Atopic dermatitis is a chronic inflammatory skin disease involving T-helper (Th) 2 cells, eosinophils, and mast cells. Although CCR4 is a major chemokine receptor expressed on Th2 cells and regarded as a potential therapeutic target for allergic diseases, its role in atopic dermatitis remains unclear. Here, by using a hydrogel patch as a transcutaneous delivery device for ovalbumin (an antigen) and Staphylococcus aureus β-toxin (a mast cell activator), we efficiently induced acute atopic dermatitis—like skin lesions in BALB/c mice, a strain prone to Th2 responses, which were characterized by increased numbers of eosinophils, mast cells, and CCR4-expressing Th2 cells in the skin lesions; elevated levels of total and ovalbumin-specific IgE in the sera; and increased expression of IL-4, IL-17A, IL-22, CCL17, CCL22, and CCR4 in the skin lesions. Of note, the same model was less efficient in C57BL/6 mice, a strain prone to Th1 responses. Using this atopic dermatitis model in BALB/c mice, we demonstrated that CCR4-deficiency or a CCR4 antagonist ameliorated the allergic responses. Collectively, these results demonstrate that CCR4 plays a pivotal role in skin allergic inflammation of BALB/c mice by recruiting CCR4-expressing Th2 cells and Th17 cells.

INTRODUCTION

Atopic dermatitis (AD) is a chronic, relapsing, and highly pruritic inflammatory skin disease considered to be caused by the influence of genetic factors and environment factors. The pathophysiology of AD primarily involves impaired skin barrier function together with dysregulated immune responses (Boguniewicz and Leung, 2011). In particular, AD patients have an increased susceptibility to colonization or infection by pathogenic microbes, most notably Staphylococcus aureus (SA) (Boguniewicz and Leung, 2011). SA is known to activate T cells and other immune cells in AD patients through the production of toxins, including superantigens, resulting in persistent skin inflammation (Schlievert et al., 2010). In the acute phase of AD, there is a marked infiltration of T helper (Th) 2 cells, eosinophils, and mast cells in the skin lesions (Brandt and Sivaprasad, 2011). Th2 cells produce cytokines, such as IL-4, IL-5, and IL-13, which are responsible for the promotion of IgE production by B cells (Kim et al., 2010). In the chronic phase of AD, on the other hand, the skin lesions also contain Th1 cells, which are considered to contribute to dermal thickening and hyperkeratosis by IFN-γ (Kim et al., 2013).

Chemokines are a group of cytokines that play important roles in inflammatory and immunological processes through recruitment of select types of leukocytes via respective chemokine receptors (Zlotnik and Yoshie, 2012). CCR4 is a chemokine receptor predominantly expressed by Th2 cells and skin homing T cells (Bonecchi et al., 1998; Campbell et al., 1999; Imai et al., 1999; Yoshie and Matsushima, 2006). However, Stutte et al. (2010) found that CCR4-deficient mice on the C57BL/6 background had no responses (Boguniewicz and Leung, 2011). In particular, AD barrier function together with dysregulated immune response (Kim et al., 2010). In the chronic phase of AD, on the other hand, the skin lesions also contain Th1 cells, which are considered to contribute to dermal thickening and hyperkeratosis by IFN-γ (Kim et al., 2013).

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significant reduction in allergen-specific Th2 responses in a mouse model of AD induced by repeated epicutaneous sensitization of ovalbumin (OVA). Similarly, Islam et al. (2011) reported that the absence of CCR4 had little impact on the development of the OVA-sensitized mouse model of AD. Thus, the role of CCR4 in animal models of AD remains controversial.

Recently, Nakamura et al. (2013) reported that SA δ-toxin is a potent inducer of mast cell degranulation and an important pathogenic factor for the development of allergic skin inflammation (Nakamura et al., 2013). On the other hand, we developed a transcutaneous immunization system using a hydrogel patch, and demonstrated that the hydrogel patch promotes skin penetration of antigenic proteins more efficiently than a gauze patch (Hirobe et al., 2012; Ishii et al., 2008; Matsuo et al., 2010; Matsuo et al., 2011a; Matsuo et al., 2011b). In this study, therefore, we combined the hydrogel patch and SA δ-toxin in the classical OVA-sensitized mouse model of AD (Spergel et al., 1998). We found that the topical application of OVA and δ-toxin by the hydrogel patch efficiently induced acute AD-like skin lesions in BALB/c mice, but less so in C57BL/6 mice. Using this model in BALB/c mice, we have demonstrated that CCR4 plays a pivotal role in the pathogenesis of AD-like skin inflammation.

RESULTS

Comparison of the hydrogen patch and the gauze patch for topical application

After tape stripping, the auricle skin of BALB/c mice was applied with a hydrogel patch or a gauze patch containing Alexa Fluor 488 (green)—conjugated OVA and Texas Red (red)—conjugated δ-toxin for 24 hours (Figure 1a). While no obvious fluorescence was observed in the skin with the gauze patch applied, both green and red fluorescence were clearly detected in the epidermal and dermal layer of the skin with the hydrogel patch applied (Figure 1b). Consequently, most of the CD11c⁺ dendritic cells took up OVA in the skin applied with the hydrogel patch, but not with the gauze patch (Figure 1c). These results confirm that the hydrogel patch is a highly efficient skin delivery method for induction of antigen-specific immune responses compared with the conventional gauze patch.

Topical application of OVA and δ-toxin by the hydrogel patch efficiently induces allergic skin inflammation in BALB/c mice

BALB/c mice were applied at the auricle skin with a hydrogel patch containing either distilled water, OVA alone, δ-toxin alone, or OVA and δ-toxin. Hematoxylin and eosin (HE) and toluidine blue staining, respectively, demonstrated mild epidermal hyperplasia and increased infiltration of mast cells in the skin applied with the hydrogel patch containing OVA and δ-toxin (Figures 1e, 1f). Because δ-toxin was reported to induce robust mast cell degranulation, resulting in increased vascular permeability (Nakamura et al., 2013), we also examined the effect of δ-toxin on the vascular leakage of Evans blue. As shown in Figure 1g, the hydrogel patches containing δ-toxin alone or OVA and δ-toxin, but not those containing distilled water or OVA alone, significantly increased Evans blue dye leakage in the skin. We also measured total and OVA-specific IgE in the blood samples of these mice. While the hydrogel patch containing δ-toxin alone substantially increased total IgE levels, the hydrogel patch containing OVA and δ-toxin increased not only total but also OVA-specific IgE at high levels (Figure 1h).

Topical application of OVA and δ-toxin by the hydrogel patch induces Th2-type skin inflammation in BALB/c mice

It is now accepted that AD is a biphasic disease with a cytokine switch from Th2 dominant in the acute stage to Th1 dominant in the chronic stage (Kim et al., 2013). In addition, it has been reported that the expression of IL-17A is increased in acute AD skin lesions (Koga et al., 2008), while the expression of IL-22 is increased in both acute and chronic AD skin lesions (Biedermann et al., 2015). This suggests that Th17 cells also play a dominant role in acute AD skin lesions. We performed real-time PCR to examine the expression of IL-4, IFN-γ, IL-17A, and IL-22 mRNAs in the skin lesions. As shown in Figure 2a, IL-4 and IL-17A mRNAs, but not IFN-γ mRNA, were highly increased in the skin lesions induced by the hydrogel patch containing OVA and δ-toxin. Although not statistically significant, IL-22 mRNA was most increased in the skin lesions induced by the hydrogel patch containing OVA and δ-toxin. Because CCR4 is known to be expressed by regulatory T cells (Iellem et al., 2001), we also examined the expression of Foxp3 mRNA, the regulatory T-cell marker. Foxp3 mRNA was not increased in any skin lesions, including those induced by the hydrogel patch containing OVA and δ-toxin. We further confirmed that IL-4, IL-17A, and IL-22 proteins were most increased in the skin lesions induced by the hydrogel patch containing OVA and δ-toxin (Figure 2b). Next, we analyzed cells infiltrating the skin lesions by flow cytometry. Eosinophils (CCR3⁺Siglec-F⁺ cells), Th2 cells (CD4⁺IL-4⁺ cells), and Th17 cells (CD4⁺IL-17⁺ cells) were highly increased in the skin lesions induced by the hydrogel patch containing OVA and δ-toxin (Figure 2c). We also confirmed that Th2 cells infiltrating the skin lesions were positive for CCR4 (Figure 2d). Of note, the surface CCR4 expression levels were quite similar in all skin samples. These results demonstrate that the topical application of the hydrogel patch containing OVA and δ-toxin in BALB/c mice efficiently induces allergic skin inflammation with pathological features quite similar to those found in the acute phase of human AD.

Expression of CCL17 and CCL22 in AD-like skin lesions in BALB/c mice

CCL17 and CCL22 are the ligands of CCR4 and known to be increased in skin lesions and serum samples of human AD patients (Fujisawa et al., 2002; Horikawa et al., 2002). Thus, the elevated expression of CCL17 and CCL22 is considered to be responsible for the infiltration of CCR4-expressing Th2 cells into AD skin lesions (Fujisawa et al., 2002; Horikawa et al., 2002). To confirm this notion, we examined the expression of CCL17 and CCL22 in our skin samples. As shown in Figure 3a and 3b, the expression of CCL17 and CCL22 mRNAs and their proteins were highly increased in skin lesions induced by the hydrogel patch containing OVA and δ-toxin. Accordingly, CCR4 mRNA levels were also most highly increased in skin lesions induced by the hydrogel patch containing OVA and δ-toxin. By
immunohistochemistry, CCL17 and CCL22 were most strongly stained in the epidermal keratinocytes of skin lesions induced by the hydrogel patch containing OVA and δ-toxin (Figure 3c). Because CCL17 was also shown to be present on the venular endothelial cells of inflamed skin and to trigger rapid adhesion of circulating skin-homing T cells expressing CCR4 (Bachererie et al., 2013), we also examined co-localization of CCL17 and CD31, an endothelial
cell marker. Indeed, CCL17 signals, but not CCL22 signals, were often co-localized with CD31 signals in skin lesions induced by the hydrogel patch containing OVA and δ-toxin (Figure 3c). Of note, weak CCL17 and CCL22 signals were also detected in the epidermal keratinocytes of skin lesions induced by the hydrogel patch containing OVA alone or δ-toxin alone, while we hardly observed any CCL17 and CCL22 signals in normal skins.

Less efficient induction of allergic skin inflammation by the topical application of OVA and δ-toxin in C57BL/6 mice

We also performed the topical application of OVA and δ-toxin in Th1-prone C57BL/6 mice. HE and toluidine blue staining demonstrated moderate epidermal hyperplasia and a slight increase in mast cell infiltration, respectively, in skin lesions applied with the hydrogel patch containing OVA and δ-toxin (Figure 4a, 4b). Although less than BALB/c mice, the treatment with the hydrogel patch containing OVA and δ-toxin also significantly increased Evans blue dye leakage in skin lesions (Figure 4c). OVA-specific IgE levels were also moderately increased by the topical application of the hydrogel patch containing OVA and δ-toxin (Figure 4d). Except for the expression of CCL17 mRNA, however, there were no significant increases in the expression levels of IL-4, IL-17A, IL-22, IFN-γ, CCL22, and CCR4 mRNAs in the skin lesions induced by the hydrogel patch containing OVA and δ-toxin (Figure 4e). Eosinophils, Th2 cells, and Th17 cells were also not significantly increased in the skin lesions induced by the hydrogel patch containing OVA and δ-toxin (Figure 4f). Collectively, compared with BALB/c mice, C57BL/6 mice are quite resistant to the induction of allergic skin inflammation, even by using the hydrogel patch containing OVA and δ-toxin.

CCR4 is involved in the development of AD-like skin lesions in BALB/c mice

To explore the role of CCR4 in the present AD model, we next employed CCR4-deficient mice on the BALB/c background. HE and toluidine blue staining revealed that the topical application of the hydrogel patch containing OVA and δ-toxin induced almost no epidermal thickness and much reduced increases of local mast cell numbers in CCR4-deficient mice (Figure 5a, 5b). Serum total and OVA-specific IgE levels were also reduced in CCR4-deficient mice (Figure 5c). CCR4-deficient mice also had significantly reduced local infiltration of eosinophils and Th2 cells (Figure 5d). We further compared the expression of CCL17, CCL22, IL-4, IL-17A, and IL-22 mRNAs and their proteins in skin lesions of wild-type and CCR4-deficient mice. As shown in Figure 5e and 5f, the topical application of the hydrogel containing OVA and δ-toxin induced minimal increases in the mRNA and protein levels of these cytokines in CCR4-deficient mice compared with wild-type mice.

Effect of Compound 22, a CCR4 antagonist, on AD-like skin lesions in BALB/c mice

We further tested Compound 22 (Purandare et al., 2007), a CCR4 antagonist, in the present AD model. As shown in Figure 6a, Compound 22 at doses 0.5 and 1.0 mg/kg
significantly reduced the epidermal thickness induced by the topical application of OVA and δ-toxin. The mast cell numbers were also reduced (Figure 6a). Compound 22 at doses 0.5 and 1.0 mg/kg also reduced infiltration of eosinophils and Th2 cells (Figure 6b) and increases in serum total and OVA-specific IgE levels (Figure 6c).

DISCUSSION

Although several mouse models of AD have been developed so far, the OVA-sensitized mouse model is one of the most standard models of AD. Repeated sensitization with a gauze patch containing OVA is used to induce allergic skin inflammation with features of skin lesions similar to the acute phase of human AD (He et al., 2007; Jin et al., 2009; Ma et al., 2002). However, the gauze patch requires multiple sensitizations over an extended period of time (usually 7 weeks) to induce the AD-like skin lesion. Previously, we described a hydrogel patch that can efficiently promote skin penetration of antigenic proteins (Hirobe et al., 2012; Ishii et al., 2008; Matsuo et al., 2010; Matsuo et al., 2011a; Matsuo et al., 2011b). We postulate that the hydrogel patch has several advantages over the conventional gauze patch. First, the highly concentrated antigenic proteins on the surface of the patch generate a strong concentration gradient of antigenic proteins, which is critical for producing the driving force necessary to accelerate passive diffusion and distribution. Second, humectation and hydration of the skin by the hydrogel patch loosen intercellular gaps in the stratum corneum, thereby contributing to enhanced penetration of water-soluble substances. In addition, our hydrogel patch contains hyaluronan (HA), an important component of extracellular matrices. Recently, it has been reported that HA fragments (low molecular weight) accumulate during tissue injury and act as an endogenous danger signal by engaging TLR2 (Scheibner et al., 2006). Although we confirmed that our hydrogel patch contained only high-molecular-weight HA and injection of high-molecular-weight HA did not affect antigen-specific IgG responses (data not shown), locally generated HA fragments may enhance the induction of AD-like skin lesions. At any rate, the period necessary to induce AD-like lesions by our hydrogel patch is much shorter than that by the classical gauze patch. Thus, by using the hydrogel patch containing OVA and SA δ-toxin, we were able to efficiently induce AD-like skin lesions only by 3 weeks in BALB/c mice (Figure 1). The skin lesions exhibited Th2-dominant inflammatory responses as characterized by the infiltration of Th2 cells, eosinophils, and mast cells, and by the increased expression of IL-4 and IL-17, but not IFN-γ (Figure 2). These features are quite similar to those found in the acute phase of human AD (He et al., 2007; Jin et al., 2009; Ma et al., 2002; Oyoshi et al., 2009). Thus, our AD mouse model employing the hydrogel patch may be quite useful for basic studies of AD, as well as for the screening and evaluation of therapeutic candidates.
Mast cell degranulation is a key step in the pathogenesis of AD (Ando et al., 2013). Furthermore, mast cells are known to secrete a number of cytokines and chemical mediators (Theoharides et al., 2007). Recently, Nakamura et al. (2013) identified SA δ-toxin as a potent mast cell degranulation-inducing factor produced by SA. Nakamura et al. (2013) also demonstrated that δ-toxin—positive SA, but not a mutant deficient in δ-toxin, promoted allergic skin disease with an increased production of IgE and IL-4. In this study, we have corroborated their findings and demonstrated that the addition of δ-toxin promotes the development of AD-like skin lesions by the hydrogel patch containing OVA; the production of total and OVA-specific IgE (Figure 1); the production of IL-4 and IL-17A (Figure 2); and the expression of CCL17 and CCL22 (Figure 3). Because we found no CCR4 expression on mast cells in the skin lesions (data not shown), CCL17 and CCL22 are likely to have little direct stimulating effect on mast cells. On the other hand, mast cell activation could be highly important for the induction of CCL17 and CCL22 and the subsequent infiltration of CCR4-expressing Th2 cells and Th17 cells in AD skin lesions.

In this study, we found that epidermal keratinocytes expressed CCL17 and CCL22, while dermal vascular endothelial cells expressed CCL17 in the skin lesions induced by the hydrogel patch containing OVA and δ-toxin (Figure 3c). This expression pattern of CCL17 and CCL22 in the skin lesions is quite similar to that found in human AD skin lesions (Bachelerie et al., 2013; Horikawa et al., 2002; Shoda et al., 2014; Vestergaard et al., 2000). The cytokines, such as TNF-α, IL-4, IL-5, IL-13, and IFN-γ, are known to induce expression of CCL17 and CCL22. For example, the combination of TNF-α and IFN-γ induces CCL17 and CCL22 in epidermal keratinocytes (Horikawa et al., 2002; Vestergaard et al., 2000); the combination of IL-4, IL-5, and IL-13 induces CCL17 and CCL22 in dendritic cells and macrophages (Fujita et al., 2005; Imai et al., 1999); and the combination of TNF-α and IL-4/IL-13 induces CCL17 in dermal microvascular endothelial cells (Shoda et al., 2014). Thus, in the present AD model, proinflammatory and Th2 cytokines derived from δ-toxin–stimulated mast cells are likely to initiate the expression of CCL17 and CCL22 in the skin. In addition, δ-toxin, which belongs to the peptide toxin family of the phenol-soluble modulins, is known to directly induce secretion of proinflammatory cytokines, including TNF-α, in monocytes and keratinocytes (Otto et al., 2004; Syed et al., 2015). Thus, δ-toxin may promote the expression of CCL17 and CCL22 not only by mast cells but also by monocytes and keratinocytes. It has also been reported that antigen-loaded dendritic cells express CCL22 and CCL17 to facilitate the formation of clusters with effector/memory T cells expressing CCR4, thereby expanding CCR4-expressing T cells in regional lymph nodes (Alferink et al., 2003; Katou et al., 2001). Since we demonstrated that our hydrogel patch delivery method can efficiently deliver antigens to dendritic cells (Figure 1), such antigen-loaded dendritic cells are considered to migrate to regional lymph nodes and to contribute to the expansion of CCR4-expressing T cells, including Th2 cells and Th17 cells.

Previous studies employing CCR4-deficient mice on the C57BL/6 background only demonstrated a minor role of
CCR4 in the development of AD-like skin lesions in mice (Islam et al., 2011; Stutte et al., 2010). In contrast, we demonstrated in this study that CCR4 deficiency on the BALB/c background mice had a great impact on the development of AD-like skin lesions (Figure 5). In this regard, we have also shown that topical application of the hydrogel patch containing OVA and δ-toxin induced only minimal skin lesions with low infiltration of eosinophils, Th2 cells, and Th17 cells in C57BL/6 mice (Figure 4). Furthermore, only the expression levels of CCL17 mRNA, but not those of CCL22 and CCR4 mRNAs, were found to be significantly increased in skin lesions of C57BL/6 mice, although the expression levels were still much lower than those of BALB/c mice. Thus, one reason for the discrepancy between the previous studies and the present one is probably the difference in the genetic background of CCR4-deficient mice. Given that our AD model represents an acute AD model, the CCR4 axis is likely to play a pivotal role in the acute phase of AD-like skin inflammation. Of note, the skin lesions of a canine AD model with features of the chronic phase of human AD also had increased expression levels of CCR4 and CCL17 (Marsella et al., 2006; Murray et al., 2016), although a CCR4 antagonist had no statistically significant therapeutic effect in this model (Murray et al., 2016). Thus, the role of CCR4 in chronic AD skin lesions, if any, remains to be seen.

In conclusion, we have demonstrated that our hydrogel patch delivery method can promote the penetration of antigenic proteins into the skin and efficiently induce Th2-dominant allergic inflammation, where δ-toxin acts as an effective adjuvant. We have also demonstrated that CCR4 plays a pivotal role in the induction of acute AD-like skin lesions at least in the BALB/c background most probably through the recruitment of CCR4-expressing Th2 cells and Th17 cells in the skin. Based on our findings, we propose that CCR4 represents a promising therapeutic target, at least in the acute phase of AD.
MATERIALS AND METHODS

Mice

BALB/c mice and C57BL/6 mice were purchased from Japan SLC (Shizuoka, Japan). CCR4-deficient mice on the C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME). To generate CCR4-deficient mice on the BALB/c background, we backcrossed CCR4-deficient mice with BALB/c mice for more than 12 generations. Mice were maintained in specific pathogen-free conditions. All animal experiments were approved by the Center of Animal Experiments, Kindai University, and performed in accordance with institutional guidelines.

Reagents

Compound 22 is a potent CCR4 inhibitor in mice (Matsuo et al., 2016). We synthesized Compound 22 with 99.5% purity based on the published information (Purandare et al., 2007) with the help of the Division of Computational Drug Design and Discovery, Kindai University Faculty of Pharmacy (Osaka, Japan) (Matsuo et al., 2016). δ-Toxin (MAQDIISTGLVKWIIDTVNKFTK) was also synthesized at the Department of Applied Chemistry, Kindai University Faculty of Science and Engineering.

Hydrogel patch formulation

The hydrogel patch formulation, comprising cross-linked HiPAS acrylate medical adhesives (CosMED Pharmaceutical Co Ltd, Kyoto, Japan): octyldodecyl lactate to glycerin to sodium HA = 100:45:30:0.2 as weight ratio of composition, was prepared as described previously (Hirobe et al., 2012; Ishii et al., 2008; Matsuo et al., 2010; Matsuo et al., 2011a; Matsuo et al., 2011b).

Analysis of antigenic protein localization in the skin

A hydrogel patch or a gauze patch containing 200 μg Alexa Fluor 488–conjugated OVA (Thermo Fisher Scientific, Waltham, MA) and 100 μg Texas Red–conjugated δ-toxin was applied on tape-stripped auricle skins of mice. After 24 hours, the auricles were removed and frozen. Thin sections (10-μm thick) of frozen tissues were photographed using fluorescence microscopy.

In vivo permeability assay

In vivo permeability assay was performed as described previously (Lee et al., 2015). In brief, mice were inoculated with an intravenous injection of 4% Evans blue in phosphate-buffered saline. One hour after the inoculation, the ears were removed to measure the amount of Evans blue dye. The dye was extracted overnight from the ears in 800 μl of formamide at 65°C. The intensity of the absorbance was measured at 620 nm.

The AD model

The auricle skin of BALB/c mice was tape-stripped with 10 strokes using adhesive tape (Scotch tape; 3M, Minneapolis, MN). A hydrogel patch containing distilled water, OVA (200 μg) alone, δ-toxin (100 μg) alone, or OVA (200 μg) and δ-toxin (100 μg) was applied to the skin, covered with a wound management film (Nichiban Co Ltd, Tokyo, Japan) to allow for better skin adherence, and left for 24 hours (Figure 1a). This sensitization procedure was done on days 0 and 7, and skin and blood samples were collected on day 21 (Figure 2a).

Histochemistry

Skin sections were stained with HE as described previously (Nakamura et al., 2013). The evaluation of acanthosis was done by measuring epidermal thickness on the ear skin sections. The skin sections were also stained with toluidine blue as described previously (Nakamura et al., 2013). We counted mast cells between the cartilage and squamous epithelium in five random sites (×400) of a toluidine blue–stained ear section under a light microscope to obtain the mean cell number per site.

ELISA

Serum total and OVA-specific IgE levels were measured using ELISA kits purchased from Biolegend (San Diego, CA) and following the manufacturer’s instructions. Cytokines were also measured in skin

Figure 6. Effect of Compound 22, a CCR4 antagonist, on allergic skin inflammation. Mice were applied with a hydrogel patch on the auricle skin for 24 hours. One week later, the same procedures were repeated. Compound 22 was intraperitoneally injected into mice every other day. Two weeks after the second patch application, skins and serum samples were obtained. (a) Epidermal thickness and numbers of mast cells in the skin. The data are expressed as mean ± SE of results from 10 mice. (b) Cells infiltrating the skin lesion were analyzed by flow cytometry using CD45 gate. Eosinophils: CCR3+ siglec-F−; Th2 cells: CD4+ IL-4+. The data are expressed as mean ± SE of results from 10 mice. (c) Quantification of serum total and OVA-specific IgE. Dots represent individual mice. OVA, ovalbumin. *P < 0.05 and **P < 0.01.
Isolation of cells
Skin samples were incubated for 60 minutes at 37°C in RPMI 1640 supplemented with 0.24 mg/ml collagenase A (Roche, Basel, Switzerland), and 40 U/ml DNase I (Thermo Fisher Scientific). After shaking vigorously, the resulting suspensions were filtered through a 70-µm cell strainer. Splenocytes were obtained by mashing spleens through a 70-µm cell strainer, followed by erythrocyte lysis with an ACK lysis buffer (150 mM NH4Cl, 10 mM KHCO3 and 0.1 mM Na2EDTA, pH 7.2).

Flow cytometric analysis
We purchased fluorescence-labeled anti-CCR4 (clone 2G12), anti-CCR3 (clone J073E5), anti-CD11c (clone N418), anti-IL-4 (clone 11B11), anti-IL-17A (clone TC11-18H10.1), anti-CD45 (clone 30-F11), and anti-CD4 (clone GK1.5) from Biolegend, and anti-siglecF (clone E50-2440) from BD Biosciences (San Diego, CA). Cells were incubated for 30 minutes with a mixture of anti-CD45 and anti-CD11c, anti-CD4, anti-CCR4, anti-CCR3, or anti-siglec-F. For intracellular staining, cells were then fixed and permeabilized (Cytofix/Cytoperm kit; BD Biosciences); and subsequently stained intracellularly with anti-IL-4 and anti-IL-17A. After washing, cells were immediately analyzed on a FACSSort (BD Biosciences) and analyzed with FlowJo software (Tree Star Inc, Ashland, OR). For the staining of IL-4 and IL-17A, cells were stimulated with phorbol 12-myristate 13-acetate and ionomycin for 4 hours.

Real-time PCR
Quantitative real-time PCR was performed using a Kapa SYBR Fast qPCR Kit (Kapa Biosystems, Woburn, MA) according to the manufacturer’s protocol. The primers for IL-17A, CCL22, CCL17, and glyceraldehyde-3-phosphate dehydrogenase were described previously (Matsuo et al., 2016). The other primers used were as follows: +5’-CACGTATGTGATCCTCTGGTCTC-3’ and −5’-GCCGATGATGTCTCTCAAGTAGA-3’ for IL-4; +5’-CACCTAGTGCCATAGTAGT-3’ and −5’-GAGATACATTGCTCTGACGATT-3’ for IFN-γ; +5’-CTACAGTGCCCATGATC-3’ and −5’-AGAAAGATTGAATCTGCTGCA-3’ for Foxp3; +5’-TCAGTCTGGTCTTCTC-3’ and −5’-CATCAGTCTGGTCTTCTC-3’ for IL-22; +5’-CTACAGGCCGATCTCCTCTCC-3’ and −5’-GATACGTCTGACTGCTGCTGCTG-3’ for CCR4. The expression was normalized to the amount of glyceraldehyde-3-phosphate dehydrogenase mRNA and quantified according to the 2−ΔΔCt method (Livak and Schmittgen, 2001).

Immunohistological analysis
Immunohistological analysis was performed essentially as described previously (Nagakubo et al., 2016). Briefly, the ear sections were blocked with 1% donkey serum in phosphate-buffered saline containing 2% BSA for 15 minutes and by a Streptavidin/Biotin Blocking Kit (Vector Laboratories, Burlingame, CA) for 15 minutes. Sections were then incubated overnight with goat anti-CLL17 (Santa Cruz Biotechnology, Dallas, TX) or rabbit anti-CCL22 (Peptrotech, Rocky Hill, NJ) at 4°C. After washing, sections were incubated either with Alexa Fluor 555–labeled donkey anti-goat IgG (Thermo Fischer Scientific) or Alexa Fluor 555–labeled donkey anti-rabbit IgG (Thermo Fischer Scientific) for 1 hour, and incubated overnight with biotinylated anti-CD31 (MEC13.3; BD Biosciences, San Jose, CA), followed by Alexa Fluor 647–labeled streptavidin (Thermo Fischer Scientific) for 1 hour. Finally, sections were mounted with ProLong Gold Antifade Reagent (Thermo Fisher Scientific) and observed under a spectrum confocal laser-scanning microscope (C1si; Nikon, Tokyo, Japan).

Statistical analysis
Student t test was performed to analyze differences between the two groups. One-way analysis of variance with the Holm post-hoc test was performed for multiple groups. We considered P < 0.05 as statistically significant.

CONFLICT OF INTEREST
The authors state no conflict of interest.

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