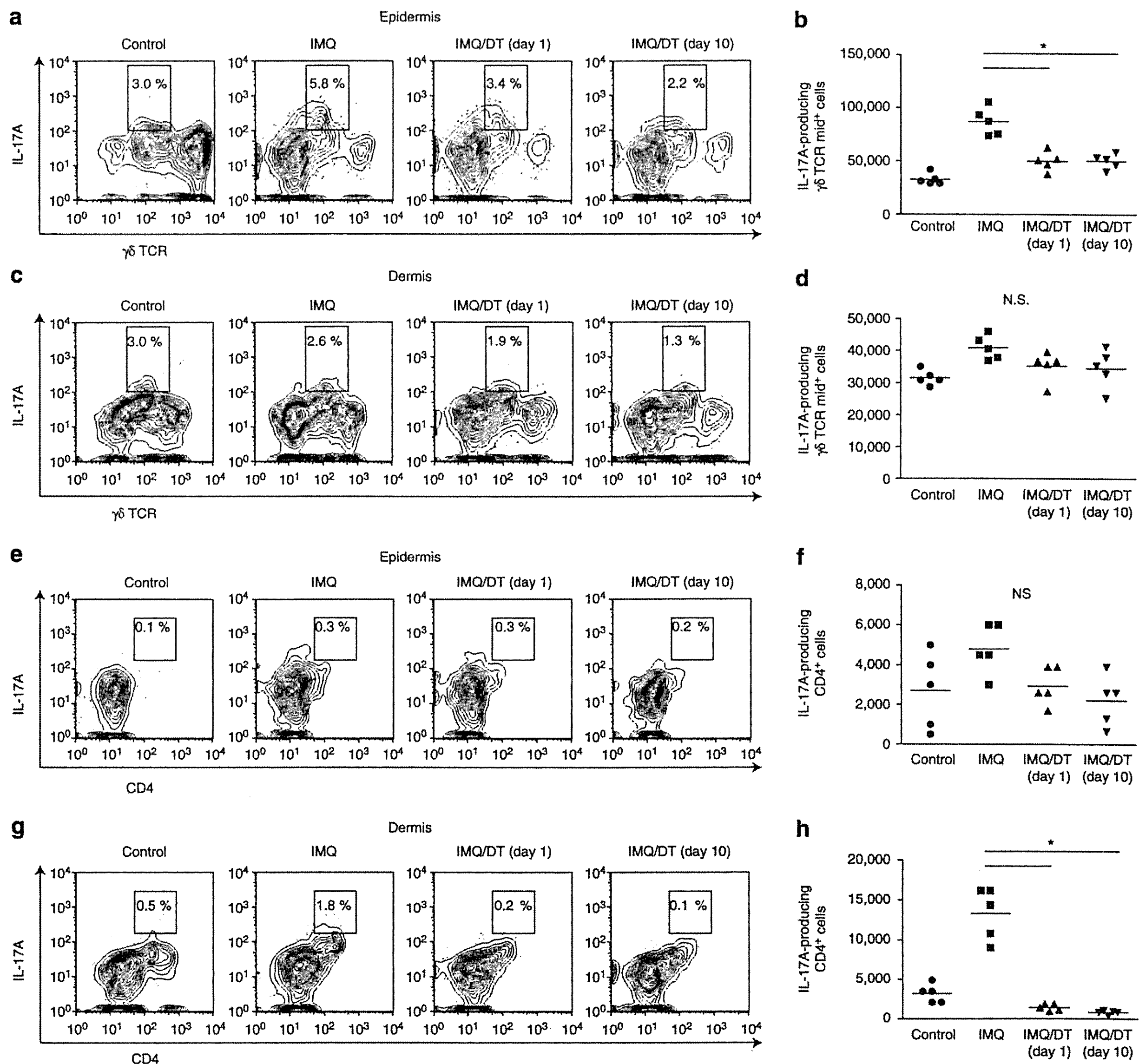

Figure 4. IL-17A-producing CCR6$^+$ γδ T cells are reduced in a Langerhans cell (LC)-depleted and imiquimod (IMQ)-applied epidermis. γδ TCR-positive cells in the epidermis and dermis. Expression of γδTCR, CD4, and intracellular IL-17A was examined in diphtheria toxin (DT)± and IMQ-applied skin by flow cytometry. (**a–d**) IMQ application induced the IL-17A-producing γδTCR mid$^+$ cells in the epidermis and LC depletion suppressed the induction of IL-17A γδTCR mid$^+$ cells. There was no change in the recruitment of IL-17A-producing γδTCR mid$^+$ cells in the dermis. (**e–h**) There was no significant increase in the number of IL-17A-producing CD4$^+$ cells in the epidermis, but IL-17A-producing CD4$^+$ cells increased in the dermis. Data are representative of four independent experiments and each group consisted of more than five mice. NS, not significant. *P<0.05 between indicated groups.

mice shown in Figure 1b. This difference was possibly due to the procedures of X-ray irradiation and BM transplantation. WT mice in Figure 6 were X-ray irradiated once and then transplanted with WT BM cells. Hence, total numbers and distribution of lymphocytes in BM-transplanted WT mice in Figure 6 are supposed to be different from those in normal WT mice shown in Figure 1. Taken together, these data indicated that IL-23 from LCs has a critical role in the IMQ-induced skin inflammation, possibly that of modulating IL-17-producing γδ T cells in the draining lymph nodes.

DISCUSSION

In this report, we analyzed the pathomechanisms of psoriasiform dermatitis caused by IMQ in Langerin-DTR–knocked-in mice. The IMQ-treated mouse skin resembles human plaque-type psoriasis with respect to erythema, skin thickening, scaling, and epidermal alterations (acanthosis, parakeratosis), but these findings were not observed in a LC-depleted state. In addition, LC-depleted mice showed decreased levels of Th17-related cytokines in IMQ-treated skin lesions. Moreover, the IMQ-treated skin of LC-depleted mice showed a decreased

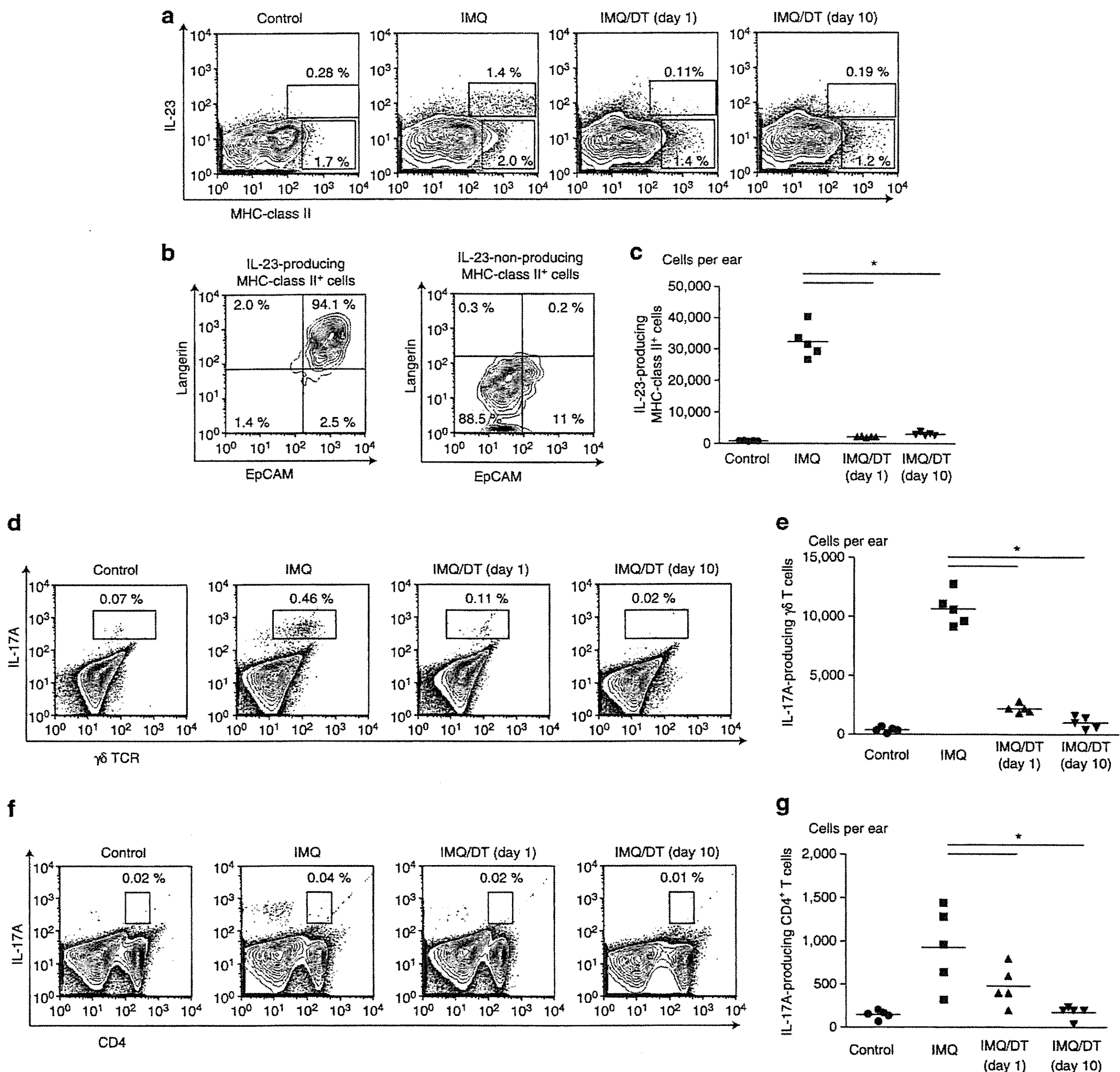

Figure 5. IL-23-producing LCs migrate to the draining lymph nodes and LCs are indispensable for induction of IL-17A-producing γδ T cells. Regional lymph nodes from IMQ-applied mice were analyzed by flow cytometry. (**a**, **c**) IMQ application induces IL-23-producing MHC class II$^+$ cells in the draining lymph nodes, but LC-depleted mice did not show induction of IL-23-producing MHC class II$^+$ cells. (**b**) IL-23-producing cells in the draining lymph node express EpCAM and Langerin, the markers for LCs. (**d–g**) Increase in the number of IL-17A-producing γδ T cells and CD4$^+$ T cells in the draining lymph node was suppressed by the depletion of LCs. Data are representative of four independent experiments and each group consisted of more than five mice. DT, diphtheria toxin; IMQ, imiquimod; LCs, Langerhans cells; MHC, major histocompatibility complex. *$P<0.05$.

number of IL-17A-producing CCR6$^+$ Vγ4$^+$ γδTCR$^+$ mid cells. These results suggest that LCs are required for the development of murine psoriasis-like lesions induced by IMQ.

The importance of T cells in the pathogenesis of psoriasis is supported by the response of patients to treatment with agents that affect T-cell functions, such as cyclosporine. Several cytokines have been implicated in the pathogenesis of psoriasis, including TNF-α, IFN-γ, IL-12, IL-17, IL-22, and IL-23 (van der Fits *et al.*, 2009). It has recently been reported that IL-23 expression is elevated in psoriatic skin lesions (Lee *et al.*, 2004), IL-23 induces psoriasis-like dermatitis (Chan *et al.*, 2006), polymorphisms in IL-23p19, IL-12/23p40, and IL-23R are associated with the development of psoriasis (Capon *et al.*, 2007), and antibody against IL-12/23p40 can ameliorate psoriasis in human skin (Krueger *et al.*, 2007). IL-23 is essential for the differentiation and survival of IL-17-producing cells (Diveu *et al.*, 2008), and IL-17 expression is augmented in psoriatic skin. In addition, anti-IL-17 antibody administration ameliorates psoriasis, indicating that IL-17 has an important role in the pathogenesis of psoriasis (Di Cesare *et al.*, 2009).

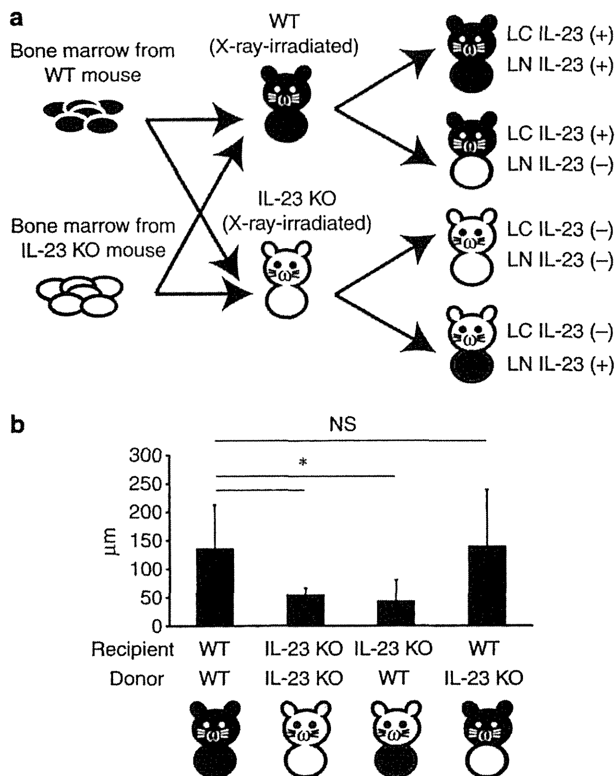

Figure 6. Skin-derived IL-23-producing LCs have a critical role in IMQ-induced skin inflammation. (**a**) Recipient WT C57BL/6J mice or IL-23 KO mice (C57BL/6J background) underwent 9 Gy total body irradiation on day −1, and received bone marrow single-cell suspensions from WT C57BL/6J mice or IL-23 KO mice on day 0. LCs remained in the skin even after the irradiation, whereas LN cells of the recipients were derived from donors. (**b**) Two months after transplantation, IMQ cream was applied on each chimera mouse and increase in ear thickness was measured on day 6. Ear thickness decreased when recipients were IL23KO mice. Data are representative of four independent experiments and each group consisted of more than five mice. IMQ, imiquimod; KO, knockout; LCs, Langerhans cells; LN, lymph node; NS, not significant; WT, wild type. *$P<0.05$.

IMQ application has been shown to induce IL-17A-producing γδ T cells in the regional lymph nodes (Roller *et al.*, 2012) and IL-23p19 mRNA in mice (van der Fits *et al.*, 2009). However, the source of IL-23 in IMQ-treated areas remained unclear. Here, we clearly demonstrated that LCs were the major DC subset to produce IL-23 in the IMQ-treated skin, and that IL-23 induced the infiltration of IL-17-producing Vγ4+ γδTCR mid$^+$ cells to the epidermis. These findings are consistent with the recent studies, which demonstrated that LCs are required for induction of IL-17-producing cells in *Candida albicans*-infected skin lesions (Igyarto *et al.*, 2011). Recently, Wohn *et al.* (2013) reported that Langerin-negative dDCs, rather than LCs, are responsible for IMQ-induced psoriatic plaque formation Their conclusion was seemingly contrary to ours in which LCs are indispensable for IMQ-induced psoriatic plaque formation. These two apparently contradictory experimental results using Langerin-DTR mice may be caused by the different IMQ-induction methods used by the two laboratories. Wohn *et al.* (2013) applied IMQ on the back of mice, whereas we painted IMQ on the ears of mice. In human beings, psoriasis is predisposed to take place on knees and elbows. This predisposition may reflect the difference in nature and the number of LCs and Langerin-negative dDCs depending on the body sites. Moreover, time course of DT application is different between the two laboratories. Wohn *et al.* applied DT on day −3 and painted IMQ, whereas we applied DT on day −1 and applied IMQ. The time course of the DT application affects the number of LCs as shown in Supplementary Figure 1 online. These differences in the IMQ-applied areas and the timing of DT application possibly gave rise to the different results. It remains unclear whether IMQ directly acts on LCs *in vivo*. Recently, it has been reported that Aldara cream, which contains IMQ, induces inflammation largely independently of TLR7, and keratinocyte death and IL-1 release also occur in response to the vehicle cream in the absence of IMQ (Walter *et al.*, 2013). However, the role of IMQ for psoriatic skin inflammation is still unclear. Intriguingly, the expression level of TLR7 is rather high in dermal DCs compared with LCs. We examined the production of IL-23 from LCs or dermal DCs by stimulating them with IMQ *in vitro*. However, we could detect no increase in IL-23 production from LCs or dermal DCs (data not shown). Therefore, there may be other pathways besides TLR7 that are involved in upregulating IL-23 production from LCs. Therefore, it should be clarified whether IMQ directly acts on LCs to produce IL-23 through TLR7 or indirectly induces LC activation possibly through other immune cells, such as plasmacytoid DCs or keratinocytes in the future.

Recently, CCR6 was shown to be a marker of peripheral IL-17A-expressing γδ T cells (Cua and Tato, 2010). These γδ T cells are observed following IL-23 intracutaneous injections (Mabuchi *et al.*, 2011). We now demonstrate that the accumulation of IL-17A-producing CCR6$^+$ γδ T cells in the epidermis and the draining lymph nodes is decreased in the LC-depleted mice. This finding supports the hypothesis that LCs are the main source of IL-23 for the induction of IL-17A-producing CCR6$^+$ γδ T cells in the IMQ-induced dermatitis model. It was reported that topical application of IMQ induces migration of LCs from treated skin into the draining lymph nodes (Suzuki *et al.*, 2000). In line with this, we demonstrated the accumulation of IL-23-producing LCs in the draining lymph nodes after IMQ application and the induction of IL-17A-producing CCR6$^+$ γδ T cells in the lymph node cell suspensions by IL-23. Therefore, LCs may function in two ways. First, IMQ treatment induces the accumulation of IL-23-producing LCs in the draining lymph nodes for the induction of IL-17A-producing γδ T cells. These γδ T cells migrate to the epidermal area of the IMQ-treated skin, possibly via CCR6, where LCs may enhance the functions or survival of IL-17A-producing γδ T cells for the development of psoriatic skin lesions.

Although IMQ-induced psoriasis-like skin lesions in mice share some clinical and histological characteristics with human psoriasis (Chan *et al.*, 2006), there are several differences between them. Most notably, the human psoriatic skin lesion contains predominantly αT cells and not many γδ T cells. However, it has recently been reported that dermal IL-17A-producing γδ T cells were significantly

increased in human psoriatic skin (Cai *et al.*, 2011), and that production of the local chemokine CCL20, a ligand for CCR6, is also increased in human psoriatic skin (Kryczek *et al.*, 2008). The increased frequency of γδ T cells in psoriatic skin suggests that γδ T cells may be crucial in the development of psoriasis not only in mice but also in humans.

In conclusion, here we demonstrate that IMQ treatment induces IL-23 production by LCs in mice, and that LC depletion resulted in attenuated IMQ-induced psoriasis-like dermatitis with decreased numbers of infiltrating IL-17A-producing γδ T cells. In addition, these IL-17A-producing γδ T cells were induced by IL-23 in the draining lymph nodes. These findings suggest that LCs seem to be the main cutaneous DC subset in the development of IMQ-induced psoriasis.

MATERIALS AND METHODS

Mice and treatments

Seven 10-week-old C57BL/6J mice were obtained from SLC (Shizuoka, Japan). Mice were maintained on a 12-hour light/dark cycle under specific-pathogen-free conditions. Langerin DTR–knocked-in mice were generated (Kissenpfennig *et al.*, 2005). To deplete Langerin^{+} cells, mice were injected intraperitoneally with DT (200 ng each; Sigma-Aldrich, St Louis, MO). IL23KO mice were obtained from Keio University (Hasegawa *et al.*, 2013). Protocols were approved by the Institutional Animal Care and Use Committee of the University of Occupational and Environmental Health. Mice at 8–11 weeks of age received a daily topical dose of 62.5 mg of commercially available IMQ cream (5%) (Mochida Pharmaceutical, Tokyo, Japan) on both ears for 5 consecutive days.

Generation of bone marrow chimera mice

To generate bone marrow chimera mice, recipient WT C57BL/6J mice or IL-23 KO mice (C57BL/6J background) underwent 9 Gy total body irradiation on day −1, and received bone marrow single-cell suspensions from WT C57BL/6J mice or IL-23 KO mice on day 0 intravenously through the tail vein. Recipient mice were treated with oral antibiotics for 2 months after transplantation.

Culture medium

RPMI-1640 (Gibco BRL Life Technology, Grand Island, NY) was supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, 10^{-5} M sodium pyruvate, 25 mM HEPES, 1% nonessential amino acids, 100 units per ml penicillin, and 100 μg per ml streptomycin (all from Gibco).

Preparation of skin suspensions

Skin sheets from mouse ears were floated in 0.2% of trypsin in PBS (pH 7.4; Sigma-Aldrich) for 30 min at 37 °C as described previously (Tokura *et al.*, 1994). The epidermis was separated from the dermis with forceps in PBS supplemented with 10% fetal calf serum. Both epidermis and dermis were minced and incubated for 1 h at 37 °C in PBS with collagenase II (Sigma-Aldrich). The obtained cells were filtered through a 40-μm filter.

Flow cytometry

Cells were immunostained with various combinations of fluorescence-conjugated mAbs and analyzed with a FACSCanto flow cytometer (BD Biosciences, San Diego, CA) and FlowJo software (Tree star, Ashland, OR). The expression levels of cell surface or intracellular molecules and intracytoplasmic cytokines were analyzed using the following antibodies: Alexa Fluor 488-conjugated anti-γδTCR (clone; eBioGL3), PE-conjugated anti-IL-12/23p40 (clone; C17.8), IL-17A (clone; eBio17B7), IL-22 (clone; 1H8PWSR), TNF-α (clone; MP6-XT22); PerCP-conjugated CD45 antibody (clone; 30-F11); biotin-conjugated anti-mouse CD207 (Langerin) (clone; eBioL31); and PE-Cy7-conjugated anti-Ly-6G (clone; RB6-8C5) (eBioscience, San Diego, CA) and anti-epithelial cell adhesion molecule (EpCAM, clone; G8.8), Vγ4 (clone; UC3-10A6), Vγ5 (clone; 536) (Biolegend, San Diego, CA), and anti-TLR7 antibody (clone; IMG4G6) (IMGENEX, San Diego, CA); APC-conjugated CCR6 antibody (clone; 140706) (BD Biosciences). All mAbs were used at a concentration of 1–5 μg per 10^{6} cells, and each incubation was performed for 30 min at 4 °C, followed by two washes in PBS supplemented with 5% fetal calf serum and 0.02% sodium azide. Viable cells were identified by 7-AAD uptake. For intracellular cytokine staining, cells were incubated in the presence of Goldi Stop (BD Biosciences) for 2 hours. Intracytoplasmic cytokines were detected in permeabilized cell suspensions using a BD cytofix/cytoperm Plus Kit (BD Biosciences).

Histology and immunohistochemistry

Paraffin-embedded skin specimens were prepared using routine methods. The sections were stained with H&E. For immunohistochemistry, sections were deparaffinized and hydrated by washing sections in xylene followed by graded alcohol series. To unmask antigens, sections were incubated in 10 mM citric acid (pH 6) at 95 °C for 30 min. Sections were blocked with normal serum for 60 min at room temperature followed by incubation with primary antibodies (Stat3, Ki67; DAKO, Glostrup, Denmark) overnight at 4 °C. Samples were washed and incubated for 60 min with secondary antibodies.

Real-time PCR

Total RNA was extracted from skin with the SVTotal RNA isolation system (Promega, Madison, WI) according to the manufacturer's protocol. Murine IL-23p19, IL-12/23p40, IL-17A, IL-22, and TNF-α gene expression levels were quantified in a two-step RT-PCR. cDNA was reverse transcribed from total RNA samples using the TaqMan RT reagents (Applied Biosystems, Foster City, CA). Target gene expression was quantified using TaqMan Gene Expression Assay (Applied Biosystems) in the ABI PRISM 7000 sequence detection system (Applied Biosystems). The probe was synthesized with VIC as a reporter dye and Tamra as the quencher dye. The forward primer, reverse primer, and Taqman probe were purchased from Applied Biosystems (IL-23p19; Mm01160011_g1, IL-12/23p40; Mm00434174_m1, IL-17A; Mm00439619_m1, IL-22; Mm00444241_m1, TNF-α; Mm00443258_m1, TLR7; Mm00446590_m1). As an endogenous control for these PCR quantification studies, GAPDH gene expression was measured using the TaqMan rodent GAPDH control reagents (Applied Biosystems). Gene expression was calculated using the ΔΔCt method.

CONFLICT OF INTEREST

The authors state no conflict of interest.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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Preferential infiltration of interleukin-4-producing CXCR4$^+$ T cells in the lesional muscle but not skin of patients with dermatomyositis

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Summary

Dermatomyositis (DM) and polymyositis (PM) are collectively termed autoimmune myopathy. To investigate the difference between muscle- and skin-infiltrating T cells and to address their role for myopathy, we characterized T cells that were directly expanded from the tissues. Enrolled into this study were 25 patients with DM and three patients with PM. Muscle and skin biopsied specimens were immersed in cRPMI medium supplemented with interleukin (IL)-2 and anti-CD3/CD28 antibody-conjugated microbeads. The expanded cells were subjected to flow cytometry to examine their phenotypes. We analysed the cytokine concentration in the culture supernatants from the expanded T cells and the frequencies of cytokine-bearing cells by intracellular staining. There was non-biased *in-vitro* expansion of tissue-infiltrating CD4$^+$ and CD8$^+$ T cells from the muscle and skin specimens. The majority of expanded T cells were chemokine receptor (CCR) type 7$^-$CD45RO$^+$ effecter memory cells with various T cell receptor (TCR) Vβs. The skin-derived but not muscle-derived T cells expressed cutaneous lymphocyte antigen (CLA) and CCR10 and secreted large amounts of IL-17A, suggesting that T helper type 17 (Th17) cells may have a crucial role in the development of skin lesions. Notably, the frequency of IL-4-producing chemokine (C-X-C motif) receptor (CXCR)4$^+$ Th2 cells was significantly higher in the muscle-derived cells and correlated inversely with the serum creatine phosphokinase (CPK) and lactate dehydrogenase (LDH) levels. stromal-derived factor (SDF)-1/CXCL12, a ligand for CXCR4, was expressed at a high level in the vascular endothelial cells between muscular fasciculi. Our study suggests that T cell populations in the muscle and skin are different, and the Th2 cell infiltrate in the muscle is associated with the low severity of myositis in DM.

Keywords: CXCR4, dermatomyositis, T cells, IL-4

Introduction

Dermatomyositis (DM) and polymyositis (PM) are two clinical phenotypes of autoimmune myopathy, and share clinical features of systemic proximal muscle weakness associated with muscle cell destruction. In addition to the muscle symptoms, DM presents with unique skin manifestations, including violaceous oedematous erythema on the upper eyelids (heliotrope rash) and hyperkeratotic erythemas that emerge symmetrically on dorsal aspects of joints (Gottron's sign) [1]. In DM and PM, impaired immunological tolerance for self-antigens causes inflammatory responses against host muscle tissues.

Previous studies have shown that the pathogenesis of DM is different from that of PM in the muscle pathophysiology [2]. In the muscle lesions, the perivascular infiltrate is composed mainly of CD4$^+$ T cells as well as B cells and macrophages in DM, whereas CD8$^+$ T cells and macrophages infiltrate predominantly in PM [3]. In the skin lesions of DM, the vast majority of infiltrating cells are mature CD4$^+$ T cells producing interleukin (IL)-2, interferon (IFN)-γ and/or IL-4 [4]. These findings suggest that

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CD4+ T helper (Th) cells are involved in the muscle and skin lesions of DM, and both Th1 and Th2 cells infiltrate at least into the skin lesions. However, the infiltrating T cells have not been characterized fully, and their subpopulations remain to be clarified. Most of the previous studies on T cells in DM and PM have been performed in peripheral blood mononuclear cells (PBMCs) because of the availability from the patients. Even when the muscle and skin tissues were used as experimental samples, T cell subsets were determined by immunohistochemistry or gene expression, and direct assessment of T cells by isolation and/or cultivation has not been performed.

To further characterize T cells in the muscle and skin lesions of DM and PM, we expanded skin- and muscle-infiltrating T cells using our established procedure. Our method enables us to expand tissue-infiltrating T cells proportionally to the original populations, and the expanded T cells are relevant to the pathogenic cells in the tissue [5]. Results suggest that T cell subpopulations in the muscle and skin are different, and the muscle infiltrate of IL-4-producing CXCR4+ T cells is associated with the low severity of myositis in DM.

Materials and methods

Patients

Enrolled into this study were 25 patients with DM and three patients with PM (Table 1). Twenty-six of these patients fulfilled Bohan and Peter's criteria of definite DM or PM [1,6]. Two patients (cases 8 and 22) were diagnosed as probable DM, because they were diagnosed clinically as having amyopathic DM, which showed typical skin manifestations and interstitial lung disease without histological myositis. The study was performed according to the Declaration of Helsinki, and the study protocol was approved by the ethical committee of Hamamatsu University School of Medicine. Written informed consent was obtained from all participants.

Reagents, antibodies and culture medium

Fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)- or peridinin chlorophyll (PerCP)-conjugated monoclonal antibodies (mAbs) against CD3, CD4, CD8, CD45RO, cutaneous lymphocyte antigen (CLA) and human leucocyte antigen D-related (HLA-DR) were purchased from BD Pharmingen (San Diego, CA, USA). A series of antibodies against 24 T cell receptor β chain variable region (TCR-Vβ) gene products (IOtest Beta Mark, TCR-Vβ repertoire kit) and FITC-conjugated TCR-Vβ2, pan-TCRγδ mAb were purchased from Beckman Coulter (Marseille, France). Chemokine receptor mAbs for CCR1–CCR10 and CXCR1–CXCR6 were obtained from R&D Systems (Minneapolis, MN, USA). FITC- or PE-conjugated mAbs against IL-4, IL-5, IL-13 and IFN-γ (BD Pharmingen), IL-17, forkhead box protein 3 (FoxP3) (eBioscience, San Diego, CA, USA) were purchased. Cells were cultured in RPMI-1640 medium (Life Technologies, Carlsbad, CA, USA) supplemented with L-glutamine, sodium pyruvate, 2-mercaptoethanol, non-essential amino acids (Life Technologies) and 10% heat-inactivated fetal calf serum or pooled human antibody serum (cRPMI), as described previously [7]. Polyclonal antibody for stromal-derived factor (SDF)-1/CXCL12 was purchased from Santa Cruz Biotech (Dallas, TX, USA).

Cell preparations

A 50–100 mm³ muscle specimen biopsied from femoral quadriceps and a 4-mm skin specimen from skin lesions of individual patients were cut into two pieces for expansion of infiltrating T cells and histological investigation. No immunosuppressive agents including corticosteroids were administered prior to the biopsy. For expansion of T cells, samples were immersed in cRPMI supplemented with 50 U/ml human recombinant IL-2 (R&D Systems) and anti-CD3/CD28 antibody-conjugated microbeads (T-cell Expander; Dynal, Copenhagen, Denmark), as reported previously [5]. We obtained $>10^7$ cells/specimen by this method.

Flow cytometric analysis (FCM) and cytokine production assessments

Aliquots of 10^6 cells were washed once with phosphate-buffered saline (PBS, pH 7·4) containing 1% bovine serum albumin (BSA) and 0·1% NaN_3, and incubated with a panel of fluorescence-conjugated mAbs for 30 min at 4°C in the dark. After washing, the harvested cells were resuspended in PBS and subjected to FCM. More than 5×10^4 cells per sample were analysed on a fluorescence activated cell sorter (FACS)caliber flow cytometer or FACSCanto2 (BD Pharmingen) by gating lymphocytes. Results were analysed using a FlowJo software (TreeStar, Ashland, OR, USA).

For the cytokine production assay, the cells (2×10^5/well) were stimulated with immobilized anti-CD3 mAb-coated 96-well plates (BD Biocoat; BD Pharmingen) for 48 h, and the culture supernatants were harvested to measure cytokine levels [IL-2, IL-4, IL-6, IL-10, IL-17, IFN-γ and tumour necrosis factor (TNF)-α] with the human Th1/Th2/Th17 Cytokine Beads Array kit (CBA; BD Pharmingen), according to the manufacturer's protocols.

Alternatively, we performed intracellular cytokine staining. The cells were incubated in cRPMI containing 10^{-8} M phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, St Louis, MO, USA), 10^{-6} M calcium ionophore (Sigma-Aldrich) and 1 μl/ml of BerGolgiStop™ (BD Pharmingen) for 6 h at 37°C. The cells were harvested and stained with fluorescent-tagged mAbs against cytokines using the Cytofix/Cytoperm Plus Kit with GolgiStop (BD Pharmingen),

Table 1. Patient profile.

No.	Age (years)	Sex	Disease duration (months)	Samples	CPK(U/l)	LDH(U/l)	Diagnosis	Interstitial lung disease	Internal malignancy	ANA
1	76	F	3	Skin/muscle	644	559	Definite DM	+		1280
2	75	F	2	Skin/muscle	200	413	Definite DM	+	Colon	40
3	75	M	2	Skin/muscle	830	467	Definite DM	+		80
4	58	M	2	Skin/muscle	2448	647	Definite DM	+		80
5	57	M	3	Skin/muscle	941	795	Definite DM	–		80
6	51	F	1	Skin/muscle	774	368	Definite DM	–	Uterus	40
7	41	F	1	Skin/muscle	2063	764	Definite DM	+		5120
8	46	F	11	Skin/muscle	32	302	Probable DM	+		40
9	33	F	2	Skin/muscle	4988	553	Definite DM	–		40
10	77	F	3	Skin/muscle	434	416	Definite DM	–	Lung	160
11	65	M	5	Skin/muscle	1415	398	Definite DM	+		–
12	32	M	1	Skin/muscle	908	435	Definite DM	+		–
13	37	F	9	Muscle	785	318	Definite PM	+		80
14	53	F	5	Muscle	274	233	Definite DM	+		40
15	23	M	1	Muscle	4387	739	Definite DM	–		–
16	43	M	5	Muscle	4957	916	Definite DM	+		40
17	51	F	10	Muscle	3923	628	Definite DM	–		640
18	81	M	1	Muscle	1532	532	Definite DM	–	Oesophagus	160
19	44	F	2	Muscle	226	274	Definite DM	+		40
20	39	F	2	Muscle	50	172	Definite PM	–		40
21	46	F	> 20	Muscle	386	331	Definite PM	+		–
22	63	F	3	Skin	64	246	Probable DM	+		40
23	50	F	6	Skin	855	456	Definite DM	+		320
24	30	M	5	Skin	82	418	Definite DM	+		–
25	45	F	1	Skin	206	298	Definite DM	+		–
26	56	F	12	Skin	384	285	Definite DM	+		–
27	55	F	3	Skin	4732	800	Definite DM	+		640
28	74	F	4	Skin	1432	437	Definite DM	–		–

ANA = anti-nuclear antibody; CPK = creatine phosphokinase (normal values <204 U/l); DM = dermatomyositis; PM = polymyositis; F = female; M = male; LDH, lactate dehydrogenase (normal value < 208 U/l).

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according to the manufacturer's protocols, followed by staining of fluorescence-tagged antibodies against CD4 and CD8.

Histopathological and immunohistochemical studies

Biopsy specimens from the lesions were fixed in 4% formalin and stained routinely with haematoxylin and eosin (H&E) for standard histopathology. Deparaffinized specimens were autoclaved in 10 mM citrate buffer (pH 6·0) for 10 min at 120°C to retrieve the antigenic epitopes and were then processed for CD4, CD8, CD3 and stromal cell-derived factor (SDF)-1 expression analysis by the avidin–biotin complex method. Nuclear staining was performed with haematoxylin. The sections were scanned by a digital image scanner, NanoZoomer (Hamamatsu Photonics, Hamamatsu, Japan), and were analysed.

Statistical analyses

For non-parametric analysis, Wilcoxon's signed-rank test and the Mann–Whitney *U*-test were used for matched-pairs analysis and non-matched-pairs analysis, respectively. Student's *t*-test was used for parametric analysis. Non-parametric correlation coefficients (R_s) were calculated using Spearman's procedure. $P < 0{\cdot}05$ was considered statistically significant.

Results

Non-biased *in-vitro* expansion of tissue-infiltrating $CD4^+$ and $CD8^+$ T cells from muscle and skin specimens

To obtain large numbers of the tissue-infiltrating T cells, we expanded cells that migrated from the specimens to the culture medium with anti-CD3/CD28 antibodies and IL-2 for 14 days, as described previously [5]. This method constantly allowed us to obtain more than 10^7 cells without a substantial phenotypical alternation. Using FCM, we found that both $CD4^+$ and $CD8^+$ T cells were expanded from the patients' muscle and skin samples, as shown in representative data (Fig. 1a). There were high variations in the CD4/CD8 ratios of both muscle- and skin-derived cells among the patients (Fig. 1b). To assess whether these cells propagated proportionally and reflected the original populations of the muscle- and skin-infiltrating T cells, we compared the CD4 : CD8 ratio of the expanded T cells (FCM analysis) with that of the tissue-infiltrating T cells (immunohistochemical analysis). Immunostaining exhibited the infiltration of $CD3^+$, $CD4^+$ and $CD8^+$ T cells in the lesional muscle (Fig. 1c) and skin (Fig. 1d) of DM. As a considerable number of $CD4^+$ dendritic cells reside in the muscle and skin lesions, we enumerated $CD3^+$ and $CD8^+$ cells and estimated the $CD4^+$ T cell number by subtracting the numbers of $CD8^+$ cells from those of $CD3^+$ cells. There was a close correlation of the CD4 : CD8 ratio between the expanded T cells and the original infiltrate in both skin (Fig. 1e) and muscle lesions (Fig. 1f). These results suggest that the expanded T cells reflect the original tissue-infiltrating T cells.

TCR Vβ usage of muscle- and skin-derived T cells

The TCR Vβ repertoire of the expanded T cells was examined using a panel of antibodies against various Vβ chains. We found deviated TCR Vβ usage in muscle- and skin-infiltrating T cells in all the patients examined. As represented by case 3 (Fig. 2a), Vβs of the preferentially expanded T cells were different between the muscle- and skin-derived T cells even in the same patients, suggesting that T cells reacted differentially to antigens in each of the tissues. In the three PM patients, as represented by case 13 (Fig. 2b), the skewed $CD4^+$ and $CD8^+$ T cell Vβ usage in the muscle lesions was more marked than that of DM patients. We analysed the mean ± standard deviation (s.d.) of the frequencies of T cells bearing individual Vβs in 15 DM/PM patients and found no common Vβ in the expanded T cells among the patients (Fig. 2c), suggesting a different T cell repertoire in each patient. To verify reflection of the original tissue-infiltrating T cells, we took two muscle biopsy samples from one patient (case 19) and obtained expanded T cells. Virtually the same distribution of Vβ usage was observed in the two samples (Fig. 2d), again validating the non-biased expansion during culture.

Chemokine receptor expressions of muscle- and skin-derived T cells

The chemokine receptor expression was investigated in the expanded T cells from muscle and skin samples. No significant differences were observed between the muscle- and skin-derived T cells in the frequency of $CD4^+CXCR3^+$ (mostly Th1) or $CD4^+CCR4^+$ (mostly Th2) cells (Fig. 3a). The majority of expanded T cells did not express CCR7 (Fig. 3a) but bore CD45RO in both tissues (Fig. 3b), indicating their effector memory phenotype. Notably, the frequencies of the CLA^+ and $CCR10^+$ cells were significantly higher in the skin-derived T cells than in the muscle-derived cells, indicating the functional relevance of these skin-homing receptors [8,9]. It has been reported that chemokines CCL2, CCL3, CCL4 and CCL5 are produced in vessels of autoimmune myopathy and their receptors, CCR1, CCR2 and CCR5, are expressed in muscle-infiltrating mononuclear cells [10]. In our study, however, the frequencies of cells expressing these chemokine receptors were not different between the muscle- and skin-derived cells (Fig. 3a). Of particular interest is the observation that the frequency of $CXCR4^+$ T cells was significantly higher in the muscle-derived cells than in the skin-derived cells (Fig. 3a). It has been shown that SDF-1/CXCL12, a ligand for CXCR4, is expressed in vascular

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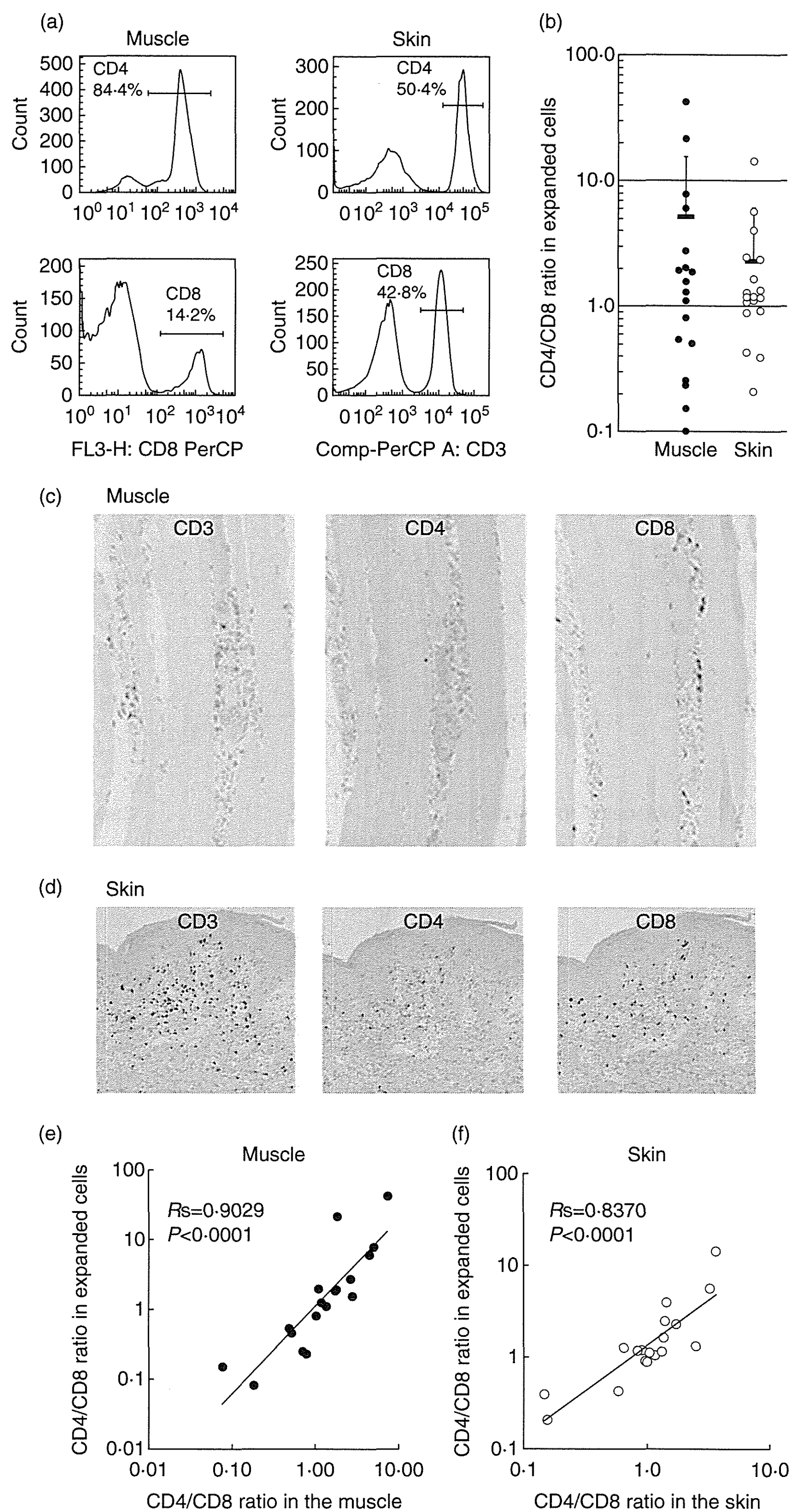

Fig. 1. CD4/CD8 ratios in muscle- and skin-derived T cells in dermatomyositis (DM) patients. (a) Flow cytometric analysis (FCM) of expanded T cells. The numbers represent $CD4^+$ or $CD8^+$ cells. (b) The CD4 : CD8 ratio of expanded T cells from muscle lesions (closed circles, $n = 18$) and skin lesions (open circles, $n = 18$) of DM patients. Horizontal bars indicate mean ± standard deviation (s.d.). (c,d) Immunohistochemical staining of muscle and skin lesions with monoclonal antibodies against CD3, CD4 and CD8. Original magnification ×200. (e) Relationships between the CD4 : CD8 ratio of the original tissue-infiltrating T cells and that of expanded T cells from muscle lesions (closed circles) and skin lesions (open circles).

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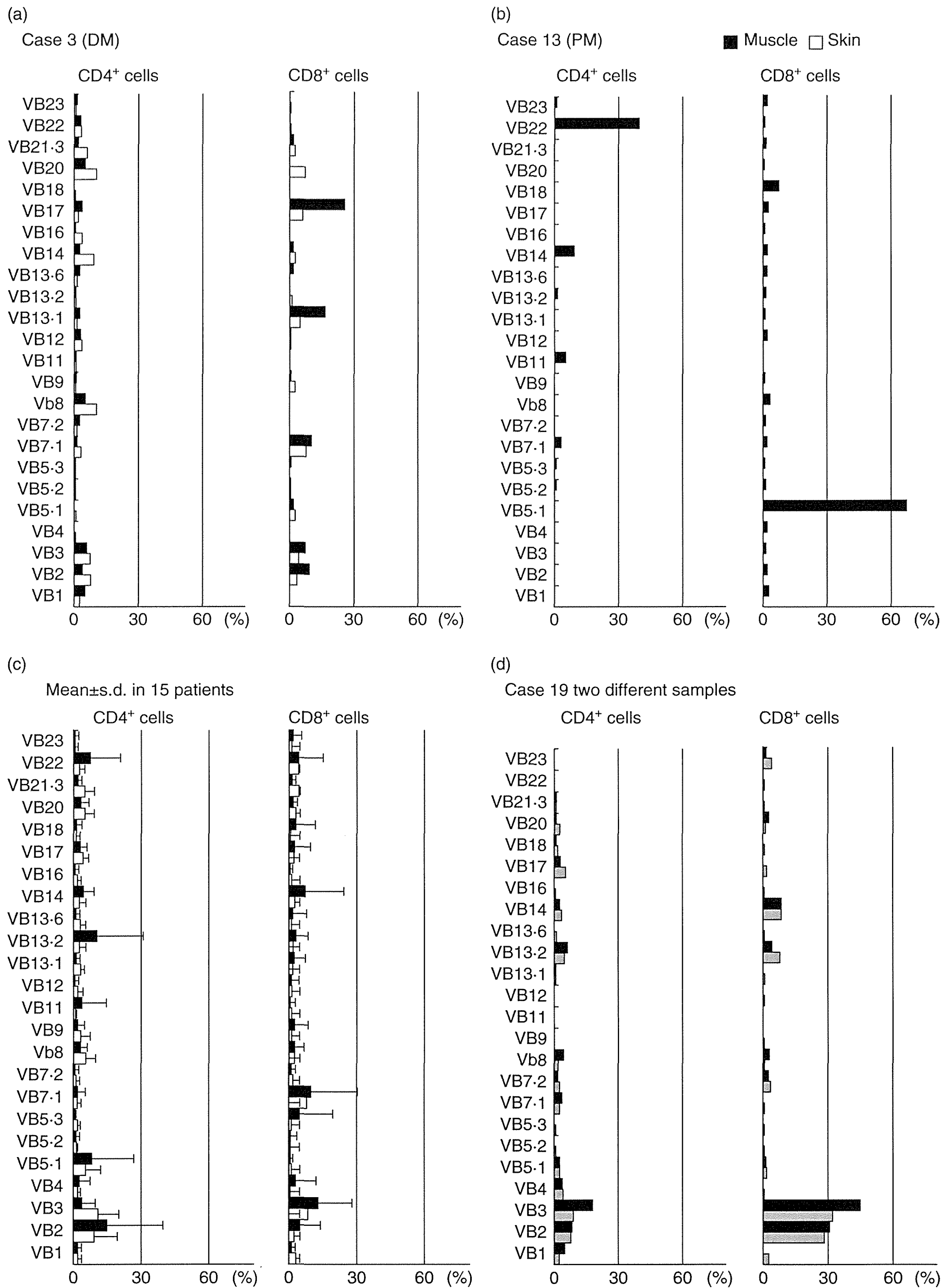

Fig. 2. T cell receptor (TCR) Vβ usage of muscle (closed)- and skin-infiltrating (open) T cells in dermatomyositis/polymyositis (DM/PM) patients. The numbers indicate the percentage of T cells bearing each TCR Vβ in total T cells ($CD4^+$ and $CD8^+$ T cells). (a) Representative data of DM patient (case 3). (b) Representative data of PM patient (case 13). (c) The mean frequencies ± standard deviation (s.d.) of T cells bearing each TCR Vβ in 15 patients. (d) TCR Vβ usage of two muscle samples (black and grey) in case 19.

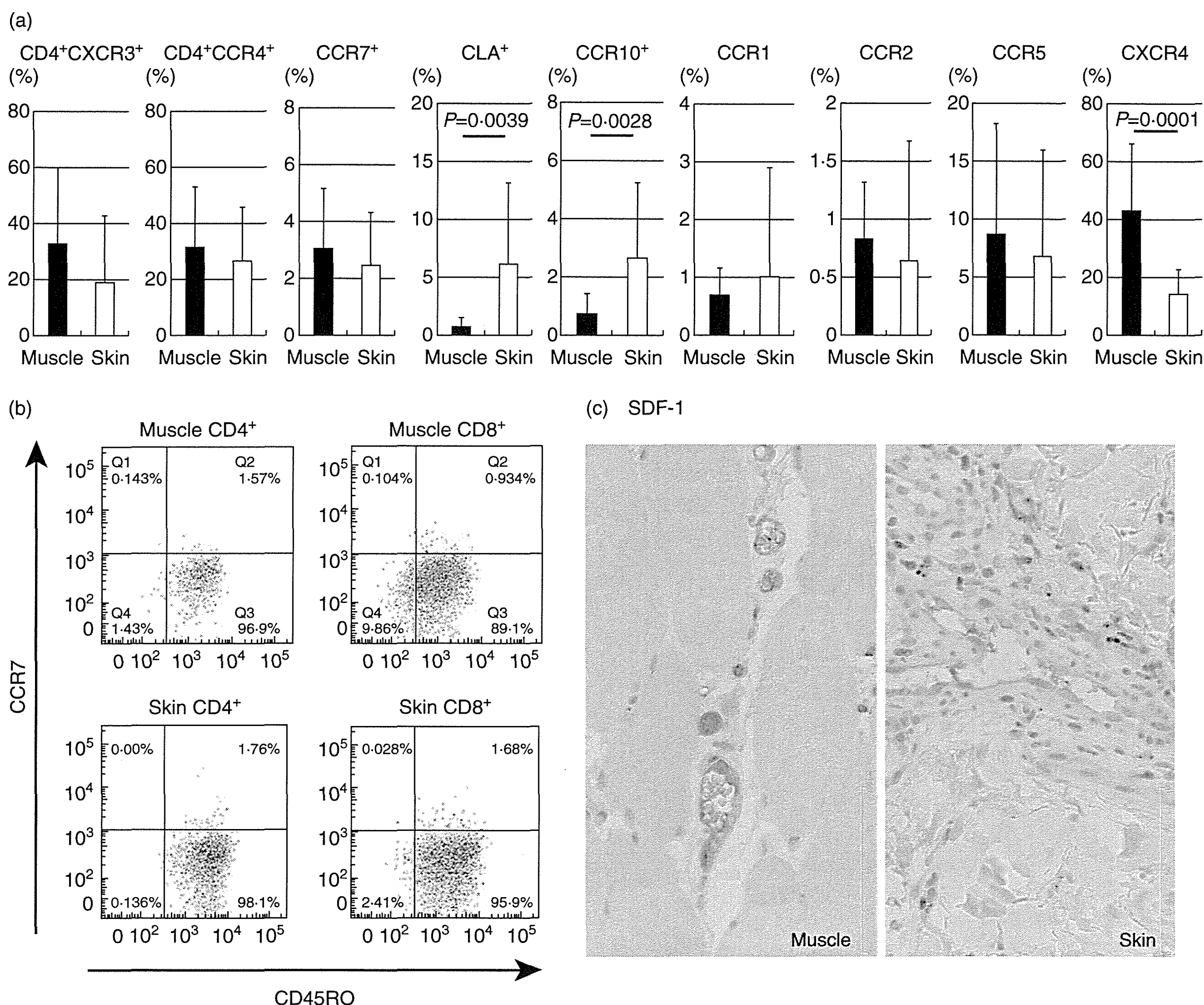

Fig. 3. Chemokine receptor expressions of T cells expanded from muscle lesions (closed) and skin lesions (open) in dermatomyositis (DM) and polymyositis (PM) patients ($n = 24$). (a) Numbers indicate mean percentages of total T cells. Mean values ± standard deviation (s.d.) are indicated. (b) Representative fluorescence activated cell sorter (FACS) dot-plots of CD45RO and CCR7. (c) Immunohistochemistry of muscle and skin lesions with antibodies against stromal-derived factor (SDF)-1, original magnification ×400.

endothelial cells of the patients' muscle of DM and PM [11]. In our study of five samples, SDF-1 expression was also observed at a high level in the vascular endothelial cells located between muscular fasciculi and at a moderate level in the dermal vessel epithelial cells (Fig. 3c).

Cytokine production patterns of muscle- and skin-derived T cells and their association with laboratory markers

Expanded T cells (2×10^5/well) were stimulated with immobilized anti-CD3 antibody for 48 h, and the concentration values of various cytokines in the culture supernatants were measured. High amounts of IL-4 were found in the supernatants of both skin- and muscle-derived T cells (Fig. 4a). When the IL-4 levels in the muscle and skin T cells were analysed in the individual patients, IL-4 was produced at significantly higher levels by the muscle T cells than by the skin T cells (Fig. 4b). There was no significant difference in IFN-γ (Fig. 4c). Notably, the skin-derived T cells produced significantly higher amounts of IL-17A than did the muscle-derived T cells (Fig. 4a). In three DM patients with severe skin manifestations (cases 22, 23 and 27) the skin-derived T cells secreted large amounts of IL-17A (2751, 21 980 and 5147 pg/ml, respectively), suggesting that IL-17A may have a crucial role in the development of skin lesions of DM.

We analysed the relationship between the levels of produced IL-4 and IFN-γ and serum creatine phosphokinase (CPK) and lactate dehydrogenase (LDH) levels, which are clinical parameters of muscle damage [1]. The levels of IL-4

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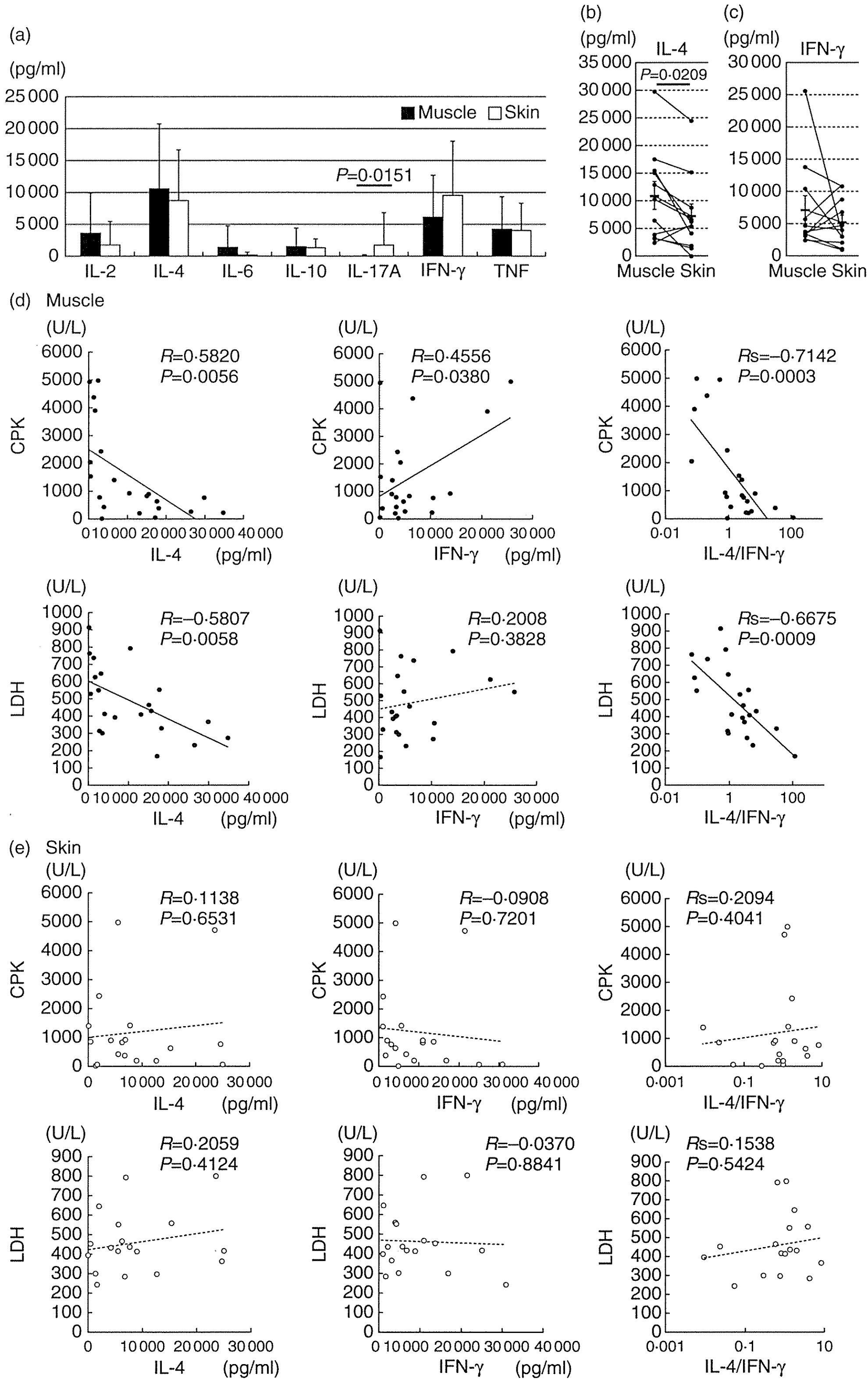

Fig. 4. Cytokine production patterns of the tissue-infiltrating T cells. (a) Cytokine concentrations in the culture supernatants from anti-CD3 antibody-stimulated expanded T cells ($n = 28$). Mean values ± standard deviation (s.d.) are indicated. (b,c) Interleukin (IL)-4 and interferon (IFN)-γ concentrations in the supernatants from the stimulated muscle (closed)- and skin (open)-derived T cells in the same patients. (d,e) Correlation between cytokine levels and clinical parameters in the muscle- and skin-derived T cells.

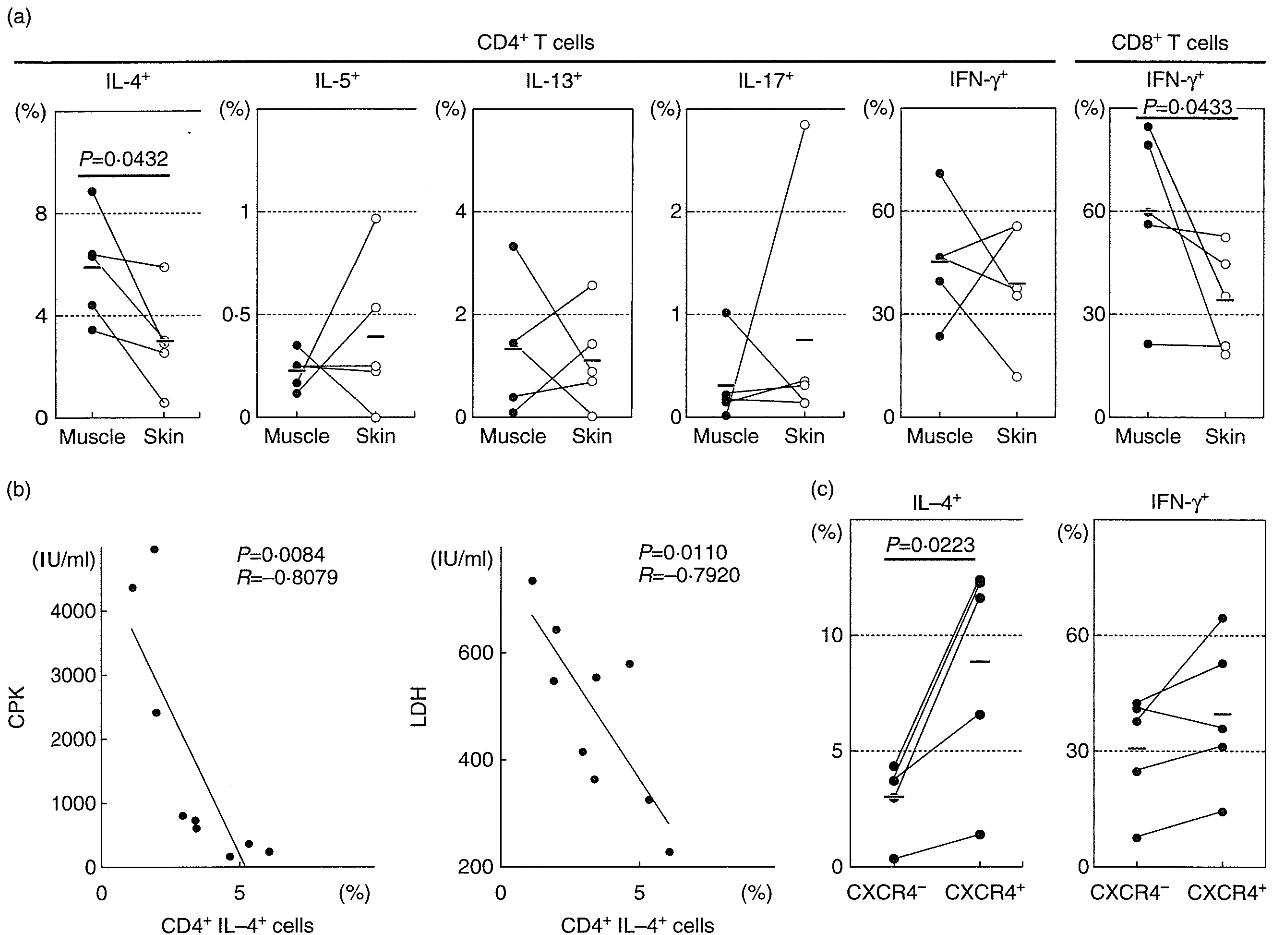

Fig. 5. Intracellular cytokine expressions of muscle- and skin-infiltrating T cells in the patients ($n = 5$). (a) The percentage of cytokine-producing T cells as assessed by intracellular cytokine staining and flow cytometric analysis (FCM). Horizontal bars indicate mean values. (b) Correlations between the percentage of interleukin (IL)-4-producing $CD4^+$ T cells and clinical parameters ($n = 9$). (c) IL-4 and interferon (IFN)-γ expression by chemokine (C-X-C motif) receptor (CXCR)4-positive or negative $CD4^+$ T cells ($n = 5$).

production by muscle-derived T cells correlated inversely with the serum CPK and LDH levels (Fig. 4d). Their IFN-γ production correlated weakly with the CPK levels, but not with the LDH levels. There were highly significant correlations between the IL-4 : IFN-γ ratio and CPK or LDH. The skin-derived T cells did not show such a correlation with CPK or LDH (Fig. 4e). Although the serum LDH level can be influenced by other organ involvements such as interstitial lung disease (ILD), the inverse correlation between the IL-4 : IFN-γ ratio and the LDH level remained even after exclusion of patients with ILD ($n = 8$, $Rs^2 = 0{\cdot}580$, $P = 0{\cdot}028$). These findings suggest that IL-4 and IFN-γ have opposite roles in the muscle damage, and IL-4 is associated with the low severity of myositis.

Intracellular cytokine staining of muscle- and skin-derived T cells

Intracytoplasmic cytokine staining of the expanded T cells was performed after stimulation with PMA and calcium ionophore, and cells were analysed by FCM. In the individual patients, IL-4-producing T cells were found at significantly higher frequencies in the muscle-derived T cells than in the skin-derived T cells (Fig. 5a), confirming the culture supernatant data. Only low percentages of $CD4^+$ T cells produced IL-5 and IL-13. One patient showed a high frequency of IL-17-producing $CD4^+$ T cells. There was a tendency that the percentage of IFN-γ-producing $CD8^+$ T cells was increased in the muscle T cells compared to the skin T cells. Again, the percentages of $IL\text{-}4^+CD4^+$ cells correlated inversely with the serum CPK and LDH levels (Fig. 5b), while there was no significant correlation between the percentages of IFN-γ-producing cells and the serum levels of CPK or LDH (data not shown).

As the muscle-derived T cells expressed CXCR4 at high frequencies, we examined the cytokine profile of $CD4^+CXCR4^+$ cells. The frequencies of IL-4-producing $CD4^+$ T cells were higher in $CXCR4^+$ cells than in $CXCR4^-$ cells (Fig. 5c), suggesting that $CXCR4^+$ T cells are Th2 cells.

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Discussion

To characterize T cells infiltrating into the lesional muscle and skin of DM and PM, we used a T cell expansion method from tissue-infiltrating T cells with immobilized anti-CD3/CD28 antibodies and 14-day cultivation with IL-2 [5]. In a comparison between the expanded T cells and the original tissue-infiltrating T cells, we observed expansion of $CCR7^-CD45RO^+$ effector memory T cells without biased propagation of $CD4^+$ and $CD8^+$ T cells. We focused upon TCR Vβ usage, chemokine receptor expression and cytokine production in a comparison between the muscle- and skin-derived T cells. We found skewed TCR Vβ usage of both the muscle- and skin-derived T cells in all the patients examined. However, the muscle lesions appear to have more marked Vβ preponderance than the skin lesions, as represented by case 13 (see Fig. 2b). No common Vβ was found in the expanded T cells among the patients (Fig. 2c), suggesting a different T cell repertoire in each patient. This suggests that T cells react differentially to antigens specific to each of the tissues, presumably in the context of major histocompatibility complex (MHC).

It is reasonable to assume that the skin-derived T cells expressed skin-homing receptors, CLA and CCR10 [8,9], at significantly higher frequencies than the muscle-derived cells. In contrast, the frequency of $CXCR4^+$ T cells was significantly higher in the muscle-derived cells than in the skin-derived cells. As reported previously [11], we observed that SDF-1, a ligand for CXCR4, was expressed at a high level in the vascular endothelial cells located between muscular fasciculi. Preferential infiltration of $CXCR4^+$ T cells may be attributable to SDF-1 produced by the endothelial cells.

By measuring the culture supernatant cytokines and the intracellular cytokine-bearing T cell frequencies, we found that the expanded T cells were comprised predominantly of IFN-γ-producing Th1 and IL-4-producing Th2 cells. In all the patients with DM, the frequencies of IL-4^+ T cells were lower than those of IFN-γ^+ cells in the lesional muscle. We observed a strong inverse correlation between IL-4 production by muscle-infiltrating T cells and the CPK- and LDH-assessed muscle damage and a weak positive correlation between IFN-γ production and muscle damage. Thus, the compartmental IL-4/IFN-γ cytokine balance was associated with the severity of muscle injury. Conversely, the skin-derived T cells contained a substantial number of IL-17A-producing Th17 cells in some of the DM patients, which are well known as pathogenic T cells for psoriasis [12,13]. Clinically, DM shares hyperkeratotic erythema with psoriasis [14]. Th17-producing cytokines, IL-17A and IL-22, may also contribute to the skin lesions of DM.

Histopathological studies of the muscle lesions demonstrated that $CD4^+$ T cells, B cells and macrophages predominantly infiltrated around endomysium capillaries with deposition of activated C5b-9 membrane attack complex [15]. A surge of circulating B cells and Th2 cells was observed frequently during the active phase of DM [3,16]. In contrast, a high number of $CD8^+$ T cells infiltrated into muscle fibres in PM, suggesting a significant contribution of autoreactive cytotoxic $CD8^+$ T cells to the pathogenesis of PM [17,18]. Although DM and PM share major clinical features, the type-2 and type-1 responses may be predominant in the development of muscle lesions of DM and PM, respectively. In certain cases of juvenile and adult DM, however, excessive tissue infiltrates of $CD8^+$ T cells were observed [4,19]. Meanwhile, recent gene expression analyses demonstrated a strong linkage to an IFN signature in the target tissues of DM [20–22], and type-I IFN-producing plasmacytoid dendritic cells markedly infiltrated the muscle of DM patients [23]. These findings indicate that both type 1 and type 2 IFN and Th2 cytokines are expressed in DM. In the muscle lesions of DM, it is possible that the infiltrating Th2 cells serve as regulators against IFN-induced inflammation. Alternatively, as IL-4 is a critical factor in muscle growth by maturating myotubules and a recruitment factor of muscle stem cells [24,25], IL-4-producing Th2 cells contribute to the muscle regeneration of DM.

In the muscle-derived $CD4^+$ T cells, the frequencies of IL-4-producing cells were higher in $CXCR4^+$ cells than in $CXCR4^-$ cells. It is therefore considered that the IL-4-producing Th2 cells preferentially express CXCR4 and can be recruited to the muscle by virtue of the tissue-producing SDF-1. The possible ameliorating or protective role of IL-4 and the CXCR4-SDF-1 engagement for T cell chemotaxis may provide a new perspective for therapeutic approaches in this intractable disease.

Acknowledgements

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Disclosure

The authors have declared no conflicts of interest.

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INVESTIGATIVE REPORT

Calcipotriol Increases hCAP18 mRNA Expression but Inhibits Extracellular LL37 Peptide Production in IL-17/IL-22-stimulated Normal Human Epidermal Keratinocytes

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Interleukins (IL)-17A and -22 are involved in the pathogenesis of psoriasis. Cathelicidin LL37 serves as not only antimicrobial peptide but also as autoinflammatory mediator. 1,25-Dihydroxyvitamin D3 analogues, such as calcipotriol, are used as topical treatment for psoriasis. However, the effect of calcipotriol on the mRNA expression/production of human cathelicidin antimicrobial protein (hCAP18) and LL37 peptide by IL-17A/IL-22-stimulated keratinocytes remains controversial. To evaluate the modulatory action of calcipotriol on the production of hCAP18 and LL37, we analysed hCAP18 mRNA expression and hCAP18/LL37 peptide production in IL-17A/IL-22-stimulated cultured human keratinocytes by real-time qPCR, ELISA, western blotting, and immunocytostaining. By western blotting, hCAP18 protein was detected in keratinocytes cultured for 72 h with IL-17/IL-22. Calcipotriol increased hCAP18 mRNA expression in IL-17/IL-22-stimulated keratinocytes. However, LL37 peptide in the culture supernatants was reduced by calcipotriol. Immunostaining revealed that the overproduced LL37 resides within the cells. LL37 promotes psoriasis via interaction with extracellular DNA, but may suppress psoriasis by interfering cytosolic DNA. ***Key words: psoriasis; cytokine; 1,25-dihydroxyvitamin D3; antimicrobial peptide; ELISA; interleukins.***

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Recent evidence has clearly shown that Th17 cells are involved in the pathogenesis of psoriasis. Th17 cells produce IL-17A and IL-22, which are crucial for the activation of STAT3 and resultant hyperproliferation of keratinocytes (1). Both cytokines cooperate with each other for Th17 to function as an immunomodulator in certain conditions (2). IL-17A and/or IL-22 induce the production of cytokines/chemokines such as interleukin (IL)-8/CXCL8 (3), and the production of antimicrobial peptides such as cathelicidin (4). Cathelicidin antimicrobial peptide (CAMP; NM 004345.4), also known as hCAP18 in humans, is an important molecule in the pathogenesis of psoriasis. hCAP18 is composed of a signal peptide domain, a cathelin domain and an LL37 domain (5), and this peptide is generated by neutrophils, monocytes, mast cells and epithelial cells. LL37 is produced when the hCAP18 protein is cleaved extracellularly by proteinase 3 or kallikrein 5 (6, 7). LL37 peptide exerts broad antimicrobial activity against bacteria and is a chemotactic agent for neutrophils, monocytes and T cells (8–10). hCAP18 protein and LL37 peptide are strongly expressed in lesional psoriatic skin and may play an important role as both an antimicrobial peptide and as an autoinflammatory mediator in psoriasis (4). Furthermore, the LL37 peptide induces multiple immunomodulatory effects on host cells (11).

1,25-Dihydroxyvitamin D3 (VitD3) regulates the proliferation and differentiation of keratinocytes, and its analogues are widely used as topical applicants for the treatment of psoriasis. While stimulating the keratinocyte differentiation, VitD3 can exert immunomodulatory actions. VitD3 (12, 13) and its analogues, 22-oxacalcitriol (13) and tacalcitol (14), inhibit the production of interleukin (IL)-6 and/or IL-8 by stimulated keratinocytes. VitD3 and calcipotriol modulate antigen-presenting dendritic cells, which consequently leads to the proliferation of regulatory T cells (15, 16). Furthermore, VitD3 regulates innate immunity by controlling the expression of antimicrobial peptides. However, the effect of VitD3 analogues on the expression and production of LL37 by stimulated keratinocytes remains controversial.

In this study, we investigated the regulatory action of calcipotriol, a synthetic VitD3 analogue with a high affinity for the vitamin D receptor, on the mRNA expression/protein production of hCAP18 and LL37 peptide in IL-17A and IL-22-stimulated human keratinocytes. We also monitored IL-8, because it targets neutrophils and participates in the epidermal collection of neutrophils and the formation of pustular psoriasis. Results suggest the unique action of the VitD3 analogue on the production and distribution of hCAP18 and LL37 peptide.

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MATERIALS AND METHODS

Cell culture

Normal human epidermal keratinocytes (NHEK) were purchased from Lifeline Cell Technology (Frederick, MD, USA). They were grown in serum-free keratinocyte growth medium Epilife (Invitrogen, Carlsbad, CA) and used at third passage in all experiments (17). Growth supplement was omitted 48 h before experiments. As a control, IL-17A and IL-22 (R&D Systems, Minneapolis, MN, USA) were either added or not added to the cells. Cultured NHEK cells were stimulated with IL-17A (200 ng/ml) and/or IL-22 (200 ng/ml) followed by co-incubation in the presence or absence of calcipotriol (Leo Pharma, Ballerup, Denmark) at 0.2–40 nM to test its modulatory effect. Cells were harvested 3 days later and subjected to real-time quantitative PCR (qPCR) as described previously (18). Culture supernatants were also collected and frozen at –80°C until use for ELISA.

Quantitative real-time qPCR for hCAP18 and IL-8 mRNA expression

Total mRNA was extracted from NHEK cells with the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. cDNA was reverse transcribed from total RNA using the TaqMan RT reagents (Applied Biosystems, Foster City, CA, USA). The evaluation of mRNA expression was analysed with SYBR®GeenER™ qPCR Reagent system (Invitrogen) using the ABI PRISM 7000 sequence detection system (Applied Biosystems). For IL-8 mRNA expression the following primer pairs were used: IL-8F; 5'-TGCAGCTCTGTGTGAAGGTG-3' and IL-8R; 5'-GGTCCACTCTCAATCACTCTCAG-3' and for hCAP18 mRNA expression the primers CAMP-F; 5'-GAAGGACGGGCTGGTGAAG-3' and CAMP-R; 5'-ACCCAGCAGGGCAAATCT-3' were used. As an endogenous reference glyceraldehydes-3-phosphate dehydrogenase (GAPDH) gene expression was measured using primers GAPDH-F; 5'-ACCACAGTCCATGCCATCAC-3' and GAPDH-R; 5'- TCCACCACCCTGTTGCTGTA-3'. The expression of the target gene normalised to the endogenous reference and relative to calibrator was given by the formula $2^{-\Delta\Delta C_T}$.

Quantification of LL37 peptide and IL-8 in culture supernatants by enzyme-linked immunosorbent assay (ELISA)

In addition, 3-day culture supernatants were measured for LL37 using LL37 ELISA kit (Hycult Biotech, Plymouth Meeting, PA, USA) and IL-8 using Quantikine® human CXCL8/IL-8 immunoassay kit (R&D Systems) according to the manufacturer's protocol. The absorbance at 450 nm was monitored with iMark microplate reader (Bio-Rad, Hercules, CA, USA).

Western blotting

Proteins were extracted from NHEK cells with cell lysis buffer (80mM Tris-HCl (pH7.6), 2% SDS, 10% glycerol, 0.1 mM PMSF, complete protease inhibitor cocktail (Roche, Mannheim, Germany)). Protein concentration was measured by Lowry assay with DCTM Protein assay kit (Bio-Rad), 40 μg sample proteins were used for the assay. CAMP 293T Cell Transient Overexpression Lysate (Abnova, Taipei, Taiwan) was used as a positive control. Proteins were separated on a 4–12% NuPAGE® Bis-Tris gel (Invitrogen) by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad). Membranes were then stained with rabbit polyclonal antibodies to human CAP18/LL37 (1:500; Hycult Biotech) or a polyclonal antibody to human β-actin (1:2,000; Cell Signalling Technology, Inc., Boston, MA, USA). Proteins were detected with the ECL plus or ECL Western blot detection system (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

Immunocytostaining for hCAP18 and LL37 peptide in NHEK cells

Cultured NHEK cells were fixed with 4% paraformaldehyde for 15 min followed by cell permeabilisation for 15 min with 0.2% TritonX-100 solution. Fixed cells were incubated for 1 h in 1% bovine serum albumin (BSA) in PBS to block non-specific binding sites. Cells were incubated for 1 h with a mouse anti-human LL37/CAP-18 monoclonal primary antibody (3D11, 1:100; HM2070, Hycult Biotech, Plymouth Meeting, PA, USA). An Alexa Fluor 594 conjugated-goat anti-mouse antibody (Molecular probes, Eugene, OR, USA) was used as a secondary antibody. Nuclei were stained with DAPI.

Statistical analysis

Student's *t*-test (impaired) was employed to determine statistical differences between means. Correlations were studied by Pearson's product-moment correlation coefficient.

RESULTS

Increased production of hCAP18 by IL-17A and IL-22

We first examined the ability of IL-17A/IL-22 to increase the production of hCAP18 protein by NHEK cells in various culture periods. We have previously reported that cytokine production by NHEK cells peaks at 72 h after the addition of IL-17A/IL-22 (3). To confirm the time course of hCAP18 production, we cultured NHEK cells with IL-17A/IL-22 for 6, 24, or 72 h, and the lysates were then subjected to western blot analysis. As positive control, CAMP 293T Cell Transient Overexpression Lysate was used. We found that hCAP18 protein was produced in 72 h cultured NHEK cells treated with IL-17A/IL-22, but not in 6 or 24 h cultured cells (Fig. S1[1]). Thus, we used 72 h as the culture period in the following experiments.

Increase of hCAP18 mRNA expression and decrease of IL-8 mRNA expression by calcipotriol in IL-17A and IL-22-stimulated NHEK cells

Given that keratinocytes are exposed to Th17-derived cytokines in the psoriatic lesion, it is reasonable to investigate the *in vitro* effect of calcipotriol on the expression of hCAP18 mRNA in keratinocytes stimulated with IL-17A and IL-22. As control, IL-8 was also monitored in parallel, because VitD3 and its analogues are known to suppress IL-8 expression (12–14). In NHEK cells that were not stimulated with IL-17A or IL-22, calcipotriol at 0.2-20 nM did not substantially affect mRNA expression of hCAP18 (Fig. 1a). The addition of IL-17A/IL-22 remarkably increased hCAP18 expression (Fig. 1b). More dramatically, further addition of calcipotriol increased the hCAP18 mRNA expression in the presence of IL-17A/IL-22 in a dose-dependent manner. Calciptriol at 20 nM yielded the maximum response.

We have shown the synergistic effects of IL-17 and IL-22 on the production of IL-8 in NHEK cells (3). When NHEK cells were not stimulated with IL-17A or IL-22, calcipotriol slightly enhanced (0.2 nM) IL-8

[1]http://www.medicaljournals.se/acta/content/?doi=10.2340/00015555-1775

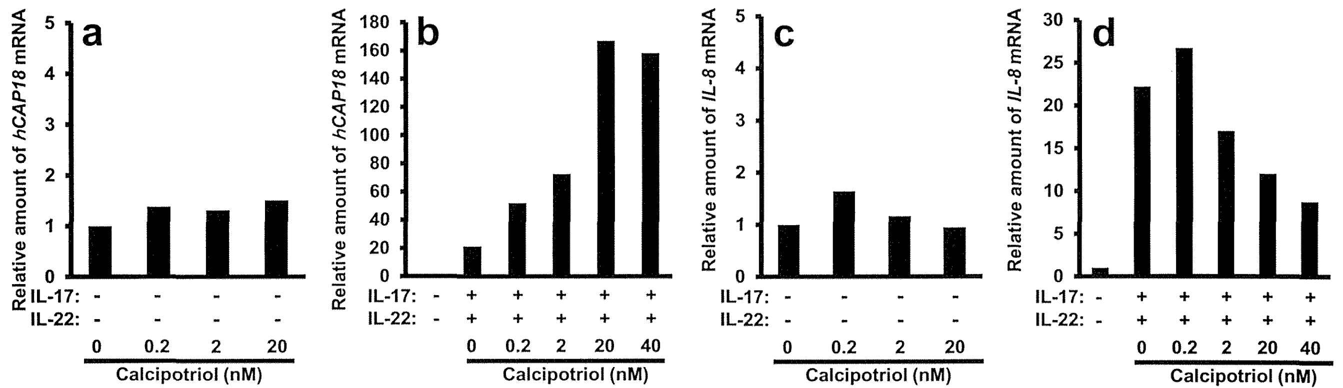

Fig. 1. Calcipotriol increases mRNA expression of hCAP18, but reduces the expression of IL-8 in IL-17A and IL-22-stimulated NHEK cells. NHEK cells were cultured with the indicated concentration of calcipotriol in the presence of IL-17A and IL-22. After a 3-day culture, they were subjected to real-time qPCR. (a, b) hCAP18 mRNA expression (n=3) and (c, d) IL-8 mRNA expression (n=3).

mRNA expression or had no effect (2–20 nM) (Fig. 1c). The addition of IL-17A and IL-22 markedly increased the mRNA expression of IL-8 (Fig. 1d), confirming our previous study (3). This enhanced IL-8 mRNA expression was suppressed by calcipotriol at 2, 20 and 40 nM in a dose dependent manner (Fig. 1d).

Thus, calcipotriol decreases IL-8 mRNA expression but increases hCAP18 mRNA expression in IL-17A/IL-22-stimulated NHEK cells.

Inhibition of extracellular production of LL37 cathelicidin peptide in IL-17A and IL-22-stimulated NHEK cells

We measured the LL37 peptide concentration by ELISA in the culture supernatants of NHEK cells after 72 h incubation with IL-17A, IL-22, and/or calcipotriol. Both cytokines enhanced the LL37 peptide production synergistically, as the LL37 concentration of supernatants from IL-17A/IL-22-stimulated NHEK cells was significantly higher than that from non-stimulated NHEK cells (Fig. 2a). This cytokine-enhanced, extracellular production of LL37 peptide was reduced by calcipotriol at 20 nM. On the other hand, the downmodulatory effect of calcipotriol on IL-8 mRNA expression was also observed at protein level (Fig. 2b). The results suggest that calcipotriol increases hCAP18 mRNA expression, but the extracellular production of LL37 peptide is inhibited in IL-17A and IL-22-stimulated NHEK cells.

hCAP18/LL37 peptide immunoreactivity in NHEK cells stimulated with IL-17A/IL-22 and/or calcipotriol

NHEK cells were cultured with IL-17A/IL-22, calcipotriol, or both, and immunoreactivity with anti-LL37 antibody was observed by immunofluorescence microscopy (Fig. 3). It is considered that both hCAP18 protein and LL37 peptides that are cleaved from hCAP18 can be detected with anti-LL37 antibody. Compared with the no treatment group (hCAP18$^+$/LL37 peptide$^+$ cells, 86/high power view), the calcipotriol-added group had a comparable number of positive cells (79/high power view). The IL-17A/IL-22-added group exhibited a high number of hCAP18$^+$/LL37 peptide$^+$ cells (182/high power view). Further addition of calcipotriol together with IL-17A/IL-22 enhanced the hCAP18$^+$/LL37 peptide$^+$ cell number (280/high power view). These findings suggest that, in calcipotriol-treated and IL-17A/IL-22-stimulated NHEK cells, the overproduced hCAP18 protein and LL37 peptide resides within the cells and its release to the culture is downmodulated, resulting in the elevation of intracellular hCAP18/LL37 peptide.

We performed western blot analysis of LL37 with anti-hCAP18/LL37 as shown in Fig. S1[1]. Although hCAP18 protein was found, LL37 peptide (4 kDa) could not be detected even at 72 h after incubation with IL-17A/IL-22. It is possible that a large part of the cleaved proteins from hCAP18 are dissimilar to LL37. Alternatively, it might be difficult to detect LL37 by western blotting because of its small molecular weight or difficulty in extraction.

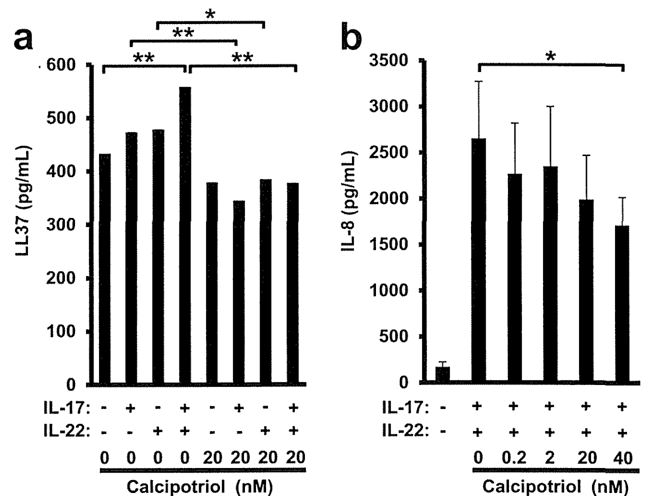

Fig. 2. Calcipotriol decreases the concentrations of hCAP18/LL37 peptide and IL-8 in the culture supernatants from IL-17A/IL-22-stimulated NHEK cells. NHEK cells were cultured with the indicated concentration of calcipotriol in the presence or absence of IL-17A and IL-22. After a 3-day culture, the supernatants were collected and subjected to ELISA. *$p<0.05$, **$p<0.01$. (a) LL37 protein concentration (n=3) and (b) IL-8 protein concentration (n=3).

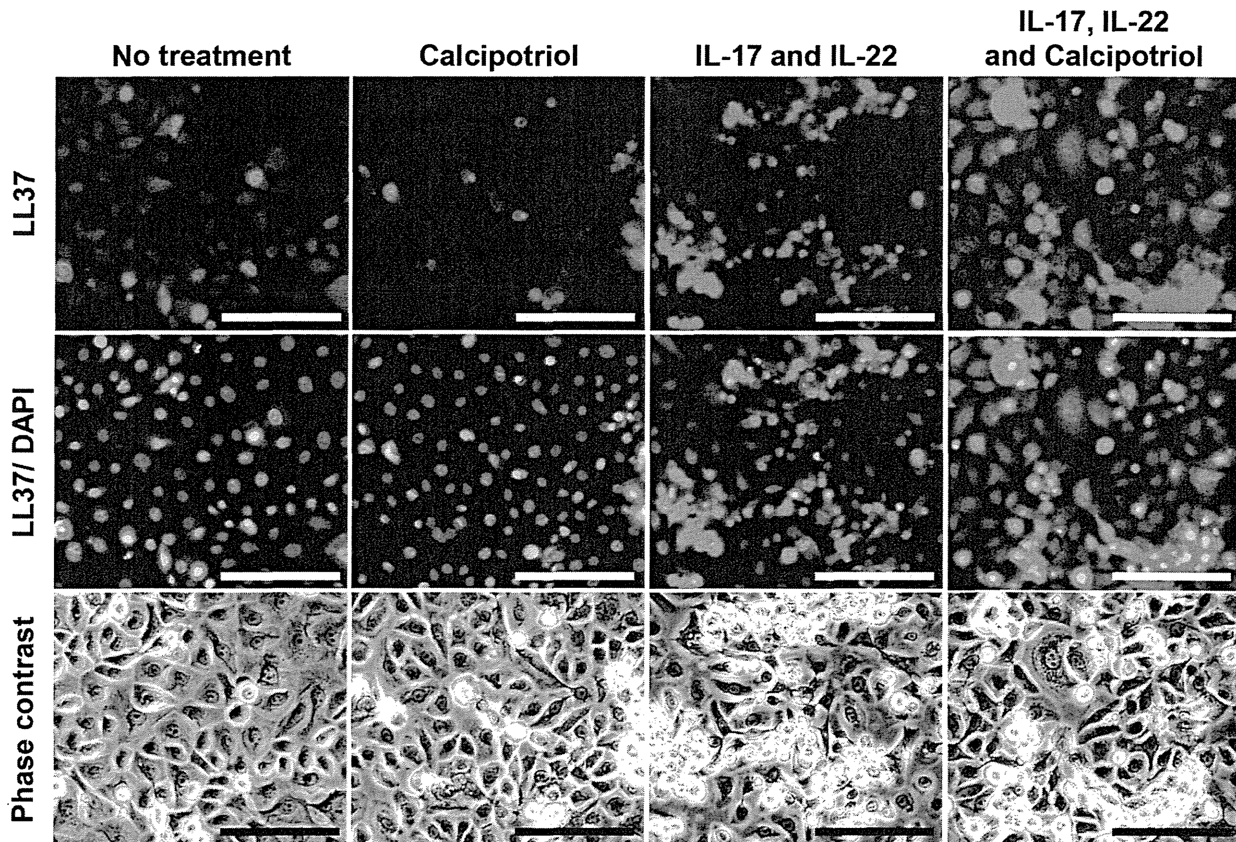

Fig. 3. Calcipotriol increases the number of intracellularly hCAP18⁺/LL37 peptide⁺ cells in IL-17A and IL-22-stimulated NHEK cells. NHEK cells were cultured with the indicated concentration of calcipotriol in the presence or absence of IL-17A and IL-22. After a 3-day culture, cells were fixed by 4% paraformaldehyde. Cells were then lysed with TritonX-100 solution. A mouse anti-human LL37/CAP-18 monoclonal antibody and an Alexa Fluor 594 conjugated-goat anti-mouse antibody were used as the primary and secondary antibodies, respectively. Nuclei were stained with DAPI. Scale bar represents 100 μm. The photograph is a representative of 3 independent experiments.

DISCUSSION

Stimulation with the combination of IL-17A and IL-22 may yield cutaneous inflammation as seen in psoriasis. By using cultured keratinocytes stimulated with IL-17A/IL-22, we found that the expressions and/or productions of hCAP18/LL37 peptide and IL-8 are differentially modulated by calcipotriol. While IL-8 was suppressed by calcipotriol at both mRNA and protein levels, hCAP18 mRNA expression was enhanced, but its extracellular release as hCAP18 or LL37 peptide was downmodulated by calcipotriol.

It has been reported that VitD3 (6, 13) and its analogues, 22-oxacalcitriol (13) and tacalcitol (14), inhibit the production of IL-8 by keratinocytes. In these previous studies, keratinocytes were stimulated with TNF-α, or synergistically with TNF-α and IFN-γ (13, 14). In addition to these well-known keratinocyte-stimulatory cytokines, we found that IL-17A and IL-22 synergistically stimulated keratinocytes to produce IL-8 (3), and that calcipotriol suppresses this overproduction. In fact, topical application of calcipotriol reduces the IL-8 concentration in the skin (19). Thus, calcipotriol is therapeutically beneficial for psoriasis by suppressing IL-8 production and resultant inhibition of neutrophil accumulation.

Of particular interest is the observation that the IL-17A/IL-22-augmented expression of hCAP18 was further enhanced by calcipotriol. Another study using different stimulants, i.e. UVB, lipopolysaccharide and TNF-α, showed that calcipotriol oppositely suppressed the expressions of LL37 and human β defensin-2 (HBD-2) (20). In agreement with our study, however, there is a report demonstrating that the simultaneous addition of IL-17A and VitD3 markedly induced the expression of cathelicidin peptide in NHEK cells (21). In their study, VitD3 blocked the induction of IL-8 and HBD-2 (21), consistent with our finding. In *in vivo* studies, topical treatment with calcipotriol enhanced cathelicidin expression, but reduced HBD-2 and HBD-3 expression in human skin (22, 23). Therefore, our data, together with previous findings, collectively suggest that calcipotriol or VitD3 decreases IL-8 but increases hCAP18 mRNA expression, when keratinocytes are stimulated especially with Th17-derived cytokines.

However, our study showed that the overexpressed LL37 cathelicidin peptide was not released extracellularly but resided within the cells. Extracellular LL37 can interact with self-DNA in psoriatic skin, and the production of self-DNA/LL37 complexes activate plasmacytoid dendritic cells, thereby inducing psoria-