RNA was isolated using an RNeasy Mini kit (Qiagen, Hilden, Germany). cDNA was synthesized using a PrimeScript RT reagent kit (TaKaRa, Ohtsu, Japan) with random hexamers as per the manufacturer's protocol. Quantitative PCR was carried out with a LightCycler 480 using a LightCycler SYBR Green I master (Roche, Lewes, UK) as per the manufacturer's protocol. The relative expression of each gene was normalized against that of Gapdh. Primer sequences were as follows: *Il1r1* forward, ATGAGTTACCCGAGGTCCAGTG, and reverse, TACTCGTGTGACCGGATATTGC; *Cxcl2* forward, CAAACCGAAGTCATAGCCAC, and reverse, TCTGGTCAGTTGGATTTGCC; *Ccr4* forward, GAAGAGCAAGGCAGCTCAAC, and reverse, GACCTCCCCAAATGCCTTGA; *Ccr8* forward, ATAATTGGTCTTCCTGCCTCGAT, and reverse CTGAGGAGGAACTCTGCGTC; *Cxcr2* forward, ACTACTGCAGGATTAAGTTTACCTC, and reverse, TCTCTGAGTGGCATGGGACA; *Cxcr3* forward, GCCATGTACCTTGAGGTTAGTGA,

and reverse ATCGTAGGGAGAGGTGCTGT; *Cxcr6* forward, ACTGGGCTTCTCTTCTGATGC, and reverse, AAGCGTTTGTTCTCCTGGCT; *Tnf* forward, CCCCAAAGGGATGAGAAGTT and reverse CACTTGGTGGTTTGCTACGA; *Nos2* forward, GTTCTCAGCCCAACAATACAAGA, and reverse, GTGGACGGGTCGATGTCAC; *Il12a* forward, CTGTGCCTTGGTAGCATCTATG, and reverse, GCAGAGTCTCGCCATTATGATTC; *Arg1* forward, ACCATAAGCCAGGGACTGAC, and reverse, AGGAGAAGGCGTTTGCTTAG; *Retnla* forward, CCAATCCAGCTAACTATCCCTCC, and reverse, ACCCAGTAGCAGTCATCCCA; *Chi313* forward, AGAAGGGAGTTTCAAACCTGGT, and reverse, GTCTTGCTCATGTGTGTAAGTGA, and *Gapdh* forward, GGCCTCACCCCATTTGATGT, and reverse, CATGTTCCAGTATGACTCCACTC.

Microarray analysis

Total RNA was isolated using the RNeasy Mini Kit (Qiagen) as per the manufacturers' protocol. An amplified sense-strand DNA product was synthesized by the Ambion WT Expression Kit (Life Technologies, Gaithersburg, MD), and was fragmented and labeled by the WT Terminal Labeling and Controls Kit (Affymetrix, Santa Clara, CA), and was hybridized to the Mouse Gene 1.0 ST Array (Affymetrix). We used the robust multi-array average algorithm for log transformation (log2) 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 determined 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. Statistical analyses were performed using Prism software (GraphPad Software Inc.). 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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AUTHOR CONTRIBUTIONS

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

COMPETENG FINANCIAL INTERESTS

The authors declare no competing financial interests.

Figure Legends

Figure 1: DC–T cell cluster formation 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**) Hematoxylin and eosin, anti-CD3, and anti-CD11c staining of the human skin biopsy sample from an eczematous legion. Asterisks and arrowheads denote epidermal vesicles and dDC–T cell clusters, respectively. Scale bar = 250 μm. (**c**) Sequential images of leukocyte clusters in the elicitation phase of CHS. White circles represent DC (green) and T cell (red) dermal accumulations. Scale bar = 100 μm. (**d**) A high magnification view of DC–T cell cluster in Fig.1c. Scale bar = 10 μm. (**e**) Intercellular edema of the epidermis overlying DC–T cell cluster in the dermis. Keratinocytes (red) are visualized with isolectin B4. The right panel shows the mean distance between adjacent keratinocytes above (+) or not above (-) DC–T cell cluster (n=20, each). Scale bar = 10 μm. (**f**) Ear swelling 24 h after CHS in subset-specific DC-depletion models (n = 5, each). *, $P < 0.001$. (**g**) The number (left) and the % frequency (right) of IFN-γ producing T cells in the ear 18 h after CHS with or without dDC-depletion (n = 5, each). *, $P < 0.05$.

Figure 2: Antigen-dependent T cell proliferation in DC–T cell clusters. (**a**) T cell proliferation in the skin. $CD4^+$ and $CD8^+$ T cells from DNFB- (red) or TNCB- (blue) sensitized mice were labeled with CellTrace™ Violet and transferred. The dilutions of tracer in the challenged sites were examined 24 h later. (**b**) Conjugation time of DNFB- (red, n = 160) or TNCB-sensitized (blue, n = 60) T cells with dDCs 24 h after DNFB challenge. *, $P < 0.05$. (**c**) Sequential images of dividing T cells (red) in DC–T cell clusters. Green represents dDCs. Arrowheads represent a dividing T cell.

Figure 3: LFA-1 is essential for the persistence of DC–T cell clustering and for T cell activation in the skin. (**a**) DC (green) and T cell (red) clusters in the DNFB-challenged site before (0 h) and 9 h after KBA or isotype-matched IgG treatment. Scale bar = 100 μm. (**b**) Fold changes of T cell velocities in DNFB-challenged sites after KBA or control IgG treatment (n = 30, each). (**c**) Ear swelling 24 h after KBA (red) or control IgG (black) treatment with DNFB challenge (n = 5, each). (**d** and **e**) IFN-γ production by $CD8^+$ T cells (d) and the number of IFN-γ producing cells in $CD4^+$ or $CD8^+$ populations (e) in KBA (red) or control IgG (black) treated mice (n = 5, each). DNFB-sensitized mice were treated with KBA or control IgG 12 h after DNFB challenge and the skin samples were obtained 6 h later. *, *P*

651 < 0.05.

652

653 **Figure 4:** Macrophages are essential for DC cluster formation. (**a**) Score of DC cluster
654 number 24 h and 48 h after DNFB application in sensitization (red) or elicitation (green)
655 phase of CHS (n=4, each)**.** (**b**) Score of DC cluster number in non-treated (NT) mice and
656 DNFB-applicated-C57BL/6 (WT), Rag2-deficient, aly/aly, MasTRECK, BasTRECK,
657 LysM-DTR, and 1A8-treated mice (n=4, each). *, $P < 0.05$. (**c**) DC clusters observed in
658 LysM-DTR BM chimeric mice with or without DT-treatment. Scale bar = 100 μm. (**d**) Ear
659 swelling 24 h after DNFB application in LysM-DTR BM chimeric mice with (red) or without
660 (black) DT-treatment (n = 5, each). (**e**) The number (left) and the % frequency (right) of
661 IFN-γ producing $CD8^+$ T cells in the ear 18 h after DNFB application in LysM-DTR BM
662 chimeric mice with (red) or without (black) DT-treatment (n = 5, each). *, $P < 0.05$.

663

664 **Figure 5**: Macrophages mediate perivascular DC cluster formation. (**a**) A distribution of
665 dDCs (green) in the steady state (left) and in the elicitation phase of CHS (right). The white
666 circles show DC clusters. Sebaceous glands visualized with BODIPY (green) are indicated by
667 arrows. Blood vessels, yellow/red; macrophages, red. (**b**) A high magnification view of
668 perivascular DC cluster. Scale bar = 100 μm.(**c**) Sequential images of dDCs (green) and
669 macrophages (red) in the elicitation phase of CHS. The white dashed line represents the track
670 of a DC. (**d**) Chemotaxis assay. % input of dDCs transmigrating into the lower chamber with
671 or without macrophages prepared from the skin.

672

673 **Figure 6:** IL-1α upregulates CXCR2 ligands expression in M2-phenotype macrophages to
674 form DC clusters. (**a**) Scores of DC cluster numbers in NT or 24 h after hapten-painted sites
675 in WT, IL-1R-, NALP3-, or caspase 1 (Casp1)-deficient mice (n=4, each). (**b**) Scores of DC
676 cluster numbers in NT or 24 h after hapten-painted sites in isotype control IgG,
677 anti-IL-α antibody, anti-IL-1β antibody, IL-1R antagonist, or pertussis toxin (Ptx)-treated
678 mice (n=4, each). (**c, d**) Ear swelling 24 h after DNFB application (**c**) and the number (left)
679 and the % frequency (right) of IFN-γ producing $CD8^+$ T cells in the ear 18 h after DNFB
680 application (**d**) in IL-1α/β-deficient (red) and WT (black) mice (n = 5, each) which were
681 adoptively transferred with DNFB-sensitized T cells. *, $P < 0.05$. (**e**, **f**) Relative amount of
682 *Il1r1* and *Cxcl2* mRNA expression. Quantitative RT-PCR analysis of mRNA obtained from
683 M1 or M2-phenotype macrophages (e), cultured with (+) or without (-) IL-1α (f) (n=4, each).

(**g**) Scores of DC cluster numbers in NT or 24 h after hapten-painted sites in the presence (SB265610) or absence (vehicle) of a CXCR2 inhibitor (n=4, each). *, $P < 0.05$. (**h, i**) Ear swelling 24 h after DNFB application (**h**) and the number (right) and the % frequency (left) of IFN-γ producing $CD8^+$ T cells 18 h after DNFB application (**i**) with (red) or without (black) SB265610-treatment (n = 5, each). *, $P < 0.05$.

Figure 7: A schema of immunological events in CHS response. (**a**) Sensitization phase. Epidermal contact with antigens triggers release of IL-1 in the skin, which activates macrophages that subsequently attract dDCs to perivascular area via CXCL2 to form clusters. In the absence of antigen-specific effector/memory T cells, DC clustering is a transient event, and hapten-carrying DCs migrate into draining LNs to establish sensitization. (**b**) Elicitation phase. In the presence of antigen-specific effector/memory T cells, the antigen is recognized efficiently in the DC clusters by antigen-specific effector T cells to form clusters, and inflammation is induced promptly via activation and proliferation of antigen-specific effector T cells.

Supplementary Figure 1: DDC motility in elicitation phase of CHS.
(**a**) Superimposed 30-min tracks of 30 randomly selected dermal DCs in the x–y plane, setting the starting coordinates to the origin. Tracks of a steady state, 6, 12, and 24 h after the elicitation with DNFB are shown. (**b** and **c**) Velocity (b) and displacement (c) of dDCs at each time point (n=30). Each bar represents the mean + SD. *, $P < 0.05$.

Supplementary Figure 2: Subset-specific depletion of cutaneous DCs.
(**a**) A schematic representation of our strategy to generate subset-specific cutaneous DC depletion models. To deplete all cutaneous DC subsets, Langerin-DTR mice were transferred with BM cells from CD11c-DTR mice, and DT was injected. To selectively deplete LCs, Langerin-DTR mice were transferred with BM cells from C57BL/6 mice, and DT was injected. To selectively deplete dDCs, C57BL/6 mice were transferred with BM cells from CD11c-DTR mice, and DT was injected. BMT; BM transplantation. (**b**) FACS plots of each group of mice after DT treatment. In dermis, the percentages in $CD45^+$ cells were indicated.

Supplementary Figure 3: Histological findings of the ear skin after CHS.
HE staining of the ears of mice 24 h after challenge with DNFB. Mice were pretreated with

or without sensitization, depleted of LCs and/or dDCs, and challenged with DFNB. Scale bar = 100 μm.

Supplementary Figure 4: The skin-infiltrating T cells exhibit $CD44^+$ $CD62L^-$ effector memory phenotype.

CHS response was induced on the ear skin, and skin-infiltrating cells were stained and analyzed with TCR beta, CD44, and CD62L antibodies by flow cytometry.

Supplementary Figure 5: DDC clusters are formed in response to various stimuli.

(**a**) The scoring criteria for DC clusters by numbers and diameters of clusters. (**b**) DC (green) cluster formation 24 h after topical application without (NT) or with acetone, olive oil, 3% TNCB, 2% DNTB, 0.3% DNFB, or *Mycobacterium bovis BCG*-inoculation (n=4, each). (**c**) Scores of DC cluster numbers of each group 24 h after each stimuli. (**d**) DC (green) cluster formation 24 h after topical application without (NT) or with 0.5% DNFB on the back skin and footpad. Scale bar = 100 μm.

Supplementary Figure 6: DC cluster formation is LFA-1-independent.

(**a**) Mobility of DCs and T cells of the cluster by treatment with anti-LFA-1 treatment. Anti-LFA-1 neutralizing antibody, KBA, was injected intravenously 14 h after elicitation. T cell (red) clustering was dissolved but DC (green) clustering persisted 10 h after KBA-treatment. Scale bar = 100 μm. (**b**) Score of DC cluster number 24 h after DNFB application with KBA (red) or control IgG (black) treatment (n=5, each).

Supplementary Figure 7: $TRITC^+$ cells in the dermis represent macrophage phenotypes.

TRITC-conjugated dextran was injected and dermal suspension was prepared 24 h later. $CD45^+$ and $TRITC^+$ cells were further analyzed with CD11b and F4/80 antibodies by flow cytometry.

Supplementary Figure 8: RT-PCR analysis for M1- and M2-phenotype macrophage markers.

M1-phetnotype macrophage markers, such as TNF-α, Nos2, and IL-12a, and M2-phenotype macrophage markers, such as arginase (Arg)-1, Retnla, and Chi313, were examined in BM-derived M1- and M2-phenotype macrophages. Each bar represents the mean + SD (n=3).

A.U., arbitrary units. *, $P < 0.05$. ***$P < 0.0001$.

Supplementary Figure 9: Neutrophils are not essential for CXCL2 expression in DNFB-painted skin.

(**a**) Relative amount of *Il1r1* 24 h after with or without DNFB-sensitization (n=5). (**b**) Relative amount of *Cxcl2* in DNFB-painted skin in 1A8- or control IgG-treated mice (n=5, each). (**c**) FACS plot of DNFB-painted skin prepared from 1A8- or control IgG-treated mice. $CD11b^+$ $Gr\text{-}1^+$ neutrophils were significantly depleted with 1A8-treatment. (**d**) Relative amount of *Il1r1* from dermal macrophages cultured with or without IL-1α (n=4, each). (**e**) RT-PCR analysis of chemokine receptor mRNA expression in BM-derived DCs.

761 **Supplementary Table 1: Chemokine expression profiles in M1- vs M2-phenotype**
762 **macrophages with or without IL-1α treatment by means of microarray analysis.**

ratio (log2)

Gene Description	Gene Symbol	M2/M1	M2_IL-1α /M1_IL-1α	M1_IL-1α /M1	M2_IL-1α /M2
chemokine (C motif) ligand 1	Xcl1	0.079755	-0.11996	0.029899	-0.16982
chemokine (C-C motif) ligand 1	Ccl1	0.001259	-0.24313	0.237145	-0.00725
chemokine (C-C motif) ligand 2	Ccl2	-0.44104	-0.33997	0.00156	0.10263
chemokine (C-C motif) ligand 3	Ccl3	-0.21153	-0.07617	0.08055	0.21591
chemokine (C-C motif) ligand 4	Ccl4	-0.56782	-0.30699	0.253582	0.514407
chemokine (C-C motif) ligand 5	Ccl5	-5.72304	-4.22995	-0.01614	1.476948
chemokine (C-C motif) ligand 6	Ccl6	1.88874	2.10452	-0.24231	-0.02653
chemokine (C-C motif) ligand 7	Ccl7	-0.2329	-0.32698	0.10564	0.01156
chemokine (C-C motif) ligand 8	Ccl8	-1.61746	-1.40666	-0.05775	0.153052
chemokine (C-C motif) ligand 9	Ccl9	0.44612	0.50154	-0.03128	0.02414
chemokine (C-C motif) ligand 11	Ccl11	0.077222	0.340821	-0.10453	0.159072
chemokine (C-C motif) ligand 12	Ccl12	-3.17708	-2.41643	-0.25347	0.507182
chemokine (C-C motif) ligand 17	Ccl17	1.713942	3.668465	-0.06557	1.888951
chemokine (C-C motif) ligand 20	Ccl20	0.160738	-0.42807	0.24176	-0.34705
chemokine (C-C motif) ligand 21a	Ccl21a	-0.09737	-0.12556	-0.03861	-0.0668
chemokine (C-C motif) ligand 22	Ccl22	-0.02726	1.771884	-0.12263	1.676516
chemokine (C-C motif) ligand 24	Ccl24	4.180073	4.708531	0.077052	0.60551
chemokine (C-C motif) ligand 25	Ccl25	-0.2785	-0.32217	0.142979	0.099304
chemokine (C-C motif) ligand 26	Ccl26	0.133507	-0.12029	0.103554	-0.15024
chemokine (C-C motif) ligand 27a	Ccl27a	0.127154	0.115419	0.007782	-0.00395

chemokine (C-C motif) ligand 27b	Ccl27b	0.246656	0.148537	0.048522	-0.0496
chemokine (C-C motif) ligand 28	Ccl28	1.03498	1.441795	-0.18907	0.217748
chemokine (C-X-C motif) ligand 1	Cxcl1	-0.04569	-0.02674	0.007147	0.026103
chemokine (C-X-C motif) ligand 2	Cxcl2	-1.61789	1.432005	0.130248	3.180143
chemokine (C-X-C motif) ligand 3	Cxcl3	0.185853	0.371034	-0.14298	0.042196
chemokine (C-X-C motif) ligand 5	Cxcl5	0.150911	0.063672	0.178769	0.09153
chemokine (C-X-C motif) ligand 9	Cxcl9	-7.44194	-6.83237	-0.00444	0.605132
chemokine (C-X-C motif) ligand 10	Cxcl10	-6.8282	-5.0165	-0.16726	1.644438
chemokine (C-X-C motif) ligand 11	Cxcl11	-4.88792	-5.05843	0.11235	-0.05816
chemokine (C-X-C motif) ligand 12	Cxcl12	0.455115	0.009638	0.245324	-0.20015
chemokine (C-X-C motif) ligand 13	Cxcl13	-0.20062	-0.25052	-0.02902	-0.07892
chemokine (C-X-C motif) ligand 14	Cxcl14	0.389156	0.354584	0.107933	0.073361
chemokine (C-X-C motif) ligand 15	Cxcl15	-0.16601	-0.05923	-0.05137	0.055403
chemokine (C-X-C motif) ligand 16	Cxcl16	-2.73158	-1.55457	0.0482	1.225208
chemokine (C-X-C motif) ligand 17	Cxcl17	0.019214	0.148531	0.002397	0.131714
chemokine (C-X3-C motif) ligand 1	Cx3cl1	0.228177	0.266712	-0.0622	-0.02366

Video 1: Leukocyte cluster formation in elicitation phase of DNFB-induced CHS response.

CMTMR-labeled DNFB-sensitized T cells were transferred into CD11c-YFP mice and then challenged with DNFB to the ear. $CD11c^+$ dermal DCs (green) and T cells (red) formed clusters approximately 6 h after hapten application. The images were taken every 7 min for 24 h.

Video 2: High magnification view of leukocyte cluster in the elicitation phase of CHS.

CMTMR-labeled DNFB-sensitized T cells were transferred into CD11c-YFP mice and then challenged with DNFB to the ear. Sixteen hours later, the established DC–T cell cluster was

observed in high magnification view for 2 h every 1 min. In this leukocyte cluster, some of T cells (red) interacted with dermal DCs (green) for more than 2 h. The pale yellow debris are melanin granules. Fragmented red and green debris seems to be indicative of dead T cells and DCs engulfed by macrophages, respectively.

Video 3: T cell division in the skin.

CMTMR-labeled DNFB-sensitized T cells divided in DNFB-challenged site. The mean frequency of T cell division was 1.67 ± 1.81 /h/mm^2 (calculated from 5 movies which recorded more than an hour).

Video 4: Macrophages attracted dermal DCs.

TRITC-conjugated dextran was intravenously injected to DNFB-sensitized CD11c-YFP mice to label skin macrophages. The next day, ear skin was challenged with DNFB and examined using two-photon microscopy. In this representative movie, a dermal DC (green) migrated toward TRITC-positive macrophages (red)**.**