

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

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29 **ABSTRACT**

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

33 Objective: To clarify whether and how the topical vitamin D analogue calcipotriol
34 (CAL) controls the interleukin-17A-mediated pathogenesis of murine psoriasis-like
35 dermatitis *in vivo*.

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

40 Results: Mice treated topically with CAL exhibited much milder IMQ-induced
41 psoriasis-like dermatitis compared to vehicle-treated mice, with impaired accumulation
42 of interleukin-17A-committed T (T17) cells in the lesional skin. The IMQ-induced
43 upregulation of *Il12b* and *Il23a* was marked in the epidermis and was abrogated by
44 CAL application, suggesting the CAL-mediated suppression of interleukin-23
45 expression. CAL inhibited *Il12b* and *Il23a* expression by Langerhans cells *ex vivo*
46 stimulated with IMQ and CD40 crosslinking. Topical CAL also inhibited T17 cell
47 expansion in the draining lymph nodes of the IMQ-treated skin, implying a possible
48 impact on T17 cell-mediated dermatitis at distant sites. In fact, topical CAL application
49 on the IMQ-treated left ear resulted in an amelioration of T17 cell accumulation and
50 psoriasis-like dermatitis in the right ear subsequently treated with IMQ.

Conclusion: Topical CAL can exert its anti-psoriatic effect on CAL-treated lesions and, concomitantly, distant lesions by attenuating the T17 cell accumulation in both CAL-treated lesions and the draining lymph nodes.

Key messages

- Topical CAL application inhibits the T17 cell accumulation and IL-23 expression in the lesional skin of murine psoriasis-like dermatitis.

- Topical CAL application also inhibits T17 cell expansion in the draining lymph nodes of 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.

Capsule Summary

The topical vitamin D analogue calcipotriol ameliorates psoriasis-like dermatitis at the treated site and, concomitantly, at a distant site by attenuating the IL-17A-committed T-cell accumulation in both the treated skin and the draining lymph nodes.

Key Words:

psoriasis, calcipotriol, vitamin D analogue, IL-17A, IL-23, imiquimod

Abbreviations:

CAL, calcipotriol; CCL, CC chemokine ligand; CXCL, C-X-C motif ligand; dLN,

- 73 draining lymph node; DC, dendritic cell; ELISA, enzyme-linked immunosorbent assay;
- 74 EtOH, ethanol; IL, interleukin; IMQ, imiquimod; LC, Langerhans cell; T17,
- 75 IL-17A-committed T

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 pathological crosstalk 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 interleukin (IL)-8, C-X-C motif ligand (CXCL) 1, CC chemokine ligand (CCL) 20, and S100A proteins.^{1,3} Conversely, activated immune cells that accumulate in the psoriatic skin form a cytokine milieu that induce psoriatic keratinocyte phenotypes. Among various pro-psoriatic cytokines, the immunological 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 psoriatic skin appearance. Understanding how current treatments inhibit the pathogenic processes of psoriasis is of theoretical as well as practical importance in improving the management of this disease.

Vitamin D analogues such as calcipotriol, 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 symptoms⁹⁻¹⁵. They are currently regarded as one of first-line treatments

in the guidelines for the management of psoriasis.¹⁶⁻¹⁸ The precise mechanism by which topical vitamin D analogues exert their anti-psoriatic effect *in vivo*, however, remains unknown. The biological effects of the active vitamin D metabolite, 1 α ,25-dihydroxyvitamin D₃ (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 may 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 monocytes²⁷⁻³⁴ 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 the attenuated expression of the 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 anti-psoriatic effect of topical vitamin D analogues may 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 human 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 topical vitamin D analogue CAL controls the IL-17A-mediated pathogenesis of psoriasis *in vivo* by utilizing 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 the T17 cell accumulation in not only CAL-treated lesions but also the 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 IMQ-induced dermatitis, mice were treated daily with 0.2 nmol of CAL (Tocris Bioscience, Bristol, United Kingdom) dissolved in 20 μ L of ethanol (EtOH) on the ear 15 min before IMQ application.

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 utilized an IMQ-induced murine psoriasis-like dermatitis model since 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 following either CAL (IMQ/CAL) or vehicle (IMQ/EtOH) on the ears for six consecutive days. Since the topical application of CAL on murine skin at the same concentration as in the clinically used CAL lotion (about 121 μ M) induced atopic dermatitis-like skin inflammation,⁵⁰ CAL was applied at a lower concentration (10 μ M) 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 to IMQ/EtOH-treated mice. Histological examination revealed that topical CAL application substantially attenuated epidermal hyperplasia, one of the histological

hallmarks of psoriatic skin lesions, in the IMQ-treated skin (Fig 1, C, upper panels and D). In addition, significantly smaller proportion of epidermal keratinocytes in the IMQ/CAL-treated skin were positive for the proliferation marker Ki-67 compared to those in the 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 IMQ-induced dermatitis (Fig 1, C, upper panels), which was further confirmed by the flow cytometric analyses of CD45⁺ leukocyte number (Fig 1, E).

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

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

By using these experimental settings, we examined whether topical CAL application inhibited the IL-17A-mediated pathogenesis of psoriasis-like dermatitis. Quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis revealed that, although the mRNA expression of *Il17a* was induced in the IMQ-treated skin, IMQ-induced *Il17a* 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 the development of the psoriatic phenotype in mice as well as in humans,^{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 whose orthologues are known to be over-expressed in human psoriatic skin and induced in keratinocytes stimulated with IL-17A^{55,56} compared to 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 γ 4⁺ subset of dermal $\gamma\delta$ T cells, but not in the $\alpha\beta$ T cell and V γ 4⁻ dermal $\gamma\delta$ 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 the previous reports.^{47,57} The IMQ-induced accumulation of V γ 4⁺ $\gamma\delta$ 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 the accumulation of pathogenic T17 cells in the lesional skin.

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

Previous studies have revealed that the expansion of T17 cells is promoted by IL-23 in both mice⁵⁸⁻⁶⁰ and humans.⁶¹⁻⁶³ Thus, we hypothesized that the CAL-mediated inhibition of T17 cell accumulation in the lesional skin of IMQ-induced dermatitis was

attributed to attenuated IL-23 expression. Quantitative RT-PCR analysis revealed that the mRNA expression of *Il12b* and *Il23a* genes, which encode the IL-12/23p40 and IL-23p19 subunit of IL-23, respectively, was markedly increased in the epidermis of IMQ/EtOH-treated skin compared to the 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 the expression of IL-23, but not IL-12, was upregulated in the epidermis of IMQ-treated skin. *Il12b* and *Il23a* expression in the epidermis was significantly lower in IMQ/CAL-treated skin than in IMQ/EtOH-treated skin (Fig 3, A). The IMQ-induced upregulation of *Il12b* and *Il23a* was also observed in the dermis, though much less markedly, and was suppressed by CAL application (Fig 3, A). An enzyme-linked immunosorbent assay (ELISA) revealed that IMQ-induced expression of IL-12/23p40 protein in the epidermis was significantly inhibited by CAL (Fig 3, B), corroborating that IL-23 expression in IMQ-induced dermatitis was suppressed by CAL application. Given the previous findings that induction of the *Il17a* expression, T17 cell accumulation, and 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 substantially contribute to the attenuation of the T17 cell accumulation in IMQ-treated skin.

To further confirm the inhibitory effect of CAL on the 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 the expression levels of *Il17a*, *Il22*, *Il23a*, and *Il12b* at day 6. The 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 the IL-23/IL-17A-mediated pathogenesis.

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

Although the predominant cell source for IL-23 in IMQ-induced dermatitis is still under debate,⁶⁴⁻⁶⁶ our previous study has indicated the essential role of IL-23 derived from epidermal 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 the 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 following 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 the expression of *Il12b*, but not of *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 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 failed to upregulate *Il12b* and *Il23a* in response to IMQ plus CD40 antibodies (Fig 4, A). This defect was due neither to insufficient activation nor to attenuated CD40 crosslinking since 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 h and stimulated for the last 5 h of culture with IMQ and CD40 antibodies (see Fig E4, B in this article's Online Repository at www.jacionline.org). Expression of *Il12b* and *Il23a*, but not of *Il12a*, was induced in mock-treated LCs by IMQ and CD40 antibodies although the concentrations of IL-23 protein in the culture supernatant were under the limit of detection of ELISA (Fig 4, B and data not shown). Induction of *Il12b* and *Il23a* expression was significantly

suppressed in CAL-treated LCs compared to mock-treated LCs (Fig 4, *B*). Induction of *Il6* was also suppressed in CAL-treated LCs, while 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).

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 can also suppress IL-23 expression by mature LCs directly.

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

Several human studies have suggested the pathogenic role of CC chemokine receptor (CCR) 6-expressing T17 cells that infiltrate from blood into psoriatic skin lesions, where CCL20, a ligand for CCR6, is highly expressed.^{4,69,70} It has been reported that, in the IMQ-induced murine psoriasis model, the CCR6⁺V γ 4⁺ $\gamma\delta$ T17 cell population expands considerably in the dLNs of IMQ-treated skin and homes to the 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 γ 4⁺ $\gamma\delta$ T17 cells markedly expanded in the dLNs of the IMQ/EtOH-treated ear, as previously reported,^{65,71} whereas the increases in the number of $\alpha\beta$ T17 and V γ 4⁻ $\gamma\delta$ T17 cell populations were minimal (Fig 5, A and B). Expansion of the CCR6⁺V γ 4⁺ $\gamma\delta$ T17 cell population was much less

vigorous in IMQ/CAL-treated mice compared to IMQ/EtOH-treated mice (Fig 5, A and B).

These results suggest that topical CAL application on the IMQ-treated skin can inhibit the expansion of the CCR6⁺V γ 4⁺ $\gamma\delta$ T17 cell population in the dLNs, which would contribute to the CAL-mediated amelioration of IMQ-induced dermatitis.

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 V γ 4⁺ $\gamma\delta$ 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 the CAL-mediated inhibition of T17 cell expansion in the dLNs could lead to the 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 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 following IMQ treatment, compared to EtOH-pretreated mice (Fig 6, A). In addition, the more severe swelling of the right ear that was observed in IMQ/EtOH-pretreated mice was associated with markedly increased accumulation of V γ 4⁺ $\gamma\delta$ T17 cells (Fig 6, B), suggesting the 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 $V\gamma 4^+ \gamma\delta$ T17 cells compared to IMQ/EtOH-pretreated mice (Fig 6, A and B) while 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 $V\gamma 4^+ \gamma\delta$ T17 cell accumulation on the right ear (Fig 6, A and B), 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 topical vitamin D analogue CAL can inhibit the IL-17A-mediated pathogenesis of psoriasis by attenuating the accumulation of pathogenic T17 cells not only in the lesional skin but also in the 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 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 a 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 have assessed the effect of CAL on IMQ-induced psoriasis-like skin inflammation and observed that CAL treatment did not inhibit, or rather exacerbated, swelling and inflammation of the IMQ-treated ear. In their study, mice were treated twice daily with 40 mg/cm² 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 develop significant ear swelling and atopic dermatitis-like skin inflammation,^{50,75,76} 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 the 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 the T17-mediated pathogenesis is significantly due to the attenuated IL-23 expression by lesional skin DCs, LCs in particular, in IMQ-induced psoriasis-like dermatitis. *Ex vivo* experiments revealed that CAL can suppress IL-23 expression in LCs by at least two 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 the direct inhibition of IL-23 expression by IL-23-competent mature LCs. Given the previous report⁷⁷ that

calcitriol-mediated IL-12 inhibition involves binding of the vitamin D receptor/retinoid X receptor complex to the NF-kappaB 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 may also be associated with the anti-psoriatic effect of CAL. Thus, topical CAL acts through multiple processes to inhibit the T17-mediated pathogenesis of psoriasis.

We have found that topical CAL inhibits the T17 cell expansion in the dLNs and ameliorates 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 a better control of the systemic disease activities in psoriasis by reducing the systemic burden of pathogenic T17 cells. We hypothesized that the CAL-mediated inhibition of the T17 cell expansion in the dLNs is attributed to the attenuated migration of LCs and DCs from the skin to the dLNs and/or their impaired IL-23 expression. 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 below 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 the dLNs remains to be addressed in future research.

In conclusion, the results of this study provide the immunological underpinnings of

384 topical vitamin D analogues in the treatment of psoriasis.

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Figure Legends

Figure 1. The effect of topical CAL on IMQ-induced psoriasis-like dermatitis.

A, Kinetics of ear swelling. B, Phenotypical appearances. C, Hematoxylin and eosin staining and immunohistochemistry of Ki-67. Scale bars = 100 μ m. D, Epidermal thickness. E, CD45⁺ cell number. All samples were collected 24 h after 6 applications of the indicated chemicals. Data are shown as each value (symbols) and their means $\pm$ standard deviations (bars). **** P < .0001.

Figure 2. The 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- (C) and IL-22- (D) committed cells in the lesional ear skin. All samples were collected 24 h after 6 applications of the indicated chemicals. Data are shown as each value (symbols) and their means $\pm$ standard deviations (bars). a.u., arbitrary unit. * P < .05. ** P < .01. **** P < .0001.

Figure 3. The effect of topical CAL on the production of IL-23 in the epidermis and dermis of the 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 in the lesional epidermis by ELISA. All samples were collected 24 h after 4 applications of the indicated chemicals. Data are shown as each value (symbols) and their means $\pm$ standard

deviations (bars). a.u., arbitrary unit. $**P < .01$. $***P < .001$. $****P < .0001$.

Figure 4. The 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 $\pm$ CD40 antibodies for 5 h. B, Migratory LCs from epidermal explant cultures were cultured in the presence or absence of CAL for 29 h and stimulated with IMQ and CD40 antibodies for the last 5 h of culture. Expression levels of *Il12b* and *Il23a* in stimulated LCs were measured. Data are shown as each value (symbols) and their means $\pm$ standard deviations (bars). a.u., arbitrary unit. $***P < .001$. $****P < .0001$.

Figure 5. Topical CAL application suppressed the expansion of CCR6⁺ T17 cells in the dLNs.

Flow cytometric analysis of the dLN cells. A, IL-17A and CCR6 expression by various T cell subsets. B, Number of CCR6⁺ T17 cells in the dLNs. All samples were collected 24 h after 6 applications of the indicated chemicals. Data are shown as each value (symbols) and their means $\pm$ standard deviations (bars). $*P < .05$. $**P < .01$. $****P < .0001$.

Figure 6. Topical CAL application on IMQ-treated left ear resulted in a concomitant amelioration of psoriasis-like inflammation in the right ear.

Mice were treated as indicated on the left (L) ear for 5 days (d-5 to d-1), followed by

668 IMQ treatment of the right (R) ear for 4 days (d0 to d3). A, Kinetics of the right ear
669 swelling. B, Number of T17 cells in the right ears at d4. Data are shown as each value
670 (symbols) and their means $\pm$ standard deviations (bars). * $P < .05$. ** $P < .01$. *** $P < .001$.
671 **** $P < .0001$.

FIG 1
Kusuba et al

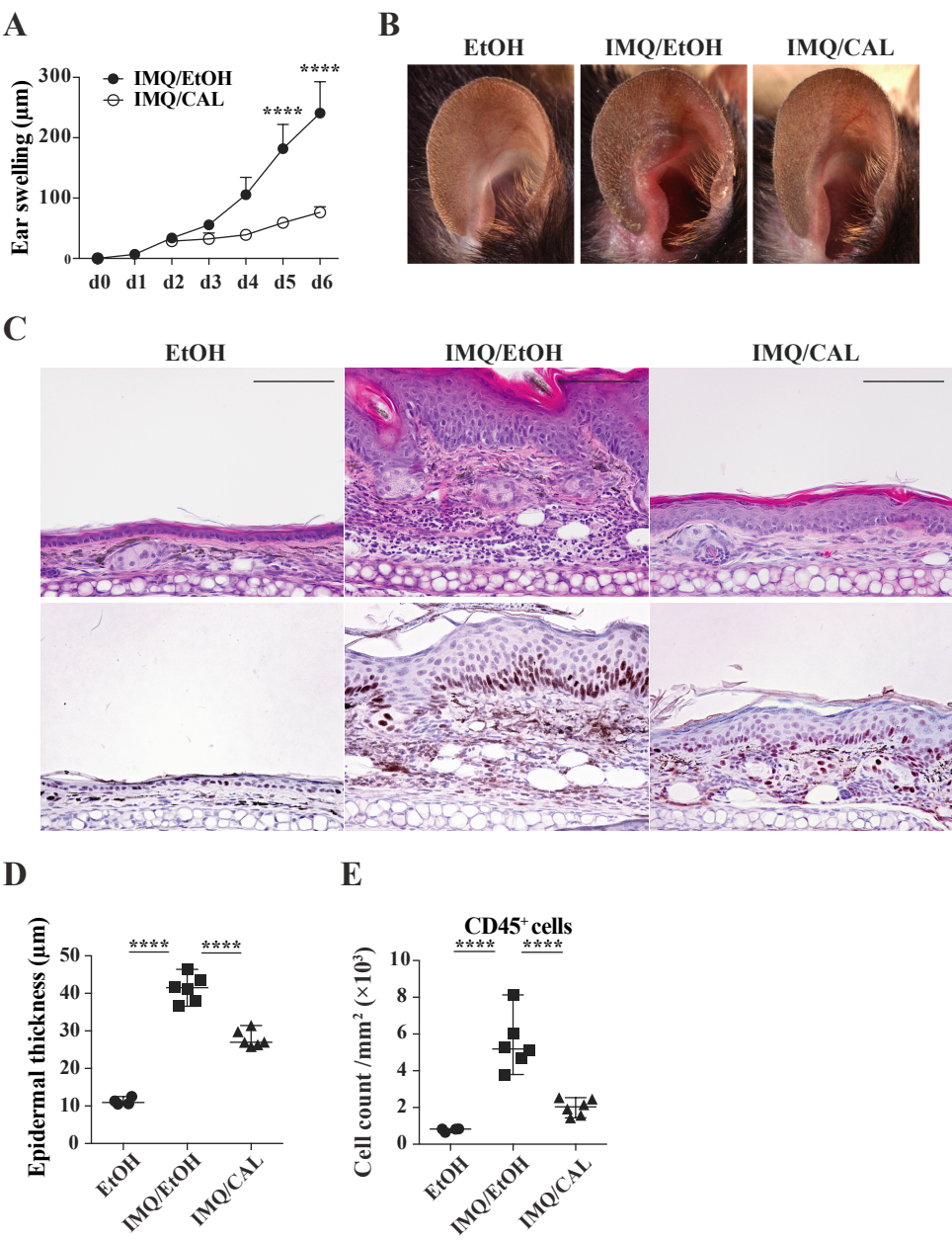


FIG 2
Kusuba et al

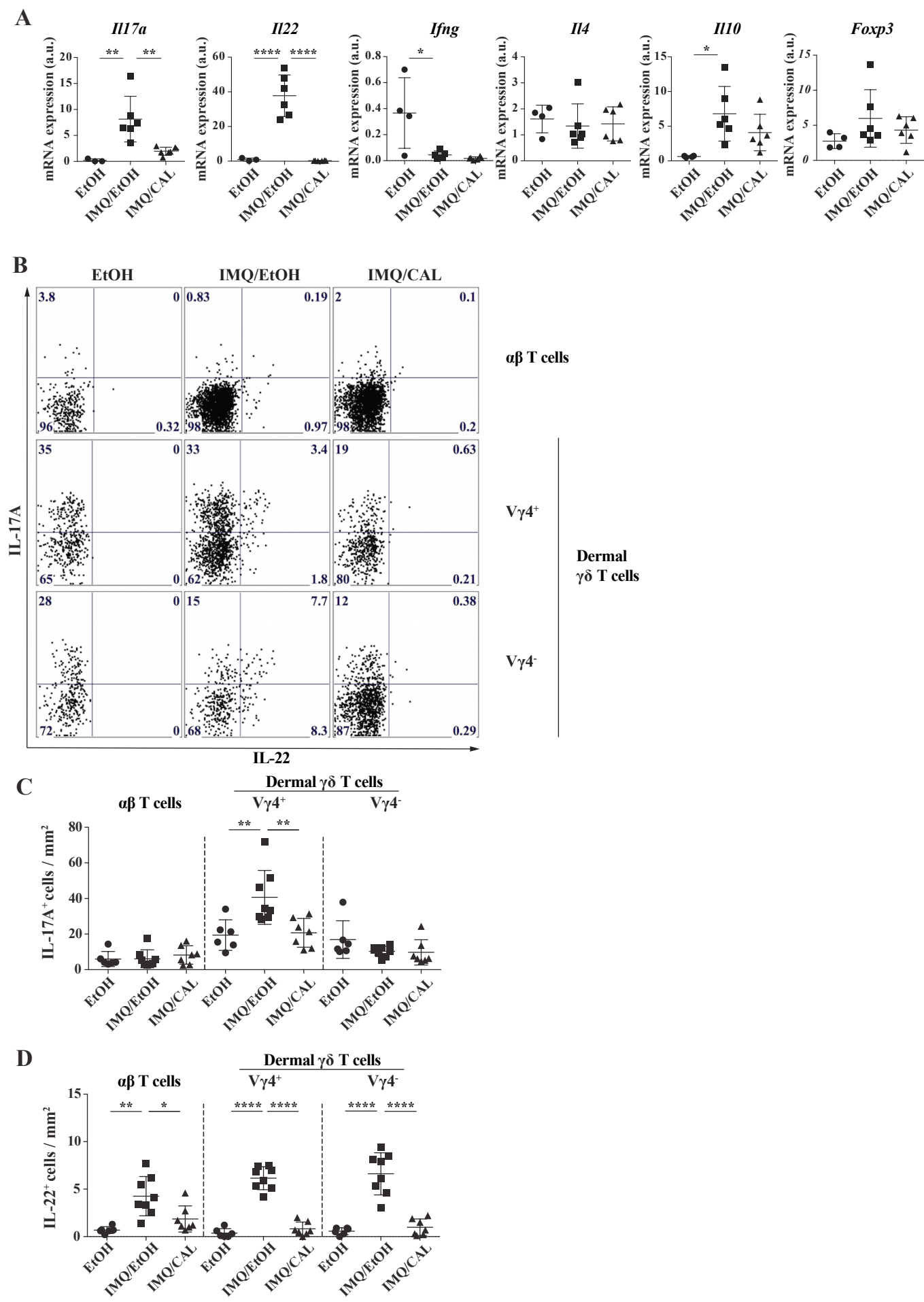


FIG 3
Kusuba et al

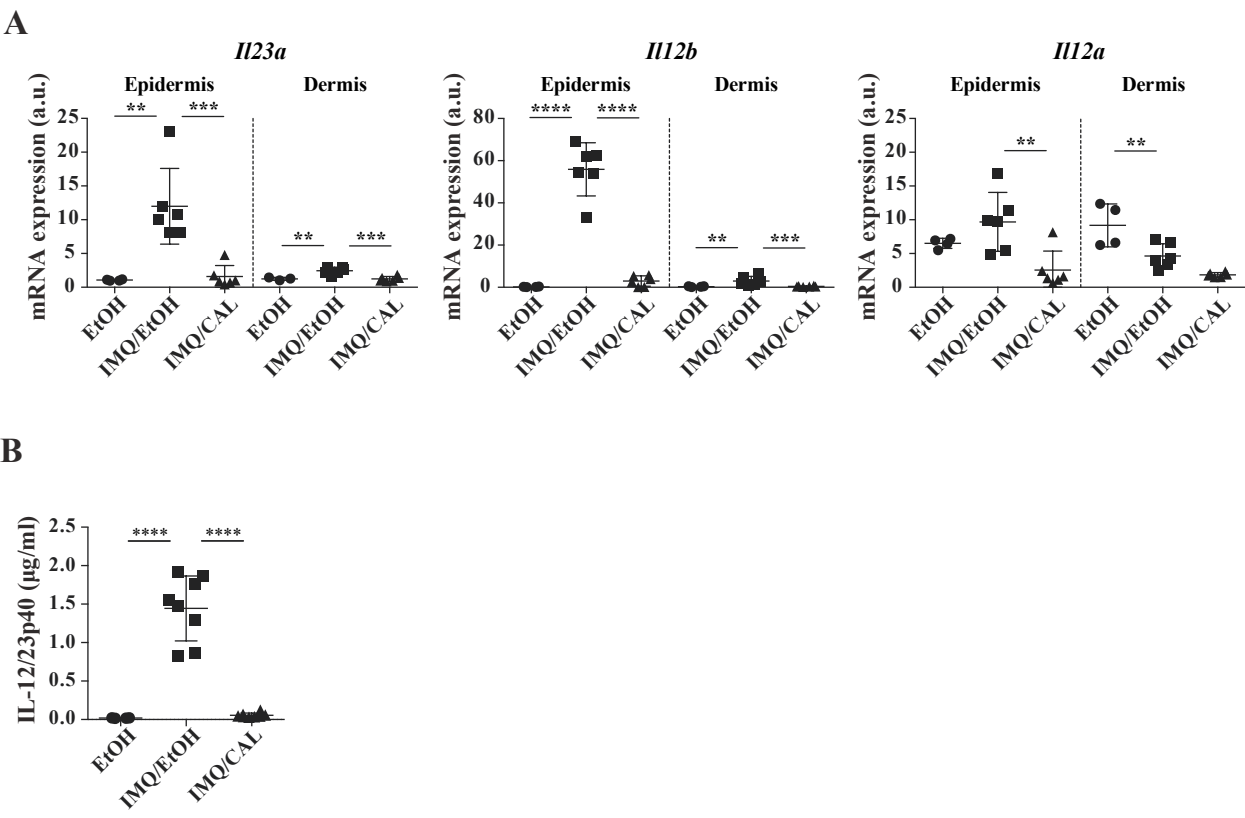


FIG 4
Kusuba et al

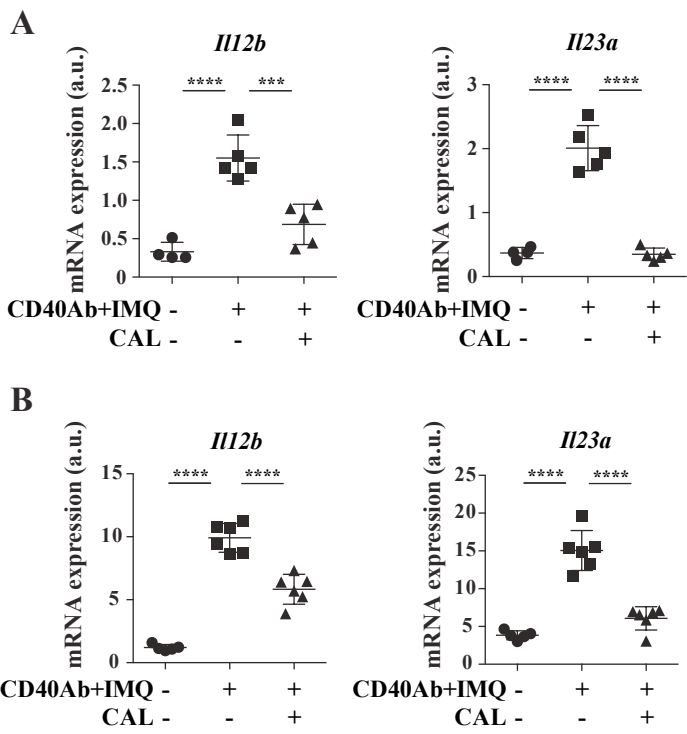


FIG 5
Kusuba et al

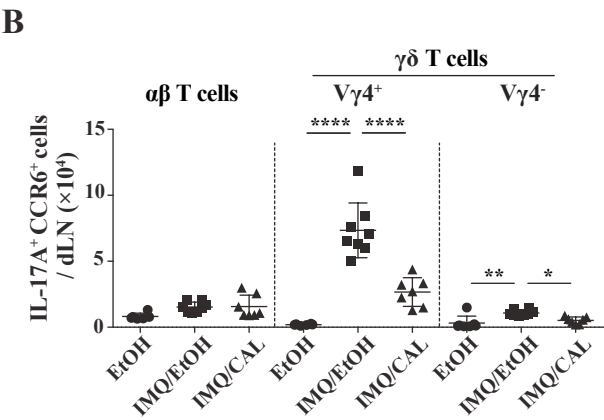
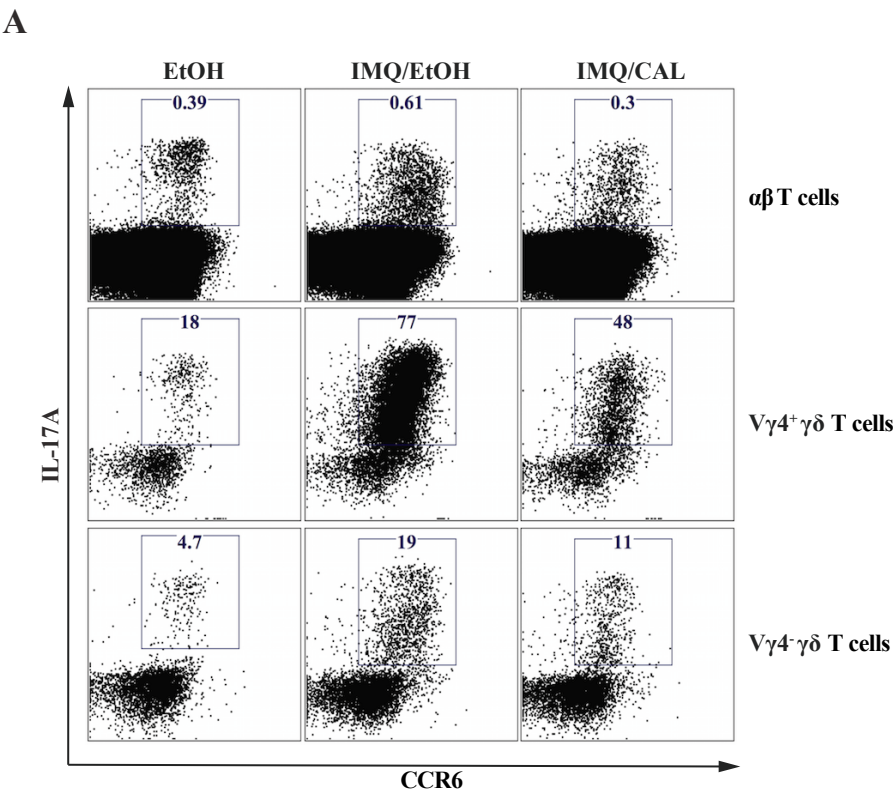
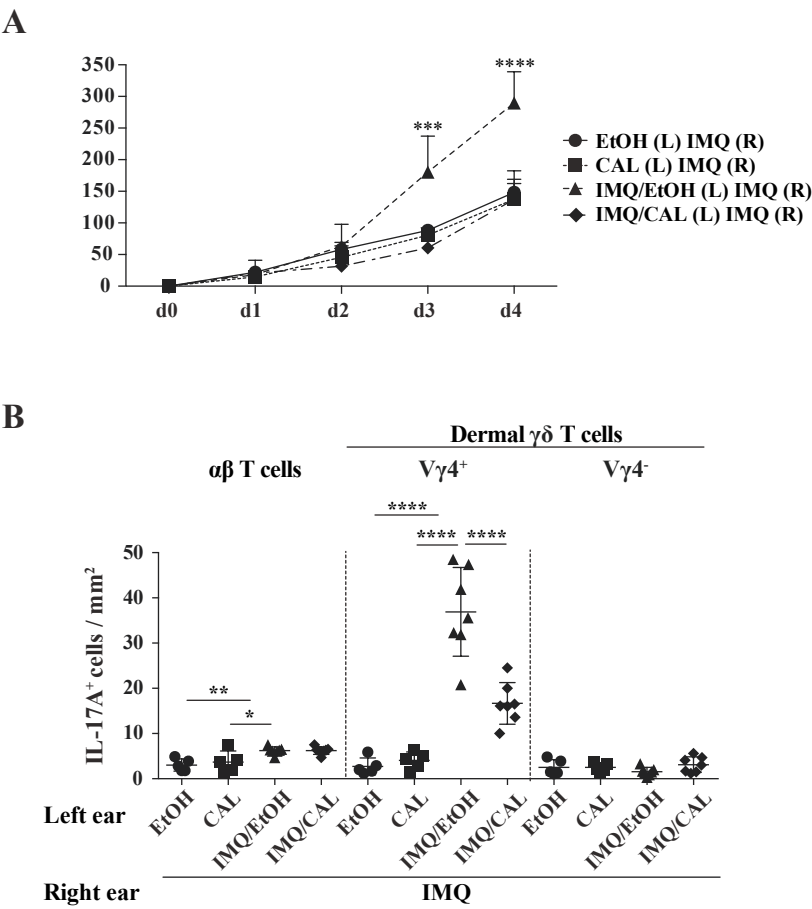


FIG 6
Kusuba et al



SUPPLEMENTAL 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 ten-week-old female B6 mice were used for all the experiments. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Kyoto University Graduate School of Medicine.

Antibodies

FITC anti-TCR β (H57-597), Pacific Blue anti-I-A/I-E (M5/114.15.2), PE-Cy7 anti-EpCAM (G8.8), Brilliant Violet 421 anti-TCR γ/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-V γ 4-TCR (UC3), APC anti-CCR6 (140706), and rat anti-mouse CD16/CD32 (2.4G) antibodies were purchased from BD Biosciences (San Diego, Calif). PerCP-Cyanine5.5 anti-CD45 (30-F11), PE-Cyanine7 anti-IL-17A (eBio17B7), APC anti-IL-22 (IL22JOP), FITC 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.

Flow cytometry

Single-cell suspensions from skin were prepared for flow cytometric analysis as described previously.¹ Briefly, ear samples were collected by using 8 mm skin biopsy specimens and separated into dorsal and ventral sides. They were cut into pieces and then digested for 90 min 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). 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 the 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 h with 50 ng/mL phorbol-12-myristate-13-acetate (Sigma-Aldrich) and 1 µM 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 using FlowJo software (TreeStar, San Carlos, Calif). The numbers of each cell subset were calculated by flow cytometry and presented as numbers per square millimeter of skin surface.

Histology and immunohistochemistry

For histological 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 (H&E). For quantification of epidermal thickness, images of two consecutive microscopic fields of H&E-stained sections were obtained by using a digital microscope (BIOREVO BZ-9000; Keyence, Osaka, Japan) with a 10 $\times$ 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 Image J software (NIH, Bethesda, Md). Quantification was performed by a dermatopathologist blind to the experimental groups analyzed.

For the immunohistochemical staining of Ki-67, sections were deparaffinized in xylene and rehydrated by washing sections in graded alcohol series. Sections were incubated in 10 mM citric acid (pH 6) at 95°C for 30 min for antigen retrieval. Sections were blocked with normal goat serum for 60 min 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 min with biotinylated goat anti-mouse IgG antibodies (Vector Laboratories, Burlingame, Calif). Next, the sections were incubated with Vectastatin ABC reagent (Vector Laboratories) for 30 min. The sections were then incubated with 3,3'-diaminobenzidine (DAB) substrate (Vector Laboratories) for 15 min. Finally, the sections were counterstained with hematoxylin solution. Images were captured using a digital microscope (BIOREVO BZ-9000). All histology and immunohistochemical

Quantitative RT-PCR analysis

Total RNA was isolated using the 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, using SYBR Green I (Roche, Basel, Switzerland) and the Light Cycler 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), 5'-GGG GTC GTT GAT GGC AAC A-3' (reverse); *Il4*, 5'- GGT CTC AAC CCC CAG CTA GT-3' (forward), 5'- GCC GAT GAT CTC TCT CAA GTG AT-3' (reverse); *Il6*, 5'- TCT ATA CCA CTT CAC AAG TCG GA-3' (forward), 5'- GAA TTG CCA TTG CAC AAC TCT TT-3' (reverse); *Il17a*, 5'- CTC CAG AAG GCC CTC AGA CTA C-3' (forward), 5'- GGG TCT TCA TTG CGG TGG-3' (reverse); *Il22*, 5'- ATG AGT TTT TCC CTT ATG GGG AC-3' (forward), 5'- GCT GGA AGT TGG ACA CCT CAA-3' (reverse); *Il12a*, 5'- AGA CAT CAC ACG GGA CCA AAC-3' (forward), 5'- CCA GGC AAC TCT CGT TCT TGT-3' (reverse); *Il12b*, 5'- GGT GTA ACC AGA AAG GTG CG-3' (forward), 5'- TAG CGA TCC TGA GCT TGC AC-3' (reverse); *Il23a*, 5'- AAC TCC TCC AGC CAG AGG ATC A-3' (forward), 5'- TCT TGG AAC GGA GAA GGG GG -3' (reverse); *Ifng*, 5'- ACA GCA AGG CGA AAA AGG ATG-3' (forward), 5'- TGG

TGG ACC ACT CGG ATG A-3' (reverse); *Foxp3*, 5'- GGC GAA AGT GGC AGA GAG G-3' (forward), 5'- AAG GCA GAG TCA GGA GAA GTT G-3' (reverse); *Tnf*, 5'- TGC CTA TGT CTC AGC CTC TTC-3' (forward), 5'- GAG GCC ATT TGG GAA CTT CT -3' (reverse); *S100a7*, 5'- GAG GAG TTG AAA GCT CTG CTC TTG-3' (forward), 5'- GTG ATG TAG TAT GGC TGC CTG CGG-3' (reverse) ; *S100a8*, 5'- AAA TCA CCA TGC CCT CTA CAA G-3' (forward), 5'- CCC ACT TTT ATC ACC ATC GCA A-3' (reverse) ; *S100a9*, 5'- GCA CAG TTG GCA ACC TTT ATG-3' (forward), 5'- TGA TTG TCC TGG TTT GTG TCC-3' (reverse) ; *Defb4*, 5'- CTC CAC TTG CAG CCT TTA CC-3' (forward), 5'-ATC TGT CGA AAA GCG GTA GGG-3' (reverse). The cycling conditions were as follows: initial enzyme activation at 95°C for 10 min, followed by 45 cycles at 95°C for 10 s and at 60°C for 20 s. Gene-specific fluorescence was measured at 60°C. For each sample, duplicate test reactions were analyzed for the expression of the genes, 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 scalpel in the cryostat at -30 °C. After shaving the epidermis, the rest of the ear skin were used as dermal samples.

ELISA

Amounts of IL-12/23p40 in the epidermis were measured by using ELISA Ready-SET-Go kit (eBioscience), according to the manufacturers instructions. Epidermal samples were homogenized in 600 μ L of PBS. The supernatants were collected for ELISA.

Purification and culture of murine epidermal Langerhans cells

Langerhans cells (LCs) were collected as described previously with modifications.² Briefly, mice 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 dermal side down on RPMI 1640 medium (Sigma Aldrich, St Louis, Mo) supplemented with 10% heat-inactivated fetal calf serum (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 granulocyte macrophage-colony stimulating factor (Peprotech, Rocky Hill, NJ) in the presence or absence of 100 nM CAL and incubated for 3 days at 37°C. Epidermal sheets were removed and supernatant was passed through a 40- μ m pore size cell strainer. LCs were isolated with MHC class II microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) using a MiniMACS separator (Miltenyi

Biotec). The purity was confirmed to be >90% by flow cytometry. All isolated LCs were matured as previously described.³ Isolated LCs were seeded at $4 \times 10^5/\text{mL}$ in round-bottom 96-well plates and stimulated with 10 $\mu\text{g/mL}$ IMQ (Tokyo Kasei Industry, Tokyo, Japan) in combination with 1 $\mu\text{g/mL}$ agonistic anti-CD40 antibody (HM40-3; BioLegend) for 5 h. In some experiments, isolated LCs were cultured with or without 100 nM CAL for 29 h and stimulated for the last 5 h of culture with IMQ and anti-CD40 antibody.

Statistical analysis

Unless otherwise indicated, results are presented as the mean $\pm$ standard deviation value and are representative of at least three independent experiments. Comparisons were analyzed for statistical significance by Student's t-test. Statistical significance between multiple groups was analyzed by the Tukey-Kramer multiple comparisons test. *P* values < .05 were considered to be significantly different.

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SUPPLEMENTAL FIGURE LEGENDS

Figure E1. CAL attenuates the 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. All samples were collected 24 h after the fourth treatment. Data are shown as each value (symbols) and their means $\pm$ standard deviations (bars). a.u., arbitrary unit. *** $P < .001$. **** $P < .0001$.

Figure E2. Gating strategy for $\alpha\beta$ T cells, dermal $V\gamma 4^+$ $\gamma\delta$ T cells, and dermal $V\gamma 4^-$ $\gamma\delta$ T cells in the ear

Figure E3. CAL attenuates the expression levels of *Il17a*, *Il22*, *Il23a*, and *Il12b* in IMQ-treated ear

Expression levels of *Il17a*, *Il22*, *Il23a*, and *Il12b* mRNA in the ear treated as indicated. CAL application started after 3 applications of IMQ. All samples were collected 24 h after the 6 applications of IMQ. Data are shown as each value (symbols) and their means $\pm$ standard deviations (bars). a.u., arbitrary unit. * $P < .05$. ** $P < .01$.

Figure 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 $\pm$ CD40 antibodies for 5 h.

B. Migratory LCs from epidermal explant cultures were cultured in the presence or

absence of CAL for 29 h and stimulated with IMQ and CD40 antibodies for the last 5 h of culture.

Figure 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 h. Data are shown as each value (symbols) and their means $\pm$ standard deviations. a.u., arbitrary unit. **** $P < .0001$.

Figure E6. The 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 h and stimulated with IMQ and CD40 antibodies for the last 5 h of culture. Expression levels of *Il6* and *Tnf* in stimulated LCs were measured. Data are shown as each value (symbols) and their means $\pm$ standard deviations (bars). a.u., arbitrary unit. * $P < .05$. ** $P < .01$. **** $P < .0001$.

Figure 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 (d-5 to d-1), followed by IMQ treatment of the right ear for 4 days (d0 to d3). The right ear was collected 24 h after the IMQ application at d3 and subjected to flow cytometric analysis.

Figure 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 (d-5 to d-1), followed by IMQ treatment of the right (R) ear for 4 days (d0 to d3). The right ear was collected 24 h after the 4time IMQ application. Data are shown as each value (symbols) and their means $\pm$ standard deviations.

FIG E1
Kusuba et al

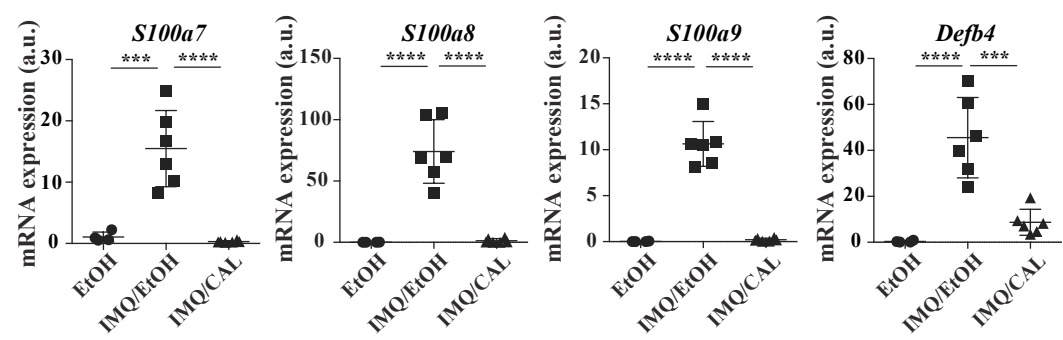


FIG E2
Kusuba et al

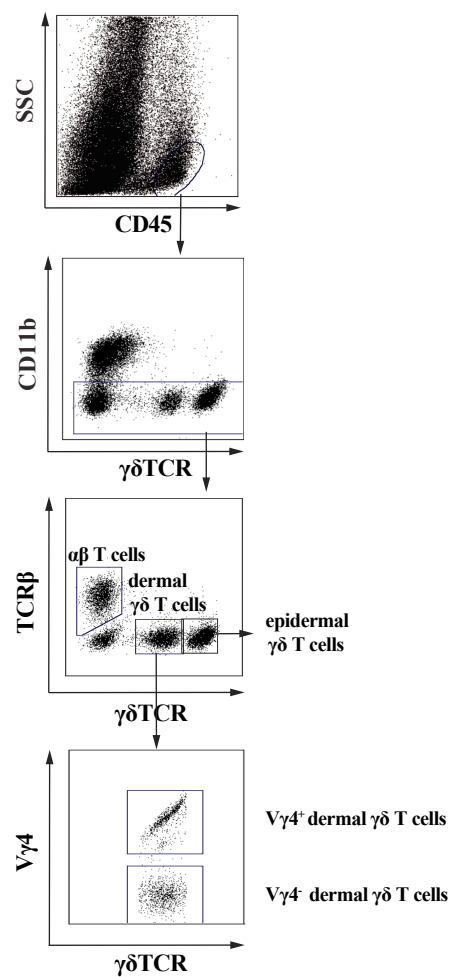


FIG E3
Kusuba et al

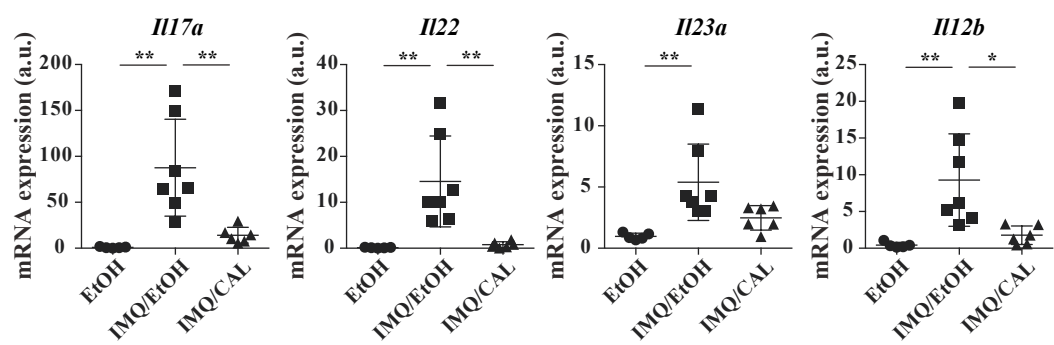


FIG E4
Kusuba et al

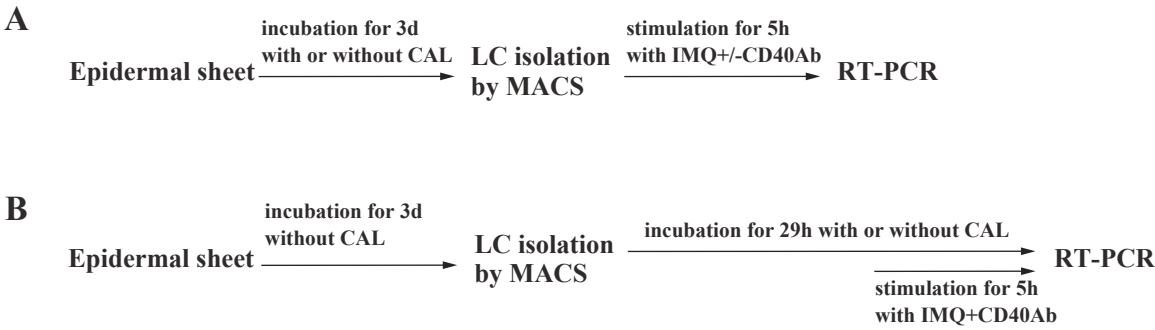


FIG E5
Kusuba et al

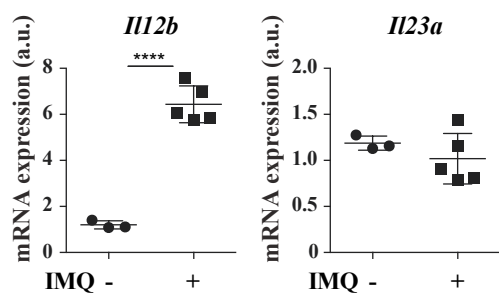


FIG E6
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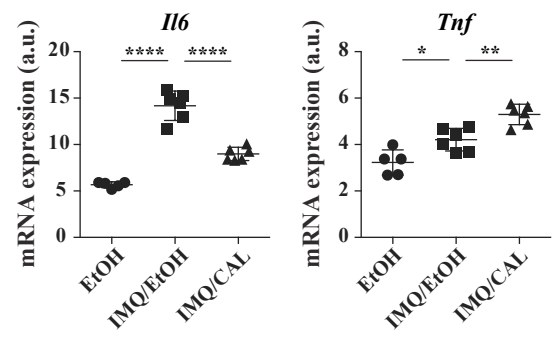


FIG E7
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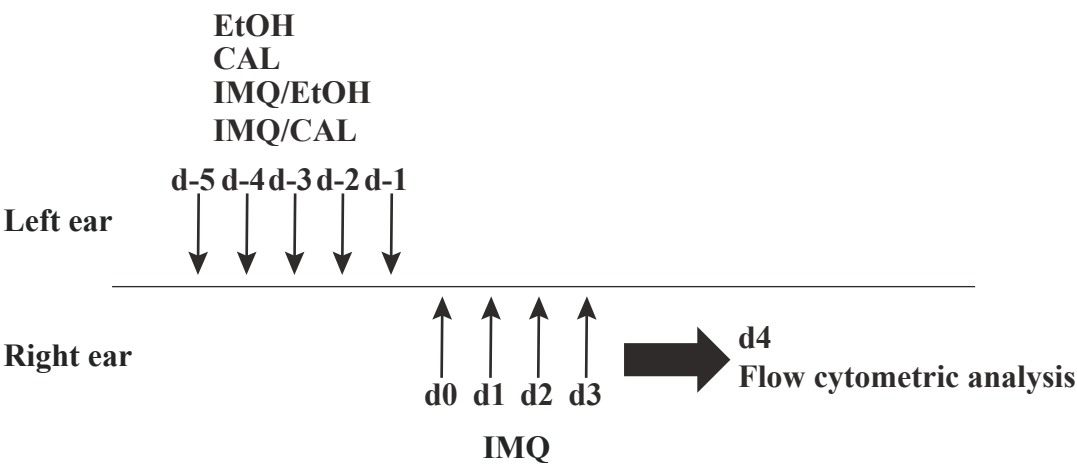


FIG E8
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