Inhibition of IL-17–committed T cells in a murine psoriasis model by a vitamin D analogue

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Background: A better understanding of the means by which topical vitamin D analogues exert their therapeutic effect on psoriasis is of theoretical and practical importance.

Objective: We sought to clarify whether and how the topical vitamin D analogue calcipotriol (CAL) controls the IL-17A–mediated pathogenesis of murine psoriasis-like dermatitis *in vivo*.

Methods: Psoriasis-like dermatitis was induced by the topical application of an imiquimod (IMQ)–containing cream on the murine ear for 4 to 6 consecutive days. For topical CAL treatment, mice were treated daily with CAL solution on the ear before IMQ application.

Results: Mice treated topically with CAL exhibited much milder IMQ-induced psoriasis-like dermatitis compared with vehicle-treated mice, with impaired accumulation of IL-17A–committed T (T17) cells in the lesional skin. The IMQ-induced upregulation of *Il12b* and *Il23a* was marked in the epidermis and was abrogated by CAL application, suggesting CAL-mediated suppression of IL-23 expression. CAL inhibited *Il12b* and *Il23a* expression by Langerhans cells *ex vivo* stimulated with IMQ and CD40 cross-linking. Topical CAL also inhibited T17 cell expansion in the draining lymph nodes of IMQ-treated skin, implying a possible effect on T17 cell–mediated dermatitis at distant sites. In fact, topical CAL application on the IMQ-treated left ear resulted in amelioration of T17 cell accumulation and psoriasis-like dermatitis in the right ear subsequently treated with IMQ.

Conclusion: Topical CAL can exert its antipsoriatic effect on CAL-treated lesions and, concomitantly, distant lesions by attenuating the T17 cell accumulation in both CAL-treated lesions and draining lymph nodes. (J Allergy Clin Immunol 2017;■■■:■■■-■■■.)

Key words: *Psoriasis, calcipotriol, vitamin D analogue, IL-17A, IL-23, imiquimod*

Psoriasis is a common chronic inflammatory skin disease characterized by red scaly papules and plaques, with a prevalence generally estimated at 1% to 3% of the population worldwide.[1-5] Although the complex pathogenesis of psoriasis remains incompletely understood, compelling evidence suggests that this skin disorder is mediated by the pathologic cross-talk between epidermal keratinocytes and immune cells.[4,6] Psoriatic keratinocytes produce proinflammatory mediators that contribute to the recruitment and activation of immune cells, such as IL-8, CXCL1, CCL20, and S100A proteins.[1,3] Conversely, activated immune cells that accumulate in psoriatic skin form a cytokine milieu that induces psoriatic keratinocyte phenotypes.

Among various propsoriatic cytokines, the immunologic pathway involving IL-23 and IL-17A (IL-23/IL-17A axis) has been demonstrated to play a pivotal role in the pathogenesis of psoriasis.[1,7,8] IL-23 derived from inflammatory dendritic cells (DCs) stimulates IL-17A–committed T (T17) cells to survive, proliferate, and produce IL-17A and IL-22, which, in turn, act on epidermal keratinocytes to undergo excessive proliferation and impaired differentiation, thereby producing the appearance of psoriatic skin. Understanding how current treatments inhibit the

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Supported in part by Grants-in-Aid for Scientific Research from the Ministries of Education, Culture, Sports, Science and Technology, Precursory Research for Embryonic Science and Technology, and Health, Labour and Welfare of Japan and Torii Pharmaceutical.

Disclosure of potential conflict of interest: N. Kusuba's institution received a grant from Torii Pharmaceutical for this work and grants from GlaxoSmithKline Japan for other works. A. Kitoh's institution received a grant from Torii Pharmaceutical for this work and received grants from the Japan Blood Products Organization, Mitsubishi Tanabe Pharma, Sanofi Japan Group, AbbVie GK, the Lydia O'leary Memorial Pias Dermatological Foundation, Actelion Pharmaceuticals Japan, and the Fujiwara Memorial Foundation for other works. T. Dainichi's institution received grants from AbbVie GK, Mitsubishi Tanabe Pharma, Sanofi Japan Group, and Novartis Pharma for other works, and the author personally received payment for lectures from Maruho, Mitsubishi Tanabe Pharma, Sanofi Japan Group, Kaken Pharmaceutical, Taiho Pharma, GlaxoSmithKline, Nihon Pharmaceutical, Novartis Pharma, and Torii Pharmaceutical. T. Honda's institution received grants from Mitsubishi Tanabe Pharma, Sanofi Japan Group, AbbVie GK, the Lydia O'leary Memorial Pias Dermatological Foundation, ROHTO Pharmaceutica, Shiseido, Novartis international AG, and Kao and payment for lectures from Novartis international AG, AbbVie GK, Sanofi Japan Group, Mitsubishi Tanabe Pharma, Janssen Pharma, Kyowa Hakko Kirin, and Torii Pharmaceutical. A. Otsuka's institution received grants and payment for lectures from Ono pharmaceutical and payment for lectures from MSD K.K., Bristol-Myers Squibb and Novartis, and GlaxoSmithKline Japan. G. Egawa's institution received grants from GlaxoSmithKline Japan and Novartis Pharma for other works. S. Nakajima's institution received grants from the Sanofi Japan Group and Kanae Foundation for the promotion of medical science. Y. Miyachi's institution received payments for lectures from Maruho, GlaxoSmithKline, POLA Pharma, Otsuka Pharmaceutical, Kinetic Concepts, Taisho Toyama Pharmaceutical, Noevir, Acseine, MSD KK, KAKEN Pharmaceutical, Shionogi, GALDERMA, Terumo Corporation, Shiseido Japan, Sato Pharmaceutical, Mitsubishi Tanabe Pharma Corporation, and Kyowa Hakko Kirin. K. Kabashima's institution received a grant from Torii Pharmaceutical for this work; grants from Sanofi Japan Group, Mitsubishi Tanabe Pharma, Chugai Pharmaceutical, POLA Pharma, and Maruho for other works, and payment for lectures from POLA Pharma, Kyowa Hakko Kirin, Maruho, and Leo Pharma.

Received for publication November 20, 2016; revised June 12, 2017; accepted for publication July 19, 2017.

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0091-6749/$36.00

http://dx.doi.org/10.1016/j.jaci.2017.07.033

Abbreviations used
CAL: Calcipotriol
DC: Dendritic cell
dLN: Draining lymph node
EtOH: Ethanol
IMQ: Imiquimod
LC: Langerhans cell
PE: Phycoerythrin
T17: IL-17A–committed T
TCR: T-cell receptor

pathogenic processes of psoriasis is of theoretical and practical importance in improving the management of this disease.

Vitamin D analogues, such as calcipotriol (CAL), tacalcitol, and maxacalcitol, have been shown to be effective and safe in the topical treatment of psoriasis by previous randomized controlled studies, although they are not effective enough to achieve almost clear psoriatic symptoms.[9-15] Currently, they are regarded as one of the first-line treatments in guidelines for the management of psoriasis.[16-18] However, the precise mechanism by which topical vitamin D analogues exert their antipsoriatic effect *in vivo* remains unknown. The biological effects of the active vitamin D metabolite 1α,25-dihydroxyvitamin D_3 (calcitriol) are mediated by the nuclear vitamin D receptor, which functions as a ligand-activated transcription factor and regulates activation or repression of gene transcription.[19] The vitamin D receptor is expressed in many cell types, including keratinocytes, DCs, and T lymphocytes,[20-23] which are significantly involved in the pathogenesis of psoriasis.[1-5] Calcitriol and its analogues suppress the proliferation of keratinocytes and their production of psoriasis-related proinflammatory molecules, including IL-6, IL-8, S100A7, and S100A15 *in vitro*.[24-26] Accordingly, topical vitamin D analogues have been considered to improve psoriatic lesions by these direct effects on psoriatic keratinocytes. In addition, it is thought that the immunomodulating properties of vitamin D analogues might also be associated with their role in treating psoriasis. A number of *in vitro* studies have demonstrated that calcitriol and its analogues inhibit the differentiation and maturation of DCs derived *ex vivo* from either human peripheral blood monocytes[27-34] or mouse bone marrow cells.[35,36] In addition, calcitriol can induce DCs to have *in vitro* and *in vivo* tolerogenic properties.[23,36-39] Their tolerogenic properties are associated at least in part with attenuated expression of costimulatory molecules, such as CD86 and CD40; decreased IL-12 and IL-23 production; and enhanced IL-10 production.[27,33,34,40] Moreover, calcitriol and its analogue CAL act directly on $CD4^+$ T cells to suppress T17 cell differentiation and induce preferential differentiation into regulatory T cells and T_H2 cells *in vitro*.[40,41]

Thus it is theoretically possible that the antipsoriatic effects of topical vitamin D analogues might be due in part to their effects on immune cells. In fact, several studies[42,43] indirectly imply a possible inhibitory effect of topical vitamin D analogues on IL-23 and IL-17A expression in lesional skin of patients with psoriasis; however, it has not yet been clearly shown whether topical vitamin D analogues actually control the immune pathogenesis of psoriasis *in vivo*. In addition, topical vitamin D analogues are generally regarded as local symptomatic treatments, and their effects on the systemic pathogenesis of psoriasis remain to be addressed.

Here we investigated whether and how the topical vitamin D analogue CAL controls the IL-17A–mediated pathogenesis of psoriasis *in vivo* by using a murine model of psoriasis in which psoriasis-like skin inflammation is critically mediated by the IL-23/IL-17A axis. We found that topical CAL application inhibited T17 cell accumulation in not only CAL-treated lesions but also draining lymph nodes (dLNs), leading to attenuated psoriasis-like skin inflammation in CAL-treated lesions and, concomitantly, distant lesions.

METHODS

IMQ-induced psoriasis-like dermatitis and topical CAL application

Murine psoriasis-like dermatitis was induced by topical application of imiquimod (IMQ), a ligand for Toll-like receptor 7, as described previously[44,45] with modifications. Briefly, B6 mice were treated topically with 10 mg of an IMQ-containing cream (5%; Beselna; Mochida Pharmaceutical, Tokyo, Japan) on each ear for 6 consecutive days, unless otherwise stated. For CAL treatment in mice with IMQ-induced dermatitis, mice were treated daily with 0.2 nmol of CAL (Tocris Bioscience, Bristol, United Kingdom) dissolved in 20 μL of ethanol (EtOH) on the ear 15 minutes before IMQ application.

Other methods

See the Methods section in this article's Online Repository at www.jacionline.org.

RESULTS

Topical CAL application ameliorated IMQ-induced psoriasis-like dermatitis

To examine whether topical CAL application can control the IL-17A–mediated pathogenesis of psoriasis *in vivo*, we used an IMQ-induced murine psoriasis-like dermatitis model because this model is critically dependent on IL-17A derived from T cells,[46,47] as is human psoriasis.[2,3] We first confirmed that the topical application of CAL ameliorated IMQ-induced psoriasis-like dermatitis, as demonstrated in human psoriatic lesions.[48,49] Mice were treated topically with an IMQ-containing cream after either CAL (IMQ/CAL) or vehicle (IMQ/EtOH) on the ears for 6 consecutive days. Because the topical application of CAL on murine skin at the same concentration as in the clinically used CAL lotion (about 121 μmol/L) induced atopic dermatitis–like skin inflammation,[50] CAL was applied at a lower concentration (10 μmol/L) in this study.

IMQ/EtOH treatment induced ear swelling (Fig 1, *A*) and psoriasis-like lesions characterized by redness, scaling, and crust formation (Fig 1, *B*), as previously described.[47,51] By contrast, mice treated with IMQ/CAL showed significantly decreased ear swelling (Fig 1, *A*) and much milder inflammation phenotypically (Fig 1, *B*) compared with IMQ/EtOH-treated mice. Histologic examination revealed that topical CAL application substantially attenuated epidermal hyperplasia, one of the histologic hallmarks of psoriatic skin lesions, in IMQ-treated skin (Fig 1, *C* and *D*, upper panels). In addition, a significantly smaller proportion of epidermal keratinocytes in IMQ/CAL-treated skin were positive for the proliferation marker Ki-67 compared with those in IMQ/EtOH-treated skin (Fig 1, *C*, lower panels), indicating that IMQ-induced hyperproliferation of keratinocytes was attenuated by topical CAL application. Moreover, CAL also suppressed inflammatory cell accumulation in the lesional skin of mice with

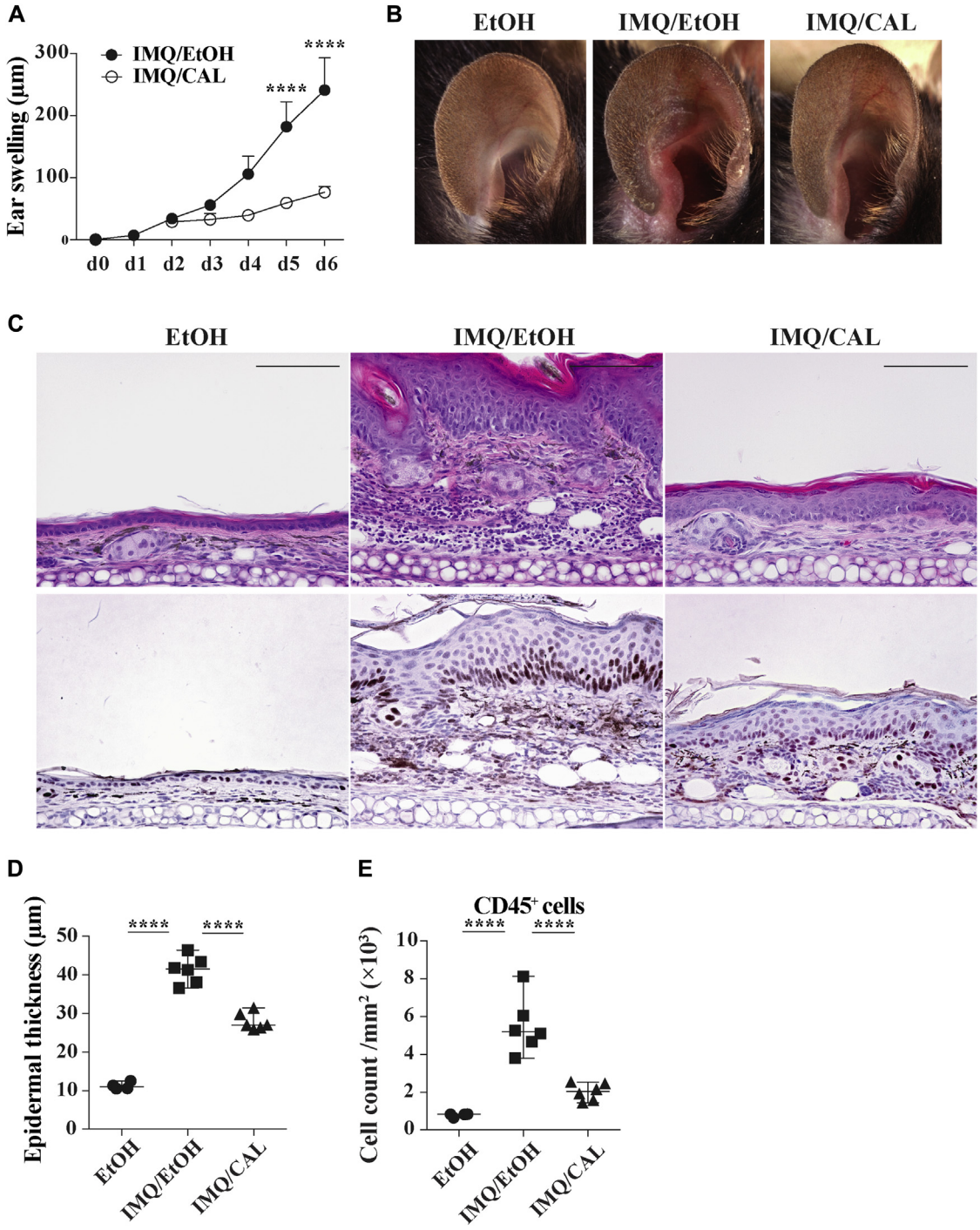

FIG 1. The effect of topical CAL on IMQ-induced psoriasis-like dermatitis. **A,** Kinetics of ear swelling. **B,** Phenotypic appearances. **C,** Hematoxylin and eosin staining and immunohistochemistry of Ki-67. *Scale bars* = 100 μm. **D,** Epidermal thickness. **E,** $CD45^+$ cell numbers. All samples were collected 24 hours after 6 applications of the indicated chemicals. Data are shown as each value *(symbols)* and their means ± SDs *(bars)*. ****$P < .0001$.

IMQ-induced dermatitis (Fig 1, *C*, upper panels), which was further confirmed by flow cytometric analyses of $CD45^+$ leukocyte numbers (Fig 1, *E*).

Taken together, these results suggest that the IMQ-induced murine psoriasis model reflects the response to topical CAL application observed in patients with psoriasis. Thus it is worthwhile to investigate how the treatment exerts its therapeutic effect on psoriasis.

Topical CAL application inhibited accumulation of T17 cells in IMQ-treated skin

Using these experimental settings, we examined whether topical CAL application inhibited the IL-17A–mediated pathogenesis of psoriasis-like dermatitis. Quantitative RT-PCR analysis revealed that although the mRNA expression of *Il17a* was induced in IMQ-treated skin, IMQ-induced *Il17a*

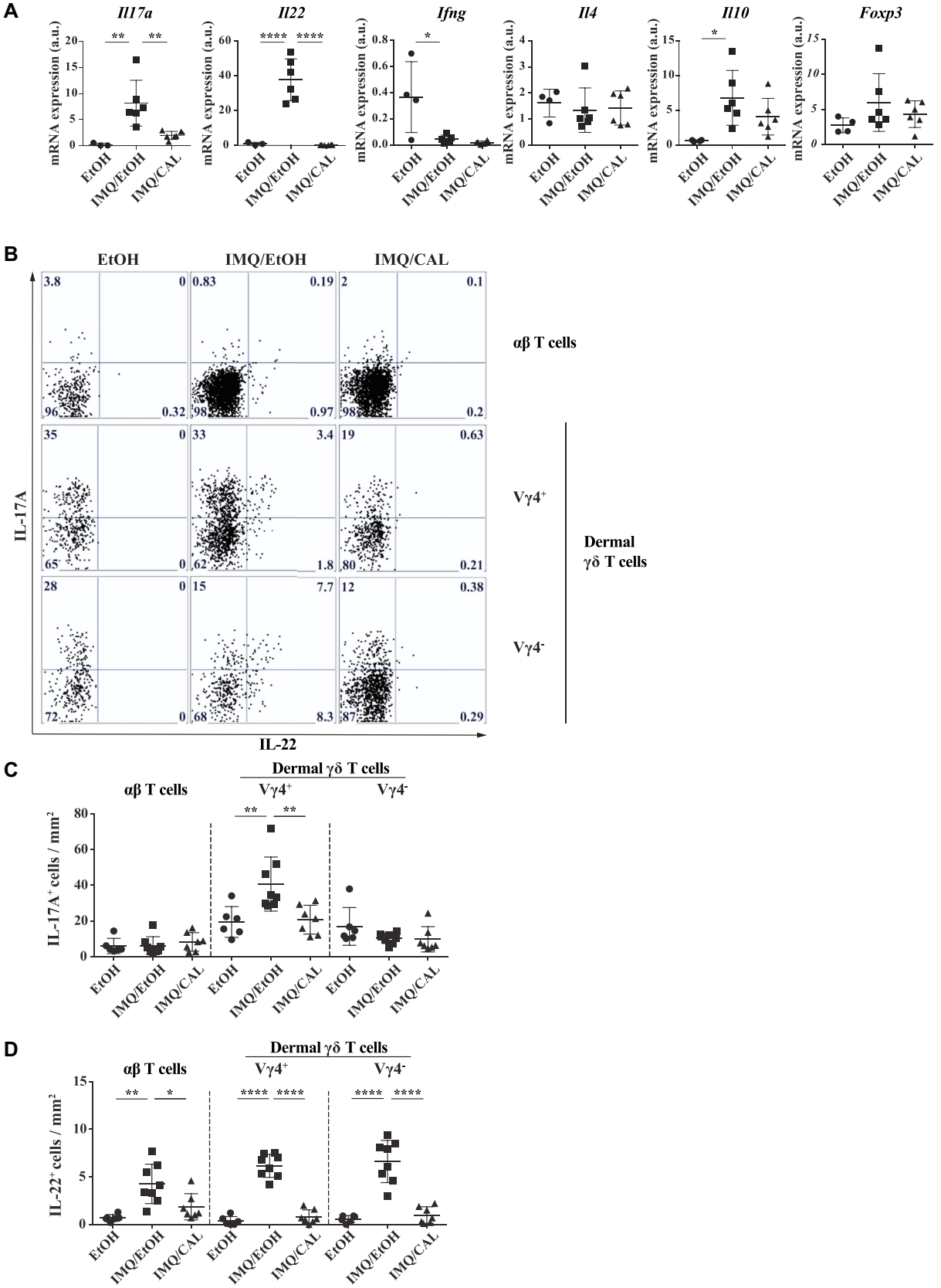

FIG 2. Effect of topical CAL on the T17 cell accumulation in IMQ-treated skin. **A,** Quantitative RT-PCR analysis of the indicated genes. **B,** Intracellular staining of IL-17A and IL-22. **C** and **D,** Number of IL-17–committed (Fig 2, *C*) and IL-22–committed (Fig 2, *D*) cells in lesional ear skin. All samples were collected 24 hours after 6 applications of the indicated chemicals. Data are shown as each value *(symbols)* and their means ± SDs *(bars)*. *a.u.*, Arbitrary units. $*P < .05$, $**P < .01$, and $****P < .0001$.

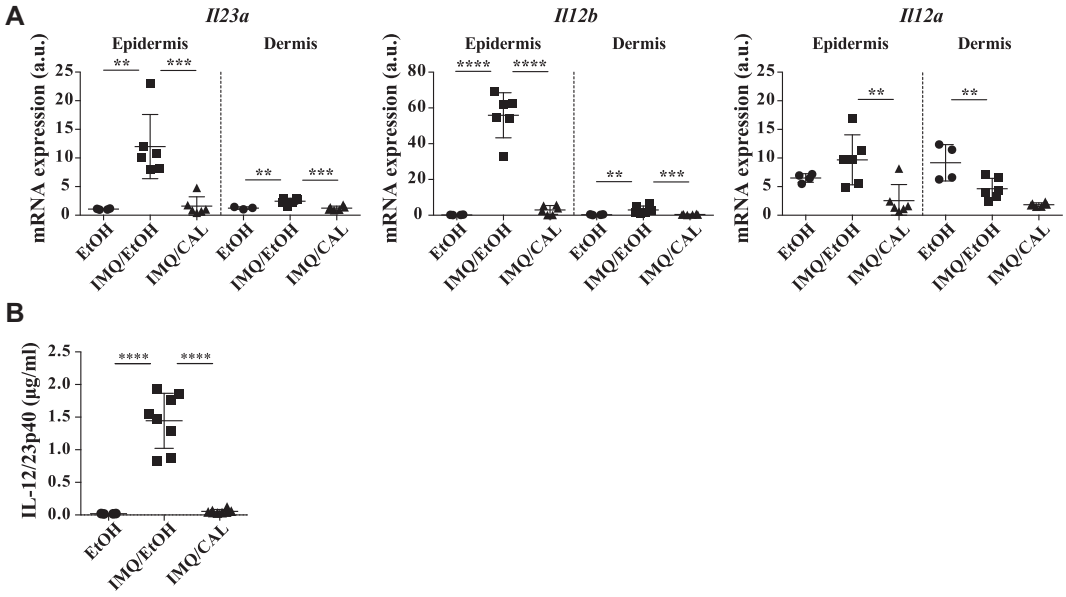

FIG 3. Effect of topical CAL on IL-23 production in the epidermis and dermis of lesional skin. **A,** Quantitative RT-PCR analysis of *Il23a*, *Il2b*, and *Il12a* in the epidermis and dermis of the lesional skin. **B,** Measurement of IL-12/23p40 protein levels in lesional epidermis by using ELISA. All samples were collected 24 hours after 4 applications of the indicated chemicals. Data are shown as each value *(symbols)* and their means ± SDs *(bars)*. *a.u.*, Arbitrary units. $^{**}P < .01$, $^{***}P < .001$, and $^{****}P < .0001$.

upregulation was markedly suppressed by topical CAL application (Fig 2, *A*). CAL also suppressed the IMQ-induced expression of IL-22, which is known to act on keratinocytes alone or in synergy with IL-17A to promote development of the psoriatic phenotype in mice, as well as in human subjects,[44,47,52-54] at the mRNA level (Fig 2, *A*). In line with the attenuated expression of *Il17a*, IMQ/CAL-treated skin showed markedly decreased expression of genes encoding the antimicrobial molecules with orthologues known to be overexpressed in human psoriatic skin and induced in keratinocytes stimulated with IL-17A[55,56] compared with IMQ/EtOH-treated skin (see Fig E1 in this article's Online Repository at www.jacionline.org). By contrast, expressions of T_H1-, T_H2-, and regulatory T cell–related genes were not affected by CAL application (Fig 2, *A*). Flow cytometric analysis showed that IMQ treatment led to a marked increase in the number of T17 cells in the $V\gamma4^+$ subset of dermal γδ T cells but not in the αβ T-cell and $V\gamma4^-$ dermal γδ T-cell subsets (Fig 2, *B* and *C*, and see Fig E2 in this article's Online Repository at www.jacionline.org), which was consistent with previous reports.[47,57] IMQ-induced accumulation of $V\gamma4^+$ γδ T17 cells was strongly attenuated by topical CAL application (Fig 2, *B* and *C*). CAL application also suppressed the increase in the number of IL-22–committed T-cell populations in IMQ-treated skin (Fig 2, *B* and *D*).

Collectively, these results suggest that topical CAL application inhibits the IL-17A–mediated pathogenesis of psoriasis-like dermatitis by attenuating accumulation of pathogenic T17 cells in lesional skin.

Topical CAL application suppressed IL-23 expression in IMQ-treated skin

Previous studies have revealed that T17 cell expansion is promoted by IL-23 in both mice[58-60] and human subjects.[61-63] Thus we hypothesized that CAL-mediated inhibition of T17 cell accumulation in lesional skin of mice with IMQ-induced dermatitis was attributed to attenuated IL-23 expression. Quantitative RT-PCR analysis revealed that mRNA expression of the *Il12b* and *Il23a* genes, which encode the IL-12/IL-23p40 and IL-23p19 subunits of IL-23, respectively, was markedly increased in the epidermis in mice with IMQ/EtOH-treated skin compared with EtOH-treated skin (Fig 3, *A*). By contrast, *Il12a*, which encodes the IL-12p35 subunit, was not significantly induced by IMQ treatment (Fig 3, *A*), suggesting that expression of IL-23, but not IL-12, was upregulated in the epidermis in mice with IMQ-treated skin. *Il12b* and *Il23a* expression in the epidermis was significantly less in IMQ/CAL-treated skin than in IMQ/EtOH-treated skin (Fig 3, *A*). IMQ-induced upregulation of *Il12b* and *Il23a* was also observed in the dermis, although much less markedly, and was suppressed by CAL application (Fig 3, *A*). An ELISA revealed that IMQ-induced expression of IL-12/IL-23p40 protein in the epidermis was significantly inhibited by CAL (Fig 3, *B*), corroborating that IL-23 expression in the setting of IMQ-induced dermatitis was suppressed by CAL application. Given the previous findings that induction of the *Il17a* expression, T17 cell accumulation, and a psoriasis-like phenotype in this model are completely abolished in *Il23a*-deficient mice,[46,51,64] the CAL-mediated suppression of IL-23 expression is likely to contribute substantially to the attenuation of the T17 cell accumulation in IMQ-treated skin.

To further confirm the inhibitory effect of CAL on IL-23/IL-17A–mediated skin inflammation, we started CAL application on IMQ-treated skin at day 3, when IMQ-induced skin inflammation had already developed, and assessed expression levels of *Il17a*, *Il22*, *Il23a*, and *Il12b* at day 6. mRNA levels of these genes were suppressed by CAL treatment (see Fig E3 in this article's Online Repository at www.jacionline.org), suggesting that topical CAL can both prevent and treat IMQ-induced dermatitis by inhibiting IL-23/IL-17A–mediated pathogenesis.

CAL inhibited the differentiation of IL-23–competent mature Langerhans cells and suppressed IL-23 expression by mature Langerhans cells *ex vivo*

Although the predominant cell source for IL-23 in the setting of IMQ-induced dermatitis is still under debate,[64-66] our previous study has indicated the essential role of IL-23 derived from epidermal Langerhans cells (LCs) in the pathogenesis of this psoriasis model.[65] Consistent with this, mRNA levels of *Il12b* and *Il23a* were markedly increased in the epidermis of IMQ-treated skin. Thus we next explored how topical CAL suppressed IL-23 expression by LCs.

To this end, we first asked whether the CAL-mediated suppression of *Il12b* and *Il23a* expression in the epidermis was due to the direct effects of CAL on epidermal cells rather than being merely secondary to attenuated dermal inflammation. We cultured epidermal sheets separated from untreated murine ear skin in the absence or presence of CAL for 3 days, isolated migratory LCs in the culture media, and assessed their *Il12b* and *Il23a* expression after stimulation (see Fig E4, *A*, in this article's Online Repository at www.jacionline.org). Although most LCs in untreated murine skin were $CD86^{-}MHCII^{low}$, migratory LCs were $CD86^{+}MHCII^{high}$, as reported previously,[67] indicating a spontaneous maturation of LCs during the *ex vivo* culture of epidermal sheets. Migratory LCs from epidermal explants cultured in the absence of CAL upregulated expression of *Il12b* but not *Il23a* after stimulation with IMQ alone (see Fig E5 in this article's Online Repository at www.jacionline.org), suggesting that LCs require additional signals for their IL-23 expression. Although *in vivo* signals that induce LCs to express IL-23 in mice with IMQ-induced dermatitis remain unknown, we found that *in vitro* stimulation with the combination of IMQ and agonistic antibodies against the costimulatory molecule CD40 induced LCs to upregulate both *Il12b* and *Il23a* (Fig 4, *A*), as previously reported in DCs.[68] By contrast, migratory LCs from epidermal explants cultured in the presence of CAL did not upregulate *Il12b* and *Il23a* in response to IMQ plus CD40 antibodies (Fig 4, *A*). This defect was due to neither insufficient activation nor attenuated CD40 cross-linking because CAL-treated LCs expressed CD86, CD80, and CD40 at equivalent amounts as control LCs (data not shown). These results suggest that CAL acts on epidermal cells, such as LCs and keratinocytes, to inhibit immature LCs from gaining IL-23 competency during their maturation.

Next, we asked whether CAL can also suppress IL-23 expression by already matured IL-23–competent LCs. Migratory LCs isolated from epidermal explants were cultured in the presence or absence of CAL for 29 hours and stimulated for the last 5 hours of culture with IMQ and CD40 antibodies (see Fig E4, *B*). Expression of *Il12b* and *Il23a*, but not *Il12a*, was induced in mock-treated LCs by IMQ and CD40 antibodies, although IL-23 protein concentrations in the culture supernatant were less than the limit of detection of ELISA (Fig 4, *B*). Induction of *Il12b* and *Il23a* expression was significantly suppressed in CAL-treated LCs compared with mock-treated LCs (Fig 4, *B*). Induction of *Il6* was also suppressed in CAL-treated LCs, whereas that of *Tnf* was not, excluding that CAL-treated LCs have a general defect in proinflammatory responses (see Fig E6 in this article's Online Repository at www.jacionline.org).

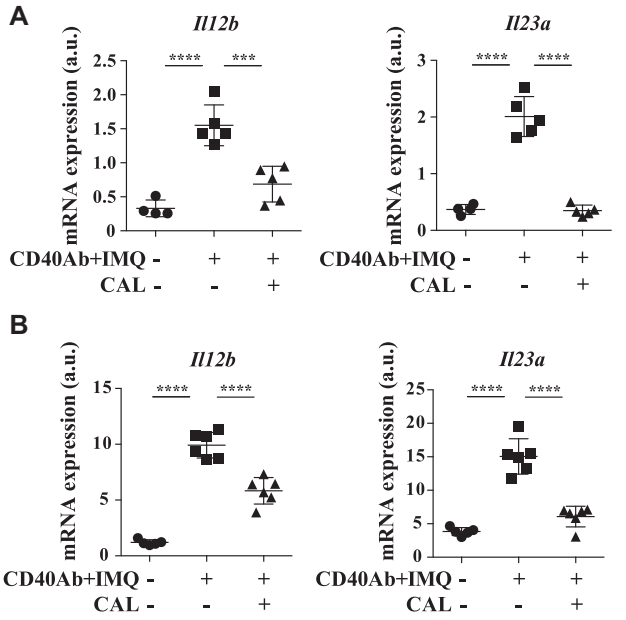

FIG 4. Effect of CAL on IL-23 expression by primary murine LCs. **A,** Migratory LCs from epidermal explant cultures in the presence or absence of CAL were stimulated with IMQ with or without CD40 antibodies for 5 hours. **B,** Migratory LCs from epidermal explant cultures were cultured in the presence or absence of CAL for 29 hours and stimulated with IMQ and CD40 antibodies for the last 5 hours of culture. Expression levels of *Il12b* and *Il23a* in stimulated LCs were measured. Data are shown as each value *(symbols)* and their means ± SDs *(bars)*. *a.u.*, Arbitrary units. ***$P < .001$ and ****$P < .0001$.

Taken together, we established an *in vitro* assay for assessing IL-23 expression by murine LCs, which revealed that CAL can inhibit the differentiation of IL-23–competent mature LCs by acting on epidermal cells and also can suppress IL-23 expression by mature LCs directly.

Topical CAL application on IMQ-treated skin resulted in impaired expansion of T17 cells in dLNs

Several human studies have suggested a pathogenic role of CCR6-expressing T17 cells that infiltrate from the blood into psoriatic skin lesions, where CCL20, a ligand for CCR6, is highly expressed.[4,69,70] In the IMQ-induced murine psoriasis model, it has been reported that the $CCR6^{+}V\gamma4^{+}$ γδ T17 cell population expands considerably in dLNs of IMQ-treated skin and homes to inflamed skin, which can accelerate psoriasis-like dermatitis.[45] Thus we next examined whether topical CAL application could control the IMQ-induced expansion of this pathogenic T-cell population in the dLNs. $CCR6^{+}V\gamma4^{+}$ γδ T17 cells markedly expanded in the dLNs of the IMQ/EtOH-treated ear, as previously reported,[65,71] whereas increases in the numbers of αβ T17 and $V\gamma4^{-}$ γδ T17 cell populations were minimal (Fig 5). Expansion of the $CCR6^{+}V\gamma4^{+}$ γδ T17 cell population was much less vigorous in IMQ/CAL-treated mice compared with that in IMQ/EtOH-treated mice (Fig 5).

These results suggest that topical CAL application on IMQ-treated skin can inhibit the expansion of the $CCR6^{+}V\gamma4^{+}$ γδ T17 cell population in dLNs, which would contribute to the CAL-mediated amelioration seen in the setting of IMQ-induced dermatitis.

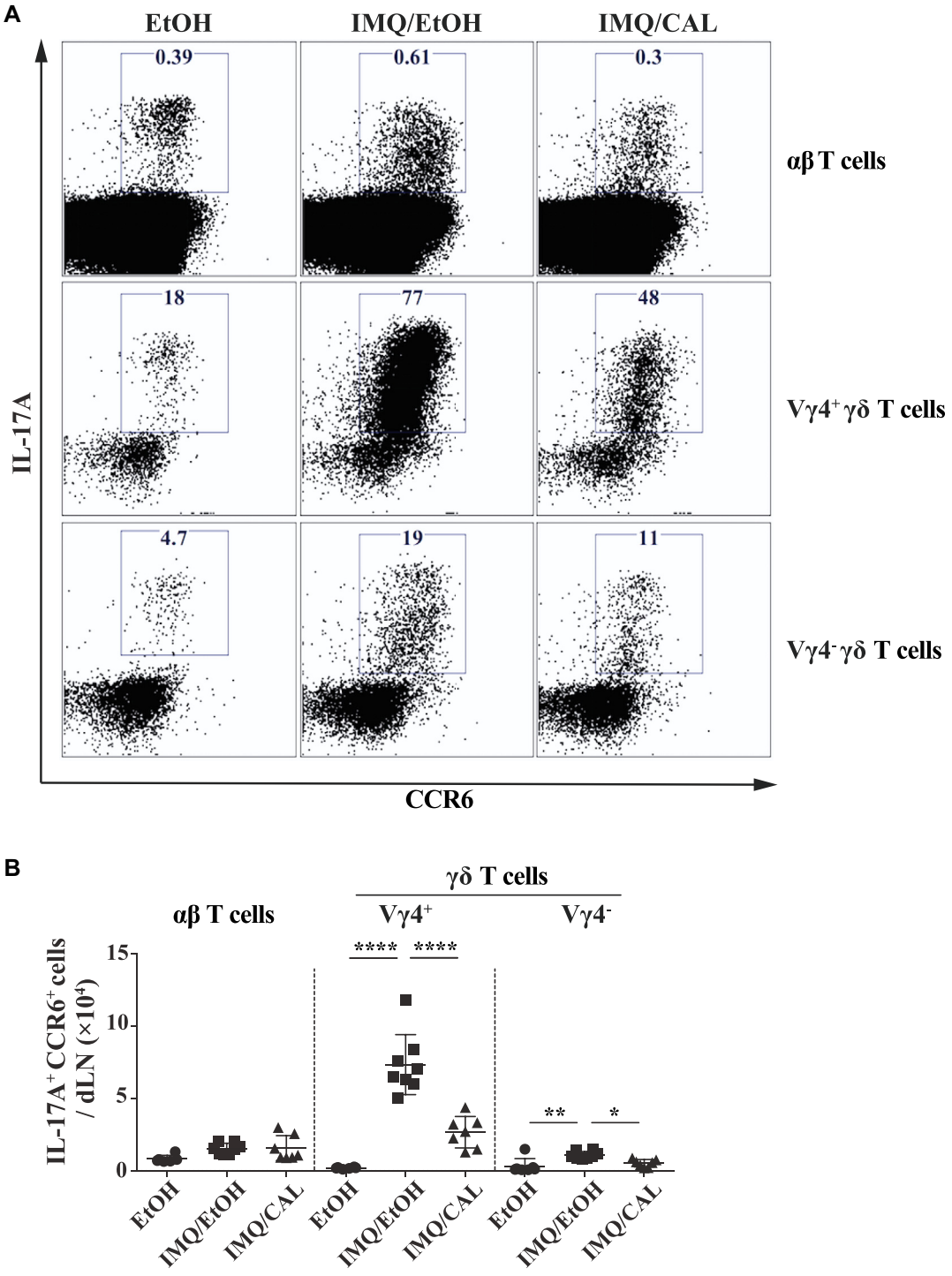

FIG 5. Topical CAL application suppressed CCR6$^+$ T17 cell expression in dLNs. Flow cytometric analysis of dLN cells is shown. **A,** IL-17A and CCR6 expression by various T-cell subsets. **B,** Numbers of CCR6$^+$ T17 cells in dLNs. All samples were collected 24 hours after 6 applications of the indicated chemicals. Data are shown as each value *(symbols)* and their means ± SDs *(bars)*. *$P < .05$, **$P < .01$, and ****$P < .0001$.

Topical CAL application on IMQ-treated skin showed a concomitant suppressive effect on psoriasis-like inflammation at a distant site

It has been shown that Vγ4$^+$ γδ T17 cells expanding in the dLNs of one psoriasis-like skin lesion can not only home to the initiating site of skin inflammation but can also accumulate in a distant site, where they can accelerate the development of psoriasis-like skin inflammation.[72,73] Thus we reasoned that CAL-mediated inhibition of T17 cell expansion in the dLNs could lead to amelioration of psoriasis-like skin inflammation at a distant site. To test this hypothesis, mice that had been pretreated with EtOH, CAL, IMQ/EtOH, or IMQ/CAL for 5 days on the left

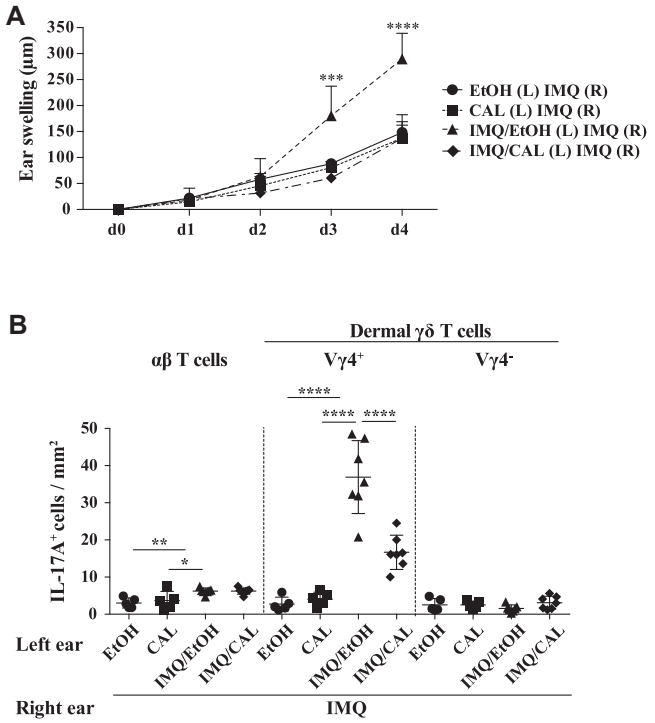

FIG 6. Topical CAL application on IMQ-treated left ears resulted in a concomitant amelioration of psoriasis-like inflammation in right ears. Mice were treated as indicated on the left *(L)* ear for 5 days (day −5 to day −1), followed by IMQ treatment of the right *(R)* ear for 4 days (day 0 to day 3). **A,** Kinetics of right ear swelling. **B,** Number of T17 cells in right ears at day 4. Data are shown as each value *(symbols)* and their means ± SDs *(bars)*. *$P < .05$, **$P < .01$, ***$P < .001$, and ****$P < .0001$.

ear were treated with IMQ for 4 days on the right ear, and IMQ-induced dermatitis on the right ear was assessed (see Fig E7 in this article's Online Repository at www.jacionline.org). Mice pretreated with IMQ/EtOH on the left ear exhibited more rapid swelling of the right ear after IMQ treatment compared with EtOH-pretreated mice (Fig 6, *A*). In addition, the more severe swelling of the right ear observed in IMQ/EtOH-pretreated mice was associated with markedly increased accumulation of $V\gamma4^+$ γδ T17 cells (Fig 6, *B*), suggesting exacerbation of T17 cell–mediated psoriasis-like inflammation. By contrast, mice pretreated with IMQ/CAL on the left ear showed significantly attenuated swelling of the IMQ-treated right ear and decreased accumulation of $V\gamma4^+$ γδ T17 cells compared with IMQ/EtOH-pretreated mice (Fig 6, *A* and *B*), whereas expression levels of *Il23a* and *Il12b* were equivalent (see Fig E8 in this article's Online Repository at www.jacionline.org). Pretreatment on the left ear with CAL alone did not ameliorate IMQ-induced dermatitis and $V\gamma4^+$ γδ T17 cell accumulation on the right ear (Fig 6), further confirming that the concomitant suppressive effect of CAL on psoriasis-like dermatitis at a distant site was not due to systemic immunosuppression by CAL absorbed from the left ear skin into the circulation.

DISCUSSION

The present study provides *in vivo* evidence that the topical vitamin D analogue CAL can inhibit the IL-17A–mediated pathogenesis of psoriasis by attenuating accumulation of pathogenic T17 cells not only in lesional skin but also in dLNs. The CAL-mediated inhibition of T17 cell accumulation in human psoriatic lesions has recently been implicated by analyses using the *ex vivo* expansion of skin-infiltrating T cells with anti-CD3/CD28 antibodies and IL-2.[74] Our results obtained by means of unbiased identification of T17 cells in skin corroborate the *in vivo* suppressive effect of topical CAL on T17 cell accumulation in psoriatic lesions. In addition, we found that topical CAL application suppresses the expansion of T17 cells in the dLNs and leads to concomitant amelioration of psoriasis-like inflammation at a distant untreated site. These results suggest that topical CAL treatment has the potential to prevent the development, exacerbation, and recurrence of psoriatic lesions, even at untreated sites, by reducing the systemic burden of pathogenic T17 cells.

Sun and colleagues[75] have assessed the effect of CAL on IMQ-induced psoriasis-like skin inflammation and observed that CAL treatment did not inhibit but rather exacerbated swelling and inflammation of the IMQ-treated ear. In their study mice were treated twice daily with 40 mg/cm^2 of 0.005% CAL ointment (about 4.85 nmol on each ear). Given that mice treated once daily with 2 to 4 nmol of CAL on each ear have significant ear swelling and atopic dermatitis–like skin inflammation,[50,76,77] it is highly likely that CAL-induced skin inflammation compensated the inhibitory effect of CAL on IMQ-induced psoriasis-like inflammation. By contrast, in this study we treated mice once daily with a much lower amount (0.2 nmol on each ear) of CAL to avoid development of CAL-induced skin inflammation and observed a significant inhibitory effect on IMQ-induced psoriasis-like inflammation.

Our results suggest that the CAL-mediated inhibition of T17-mediated pathogenesis is significantly due to the attenuated IL-23 expression by lesional skin DCs, LCs in particular, in the setting of IMQ-induced psoriasis-like dermatitis. *Ex vivo* experiments revealed that CAL can suppress IL-23 expression in LCs through at least 2 different mechanisms. One mechanism involves inhibiting immature LCs from gaining IL-23 competency during their maturation by acting on epidermal cells, such as keratinocytes, intraepidermal T cells, and LCs. Another mechanism is through direct inhibition of IL-23 expression by IL-23–competent mature LCs. Given the previous report[78] that calcitriol-mediated IL-12 inhibition involves binding of the vitamin D receptor/retinoid X receptor complex to the nuclear factor κB site in the promoter of *Il12b*, this mechanism likely underlies the inhibition of IL-23 expression by CAL. In addition to the effect on IL-23 expression, the suppressive effect of CAL on IL-17A production from human T cells *ex vivo* stimulated with anti-CD3 antibodies has been previously described.[74] It is possible that this suppressive activity can also be associated with the antipsoriatic effect of CAL. Thus topical CAL acts through multiple processes to inhibit the T17-mediated pathogenesis of psoriasis.

We have found that topical CAL inhibited T17 cell expansion in the dLNs and ameliorated psoriasis-like inflammation at a distant untreated site. These results suggest that topical CAL not only provides local symptomatic benefit but can also contribute to better control of systemic disease activities in patients with psoriasis by reducing the systemic burden of pathogenic T17 cells. We hypothesized that CAL-mediated inhibition of T17 cell expansion in dLNs is attributed to the attenuated migration of LCs and DCs from the skin to the dLNs, their impaired IL-23 expression, or both. However, the IMQ-induced increase in the number of $CD11c^+MHCII^{high}$ migratory LCs and

DCs in the dLNs was not affected by topical CAL treatment (data not shown). mRNA amounts of *Il12b* and *Il23a* were less than the detection limit of quantitative RT-PCR, even in the dLNs of IMQ-treated skin (data not shown). Thus the mechanism underlying the CAL-mediated inhibition of the T17 cell expansion in dLNs remains to be addressed in future research.

In conclusion, the results of this study provide the immunologic underpinnings of topical vitamin D analogues in the treatment of psoriasis.

We thank Dr Yo Kaku for reading histologic slides and Dr Yosuke Yamamoto for suggestions on statistical analyses.

Key messages

- **Topical CAL application inhibits T17 cell accumulation and IL-23 expression in the lesional skin of mice with psoriasis-like dermatitis.**
- **Topical CAL application also inhibits T17 cell expansion in the dLNs of mice with psoriasis-like dermatitis.**
- **Topical CAL application results in a concomitant amelioration of psoriasis-like dermatitis at a distant untreated site in a murine psoriasis model.**

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METHODS

Mice

C57BL/6N (B6) mice were purchased from Japan SLC (Shizuoka, Japan) and maintained under specific pathogen-free conditions in the Institute of Laboratory Animals at Kyoto University Graduate School of Medicine. Six- to 10-week-old female B6 mice were used for all experiments. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Kyoto University Graduate School of Medicine.

Antibodies

Fluorescein isothiocyanate anti–T-cell receptor (TCR) β (H57-597), Pacific Blue anti–I-A/I-E (M5/114.15.2), phycoerythrin (PE)-Cy7 anti–epithelial cell adhesion molecule (G8.8), Brilliant Violet 421 anti-TCRγ/δ (GL3), Brilliant Violet 605 anti-CD11c (N418), and PE anti-CD11c (N418) antibodies were purchased from BioLegend (San Diego, Calif). V450 anti-CD11b (M1/70), PE anti-Vγ4–TCR (UC3), allophycocyanin anti-CCR6 (140706), and rat anti-mouse CD16/CD32 (2.4G) antibodies were purchased from BD Biosciences (San Diego, Calif). Peridinin-chlorophyll-protein complex–Cyanine5.5 anti-CD45 (30-F11), PE-Cyanine7 anti–IL-17A (eBio17B7), allophycocyanin anti–IL-22 (IL22JOP), fluorescein isothiocyanate anti-CD40 (HM40-3), PE anti-CD80 (16-10A1), and PE anti-CD86 (GL1) antibodies were purchased from eBioscience (San Diego, Calif). Anti-mouse MHC class II microbeads were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany).

Flow cytometry

Single-cell suspensions from skin were prepared for flow cytometric analysis, as described previously.[E1] Briefly, ear samples were collected as 8-mm skin biopsy specimens and separated into dorsal and ventral sides. They were cut into pieces and then digested for 90 minutes at 37°C in complete RPMI containing 1000 U/mL collagenase type II (Worthington Biochemical, Freehold, NJ) and 0.3 mg/mL DNase I (Sigma-Aldrich, St Louis, Mo). Samples were passed through a 40-μm pore-size cell strainer to obtain single-cell suspensions. Cells were stained with fixable viability dye eFluor 780 (eBioscience), according to the manufacturer's instructions, for exclusion of dead cells. Next, nonspecific antibody binding was blocked with anti-CD16/32 antibody (BD Biosciences), and then cells were stained for surface antigens. For intracellular staining of IL-17A and IL-22, single-cell suspensions of ear and dLNs were stimulated for 2 hours with 50 ng/mL phorbol 12-myristate 13-acetate (Sigma-Aldrich) and 1 μmol/L ionomycin (Wako, Tokyo, Japan) in the presence of GolgiStop (BD Biosciences). After surface staining, cells were fixed and permeabilized with Cytofix/Cytoperm solution (BD Biosciences) and stained intracellularly. Samples were acquired on a BD LSRFortessa cell analyzer (BD Biosciences) and analyzed with FlowJo software (TreeStar, San Carlos, Calif). Numbers of each cell subset were calculated by means of flow cytometry and presented as numbers per square millimeter of skin surface.

Histology and immunohistochemistry

For histologic examination, ear skin samples were fixed with 10% formalin and then embedded in paraffin. Sections along the median plane of the ear with a thickness of 5 μm were prepared and subjected to staining with hematoxylin and eosin. For quantification of epidermal thickness, images of 2 consecutive microscopic fields of hematoxylin and eosin–stained sections were obtained by using a digital microscope (BIOREVO BZ-9000; Keyence, Osaka, Japan) with a ×10 magnification lens. Epidermal thickness was calculated by averaging the distances between the stratum corneum and the bottom of the basal layer measured at 10 locations at 0.5-mm intervals in each image by using ImageJ software (National Institutes of Health, Bethesda, Md). Quantification was performed by a dermatopathologist blind to the experimental groups analyzed.

For immunohistochemical staining of Ki-67, sections were deparaffinized in xylene and rehydrated by washing sections in graded alcohol series. Sections were incubated in 10 mmol/L citric acid (pH 6) at 95°C for 30 minutes for antigen retrieval. Sections were blocked with normal goat serum for 60 minutes at room temperature, followed by incubation with mouse anti-Ki67 antibodies (MM1; Leica Biosystems, Newcastle, United Kingdom) overnight at 4°C. Samples were washed and incubated for 40 minutes with biotinylated goat anti-mouse IgG antibodies (Vector Laboratories, Burlingame, Calif). Next, the sections were incubated with Vectastatin ABC reagent (Vector Laboratories) for 30 minutes. The sections were then incubated with 3,3′-diaminobenzidine substrate (Vector Laboratories) for 15 minutes. Finally, the sections were counterstained with hematoxylin solution. Images were captured with a digital microscope (BIOREVO BZ-9000; Keyence, Itasca, Ill).

Quantitative RT-PCR analysis

Total RNA was isolated with TRIzol reagent (Invitrogen, Grand island, NY) and RNeasy kits (Qiagen, Hilden, Germany) and digested with DNase I (Qiagen). cDNA was reverse transcribed from total RNA samples by using a Prime Script RT reagent kit (Takara Bio, Otsu, Japan). Quantitative RT-PCR was performed by monitoring the synthesis of double-stranded DNA during the various PCR cycles with SYBR Green I (Roche, Basel, Switzerland) and the LightCycler real-time PCR apparatus (Roche), according to the manufacturer's instructions. All primers were obtained from Greiner Japan (Tokyo, Japan). The primer sequences were as follows: *Gapdh*, 5′-AGG TCG GTG TGA ACG GAT TTG-3′ (forward) and 5′-GGG GTC GTT GAT GGC AAC A-3′ (reverse); *Il4*, 5′-GGT CTC AAC CCC CAG CTA GT-3′ (forward) and 5′-GCC GAT GAT CTC TCT CAA GTG AT-3′ (reverse); *Il6*, 5′-TCT ATA CCA CTT CAC AAG TCG GA-3′ (forward) and 5′-GAA TTG CCA TTG CAC AAC TCT TT-3′ (reverse); *Il17a*, 5′-CTC CAG AAG GCC CTC AGA CTA C-3′ (forward) and 5′-GGG TCT TCA TTG CGG TGG-3′ (reverse); *Il22*, 5′-ATG AGT TTT TCC CTT ATG GGG AC-3′ (forward) and 5′-GCT GGA AGT TGG ACA CCT CAA-3′ (reverse); *Il12a*, 5′-AGA CAT CAC ACG GGA CCA AAC-3′ (forward) and 5′-CCA GGC AAC TCT CGT TCT TGT-3′ (reverse); *Il12b*, 5′-GGT GTA ACC AGA AAG GTG CG-3′ (forward) and 5′-TAG CGA TCC TGA GCT TGC AC-3′ (reverse); *Il23a*, 5′-AAC TCC TCC AGC CAG AGG ATC A-3′ (forward) and 5′-TCT TGG AAC GGA GAA GGG GG-3′ (reverse); *Ifng*, 5′-ACA GCA AGG CGA AAA AGG ATG-3′ (forward) and 5′-TGG TGG ACC ACT CGG ATG A-3′ (reverse); *Foxp3*, 5′- GGC GAA AGT GGC AGA GAG G-3′ (forward) and 5′-AAG GCA GAG TCA GGA GAA GTT G-3′ (reverse); *Tnf*, 5′-TGC CTA TGT CTC AGC CTC TTC-3′ (forward) and 5′-GAG GCC ATT TGG GAA CTT CT-3′ (reverse); *S100a7*, 5′-GAG GAG TTG AAA GCT CTG CTC TTG-3′ (forward) and 5′-GTG ATG TAG TAT GGC TGC CTG CGG-3′ (reverse); *S100a8*, 5′-AAA TCA CCA TGC CCT CTA CAA G-3′ (forward) and 5′-CCC ACT TTT ATC ACC ATC GCA A-3′ (reverse); *S100a9*, 5′-GCA CAG TTG GCA ACC TTT ATG-3′ (forward) and 5′-TGA TTG TCC TGG TTT GTG TCC-3′ (reverse); and *Defb4*, 5′-CTC CAC TTG CAG CCT TTA CC-3′ (forward) and 5′-ATC TGT CGA AAA GCG GTA GGG-3′ (reverse). The cycling conditions were as follows: initial enzyme activation at 95°C for 10 minutes, followed by 45 cycles at 95°C for 10 seconds and 60°C for 20 seconds. Gene-specific fluorescence was measured at 60°C. For each sample, duplicate test reactions were analyzed for gene expression, and results were normalized to those of the housekeeping *Gapdh* gene.

Preparation of epidermal and dermal samples for RNA extraction and ELISA

Mouse ears were cut off at the base and separated into dorsal and ventral halves by using forceps. The dorsal halves were frozen in liquid nitrogen and used in the experiments. Epidermal samples were taken by shaving the surface of the ear skin with a scalpel in the cryostat at −30°C. After shaving the epidermis, the rest of the ear skin was used as dermal samples.

ELISA

Amounts of IL-12/IL-23p40 in the epidermis were measured by using the ELISA Ready-SET-Go kit (eBioscience), according to the manufacturer's instructions. Epidermal samples were homogenized in 600 μL of PBS. Supernatants were collected for ELISA.

Purification and culture of murine epidermal LCs

LCs were collected, as described previously with modifications.[E2] Briefly, mouse ears were cut and rinsed with 70% EtOH. Ears were separated into dorsal and ventral halves, and cartilage was removed with forceps. Then ears were floated with the dermal side down on RPMI 1640 medium (Sigma-Aldrich) and supplemented with 10% heat-inactivated FCS (Invitrogen, Carlsbad, Calif), 0.05 mmol/L 2-mercaptoethanol, 2 mmol/L L-glutamine, 25 mmol/L N-2-hydroxyethylpiperazine-N9-2-ethanesulfonic acid, 1 mmol/L nonessential amino acids, 1 mmol/L sodium pyruvate, 100 U/mL penicillin, and 100 mg/mL streptomycin (complete RPMI) containing 0.9 U/mL Dispase II (Roche, Mannheim, Germany) overnight at 4°C. Split epidermal sheets were floated on complete RPMI containing 20 ng/mL recombinant mouse GM-CSF (PeproTech, Rocky Hill, NJ) in the presence or absence of 100 nmol/L CAL and incubated for 3 days at 37°C. Epidermal sheets were removed, and supernatants were passed through a 40-μm pore-size cell strainer. LCs were isolated with MHC class II microbeads (Miltenyi Biotec) by using a MiniMACS separator (Miltenyi Biotec). The purity was confirmed to be greater than 90% by using flow cytometry. All isolated LCs were matured, as previously described.[E3] Isolated LCs were seeded at 4×10^5/mL in round-bottom 96-well plates and stimulated with 10 μg/mL IMQ (Tokyo Kasei Industry, Tokyo, Japan) in combination with 1 μg/mL agonistic anti-CD40 antibody (HM40-3; BioLegend) for 5 hours. In some experiments isolated LCs were cultured with or without 100 nmol/L CAL for 29 hours and stimulated for the last 5 hours of culture with IMQ and anti-CD40 antibody.

Statistical analysis

Unless otherwise indicated, results are presented as mean ± SD values and are representative of at least 3 independent experiments. Comparisons were analyzed for statistical significance by using the Student *t* test. Statistical significance between multiple groups was analyzed by using the Tukey-Kramer multiple comparisons test. *P* values of less than .05 were considered significantly different.

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E1. Nakashima C, Otsuka A, Kitoh A, Honda T, Egawa G, Nakajima S, et al. Basophils regulate the recruitment of eosinophils in a murine model of irritant contact dermatitis. J Allergy Clin Immunol 2014;134:100-7.e12.
E2. Larsen CP, Steinman RM, Witmer-Pack M, Hankins DF, Morris PJ, Austyn JM. Migration and maturation of Langerhans cells in skin transplants and explants. J Exp Med 1990;172:1483-93.
E3. Romani N, Holzmann S, Tripp CH, Koch F, Stoitzner P. Langerhans cells–dendritic cells of the epidermis. APMIS 2003;111:725-40.

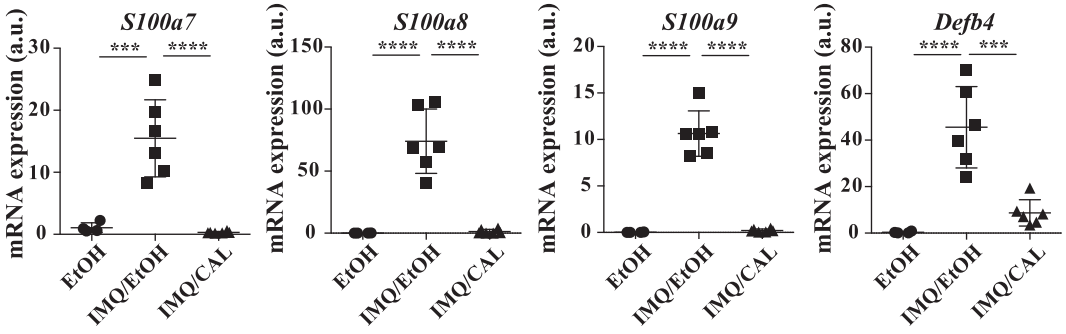

FIG E1. CAL attenuates expression levels of antimicrobial peptides in IMQ-treated ear. Expression levels of *S100a7*, *S100a8*, *S100a9*, and *Defb4* mRNA in the ear treated, as indicated for 4 days, is shown. All samples were collected 24 hours after the fourth treatment. Data are shown as each value *(symbols)* and their means ± SDs *(bars)*. *a.u.*, Arbitrary units. ***$P < .001$ and ****$P < .0001$.

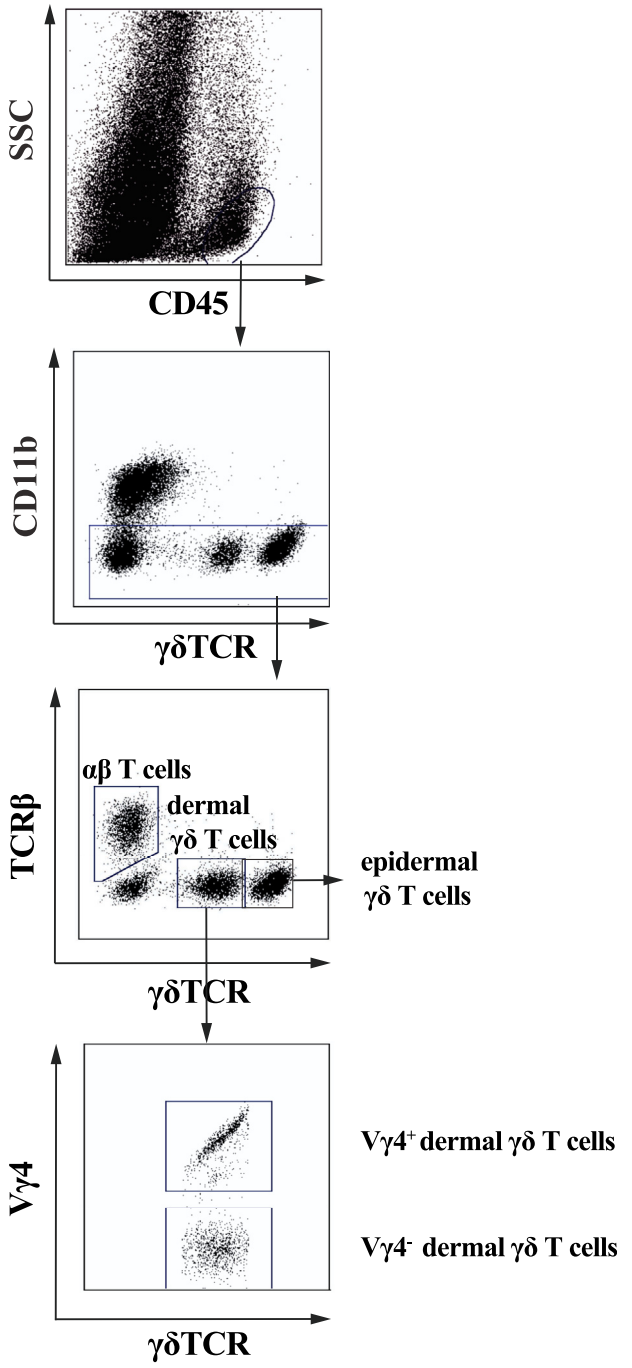

FIG E2. Gating strategy for αβ T cells, dermal $V\gamma4^+$ γδ T cells, and dermal $V\gamma4^-$ γδ T cells in the ear. *SSC*, Side scatter.

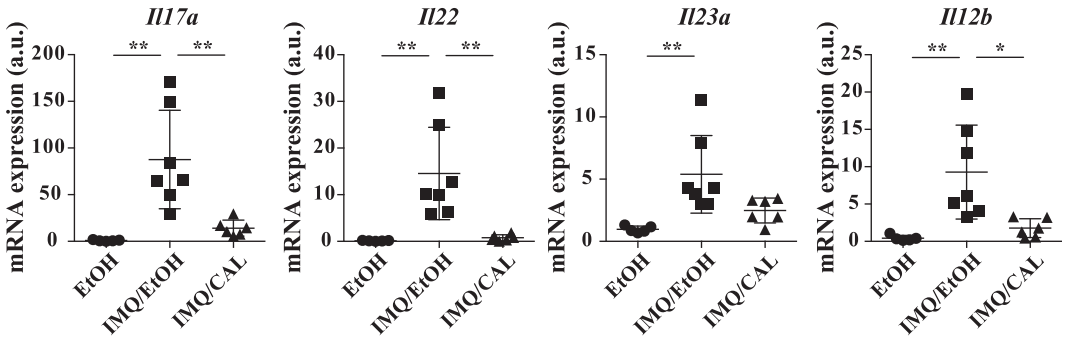

FIG E3. CAL attenuates expression levels of *Il17a*, *Il22*, *Il23a*, and *Il12b* in IMQ-treated ear. Expression levels of *Il17a*, *Il22*, *Il23a*, and *Il12b* mRNA in the ears treated as indicated are shown. CAL application started after 3 applications of IMQ. All samples were collected 24 hours after 6 applications of IMQ. Data are shown as each value *(symbols)* and their means ± SDs *(bars)*. *a.u.*, Arbitrary units. $*P < .05$ and $**P < .01$.

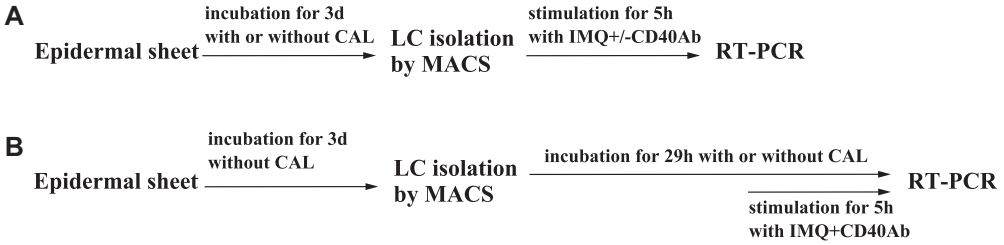

FIG E4. Protocols for LC isolation and *ex vivo* stimulation. **A,** Migratory LCs from epidermal explant cultures in the presence or absence of CAL were stimulated with IMQ with or without CD40 antibodies for 5 hours. **B,** Migratory LCs from epidermal explant cultures were cultured in the presence or absence of CAL for 29 hours and stimulated with IMQ and CD40 antibodies for the last 5 hours of culture. *MACS*, Magnetic cell sorting.

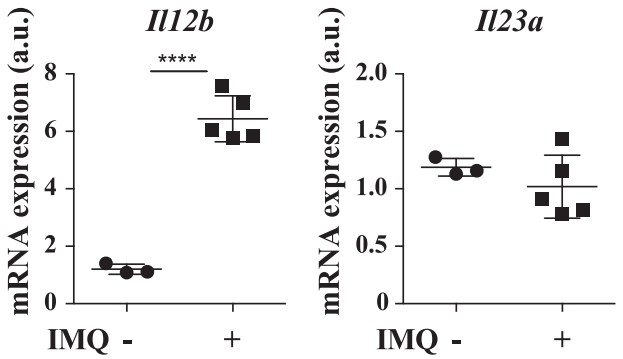

FIG E5. Expression levels of *Il12b* and *Il23a* by LCs *ex vivo* stimulated with IMQ. Migratory LCs from epidermal explant cultures were stimulated with IMQ for 4 hours. Data are shown as each value *(symbols)* and their means ± SDs. *a.u.*, Arbitrary units. ****$P < .0001$.

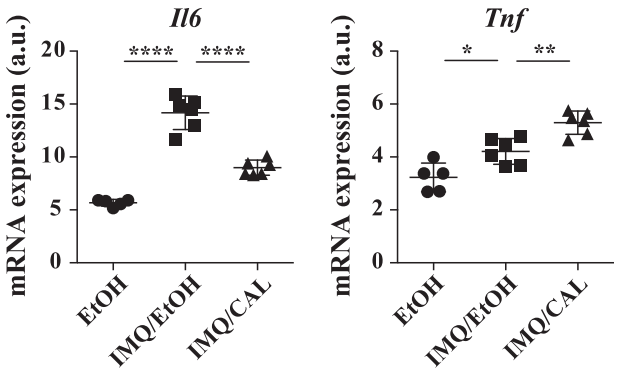

FIG E6. Effect of CAL on *Il6* and *Tnf* expression by primary murine LCs. Migratory LCs from epidermal explant cultures were cultured in the presence or absence of CAL for 29 hours and stimulated with IMQ and CD40 antibodies for the last 5 hours of culture. Expression levels of *Il6* and *Tnf* in stimulated LCs were measured. Data are shown as each value *(symbols)* and their means ± SDs *(bars)*. *a.u.*, Arbitrary units. **P* < .05, ***P* < .01, and *****P* < .0001.

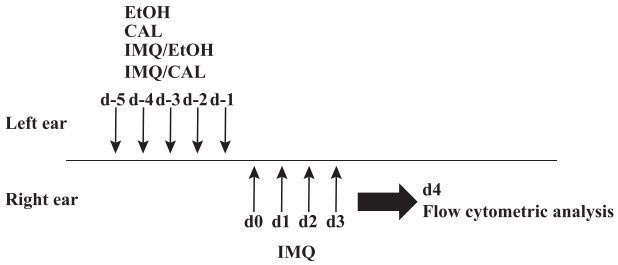

FIG E7. Experimental protocol for investigating the effect of topical CAL application to IMQ-treated left ear on IMQ-induced dermatitis in the right ear. Mice were treated with either EtOH, CAL, IMQ/EtOH, or IMQ/CAL on the left ear for 5 days (day −5 to day −1), followed by IMQ treatment of the right ear for 4 days (day 0 to day 3). The right ear was collected 24 hours after the IMQ application at day 3 and subjected to flow cytometric analysis.

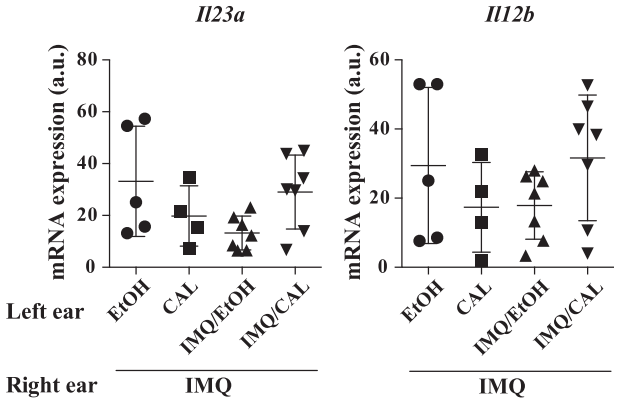

FIG E8. Expression levels of *Il23a* and *Il12b* in the right ear treated with IMQ. Mice were treated with EtOH, CAL, IMQ/EtOH, or IMQ/CAL on the left ear for 5 days (day −5 to day −1), followed by IMQ treatment of the right ear for 4 days (day 0 to day 3). The right ear was collected 24 hours after the 4 time IMQ application. Data are shown as each value *(symbols)* and their means ± SDs.