Topical application of a vitamin D3 analogue and corticosteroid to psoriasis plaques decreases skin infiltration of T_H17 cells and their *ex vivo* expansion

Toshiharu Fujiyama, MD, PhD,[a] Taisuke Ito, MD, PhD,[a] Takatsune Umayahara, MD,[a] Shigeki Ikeya, MD,[a] Kazuki Tatsuno, MD,[a] Atsuko Funakoshi, PhD,[a] Hideo Hashizume, MD, PhD,[b] and Yoshiki Tokura, MD, PhD[a]

Hamamatsu and Shimada, Japan

Background: Topical combination of a vitamin D3 analogue and corticosteroid is widely used for the treatment of psoriasis, a T_H17-mediated disorder, but the underlying mechanism remains unclear.

Objective: We investigated the effect of this topical applicant, focusing on skin-infiltrating T_H17 cells.

Methods: In 10 patients with plaque psoriasis, calcipotriol (Cal), betamethasone dipropionate (Bet), or the calcipotriol and betamethasone dipropionate 2-compound formulation (CB) was applied to 3 different psoriatic plaques with similar severity once a day for 14 days. One nonapplied lesion was used as a control. Four-millimeter biopsy specimens were taken from each site, cut into 2 pieces, and subjected to histologic examination and *ex vivo* expansion of skin-infiltrating T cells with anti-CD3/CD28 antibodies and IL-2.

Results: Clinical, histologic, and IL-17A$^+$ cell-infiltrate improvement was found in the following order: CB > Cal > Bet > control or CB > Bet > Cal > control. Numbers of *ex vivo* expanded T cells were decreased by topical application of Bet and CB, and CB exhibited the most suppressive result. Numbers and frequencies of T_H17 cells were significantly reduced by CB and Cal, suggesting that Cal has a capacity to preferentially suppress T_H17 cells. When the stocked T cells from control samples were stimulated with anti-CD3 antibodies in the presence of Bet, Cal, or both, Cal downmodulated IL-17 and IFN-γ production and tended to upregulate IL-4 and IL-6 without apoptosis, but Bet inhibited production of these cytokines with apoptosis.

Conclusion: These findings suggest that Cal and Bet have different effects on T cells to normalize psoriatic changes, with decreased T_H17 cell expansion in the skin lesions. (J Allergy Clin Immunol 2016;■■■:■■■-■■■.)

Key words: *psoriasis, IL-17A, Th17, vitamin D, corticosteroid*

Abbreviations used
7AAD: 7-Aminoactinomycin D
Bet: Betamethasone dipropionate
Cal: Calcipotriol
CB: Calcipotriol and betamethasone dipropionate, 2-compound formulation
DC: Dendritic cell
PE: Phycoerythrin
PMA: Phorbol 12-myristate 13-acetate

Psoriasis is one of the most common inflammatory keratotic skin diseases. Although the direct trigger of the onset of the disease and maintenance of its activity remains unclear, the involvement of IL-17–producing T cells in its pathogenesis has been clarified and recently proved by the therapeutic efficacy of antibodies against IL-17 and its receptors.[1-4] T_H17 cells and their cytokines, IL-17A and IL-22, play an essential role, and IL-22 might be a prerequisite for acanthosis. IL-17A and IL-22 stimulate keratinocytes to produce IL-8, vascular endothelial growth factor, and GM-CSF, thereby inducing inflammation, neutrophil accumulation, and angiogenesis.[5,6] IL-23 is required and released from TNF-α–producing inflammatory dendritic cells (DCs) for maintaining T_H17 cells.[7,8] Recently, murine epidermal Langerhans cells were found to produce IL-23.[9] In addition, plasmacytoid DCs secreting IFN-α are considered to play an initiating role in the development of psoriatic lesions.[10,11]

Although therapies targeting to TNF-α, IL-23, and IL-17 are effective for psoriasis, conventional topical treatments remain a standard choice because of their convenience, safety, and economic efficiency. The most frequently used topical drugs include corticosteroids and vitamin D3 analogues, which have different actions not only on epidermal keratinocytes[12-16] but also on immunocompetent cells.[17,18] Corticosteroids are a potent anti-inflammatory agent with broad immunosuppressive effects on a number of cell types, such as T cells, because they can block several inflammatory pathways and induce apoptosis.[19-21] Meanwhile, vitamin D3 analogues possess a more specific immunomodulatory profile characterized by the capacity to induce a tolerogenic state in DCs[22,23] and T cells, enhancing the immunosuppressive capacity of regulatory T cells[24,25] and driving T cells toward a T_H2 profile while inhibiting T_H1/T_H17 cells.[26]

It is assumed that a corticosteroid and a vitamin D3 analogue synergistically improve psoriasis when their combination is applied to the plaque lesion. However, detailed investigations on their effects on skin-infiltrating T cells, especially pathogenic T_H17 cells, have not been performed. In this study we sought to

From [a]the Department of Dermatology, Hamamatsu University School of Medicine, and [b]the Department of Dermatology, Shimada Municipal Hospital.

Supported by Leo Pharma.

Disclosure of potential conflict of interest: Y. Tokura has received a grant from LEO Pharma. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication October 7, 2015; revised March 8, 2016; accepted for publication March 31, 2016.

Corresponding author: Toshiharu Fujiyama, MD, PhD, Hamamatsu University School of Medicine, Department of Dermatology, Japan. E-mail: fujiyama@hama-med.ac.jp.

0091-6749/$36.00

http://dx.doi.org/10.1016/j.jaci.2016.03.048

explore the effects of topical calcipotriol (Cal), betamethasone dipropionate (Bet), and their combination on skin-infiltrating T cells. This *ex vivo* expansion study clarifies the differences between the 2 drugs in the inhibitory effects on T-cell populations.

METHODS

Patients and topical application of Cal and Bet

Ten patients with psoriasis vulgaris were enrolled in this study. The diagnosis was made based on clinical and histologic findings. None of the patients were receiving any systemic medication or phototherapy for psoriasis. Four different psoriasis plaques that had not been topically treated with any drugs for more than 2 weeks were chosen, and calcipotriol ointment (Cal; Dovonex, LEO pharma, Ballerup, Denmark), betamethasone dipropionate ointment (Bet; Rinderon DP, Shionogi, Japan), or calcipotriol and betamethasone dipropionate 2-compound formulation (CB; Dovobet, LEO pharma) was applied once a day to 3 different sites with similar severity of psoriatic plaques for 14 days, according to the fingertip unit method.[27] One nonapplied lesion was used as a control. After 2 weeks of topical treatment, 4-mm punch skin biopsy specimens were taken from the 4 sites for histologic and immunologic studies.

This study was performed according to the Declaration of Helsinki, and the study protocol was approved by the ethical committee of Hamamatsu University School of Medicine. Written informed consent was obtained from all participants.

Cell preparations

A 4-mm skin biopsy specimen was cut into 2 pieces, with 1 specimen used for expansion of skin-infiltrating T cells and another for histologic investigation. We previously established a method of expanding skin-infiltrating T cells using anti-CD3/CD28 antibody–coated microbeads and IL-2.[28] Samples were immersed in cRPMI supplemented with 50 U/mL human recombinant IL-2 (R&D Systems, Minneapolis, Minn) and anti-CD3/CD28 antibody–conjugated microbeads (Human T-Activator; Dynal, Copenhagen, Denmark), as previously reported. Cells were cultivated in 6-well plates for 2 weeks, and the culture media were changed or added every day to make sure half of the total volume of media was fresh. We obtained more than 10^6 cells per specimen by means of 2-week cultivation, and all cells were collected and counted for the cytokine production assay and intracytoplasmic cytokine staining assay. The rest of the cells were stored at −80°C to use the apoptosis and drug-stimulated cytokine production assays.

Flow cytometric analysis and cytokine production assessments

Aliquots of 10^6 cells were washed once with PBS (pH 7.4) with a panel of fluorescence-conjugated mAbs for 15 minutes at room temperature in the dark. After washing, the harvested cells were resuspended in PBS and subjected to flow cytometric analysis. More than 5×10^4 cells per sample were analyzed on a FACSCanto II (BD Biosciences, San Jose, Calif). Results were analyzed with FlowJo software (TreeStar, Ashland, Ore). For the intracellular cytokine staining assay, 10^6 cells/well in 24-well plates were incubated in cRPMI containing 10^{-8} mol/L phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, St Louis, Mo), 10^{-6} mol/L calcium ionophore (Sigma-Aldrich), and 1 μL/mL GolgiStop (BD) for 6 hours at 37°C. Cells were harvested and stained with fluorescence-tagged mAbs against cytokines by using the Cytofix/Cytoperm Plus Kit with GolgiStop (BD), according to the manufacturer's protocols, after staining of fluorescence-tagged antibodies against CD3 and CD8.

Histopathologic and immunohistochemical studies

Half of the biopsy specimens from the lesions were fixed in 4% formalin and routinely stained with hematoxylin and eosin for standard histopathology. Deparaffinized specimens were autoclaved in 10 mmol/L citrate buffer (pH 6.0) for 10 minutes at 120°C to retrieve the antigenic epitopes and then subjected to CD3 expression analysis by using the avidin-biotin complex method and to IL-17A expression analysis by using immunofluorescent staining. Nuclear staining was performed with hematoxylin or 4′-6-diamidino-2-phenylindole dihydrochloride. Sections were scanned with a digital image scanner (NanoZoomer; Hamamatsu Photonics, Hamamatsu, Japan) and analyzed by using its software. The epidermal thickness or thickness of the stratum corneum was calculated by measuring the area and length of the epidermal surface line of the whole tissue image.

Cytokine production assay

Cells (2×10^5 cells/well) were stimulated with immobilized anti-CD3 mAb–coated 96-well plates (BD BioCoat; BD) for 48 hours in the presence or absence of drugs, and the culture supernatants were harvested to measure cytokine levels (IL-2, IL-4, IL-6, IL-10, IL-17, IFN-γ, and TNF-α) with the Human $T_H1/T_H2/T_H17$ Cytokine Beads Array kit (BD), according to the manufacturer's protocols.

Statistical analyses

We applied the Friedman test, and if there were significant differences, *post hoc* pairwise multiple comparisons (Dunn-Bonferroni tests) were performed with SPSS software (version 21; IBM, Armonk, NY). *P* values of less than .05 were considered statistically significant.

RESULTS

Combination of Cal and Bet synergistically improves psoriatic lesions

We selected 4 different psoriasis plaques of similar severities that had not been treated for more than 2 weeks. Cal, Bet, or CB was applied once a day to the 3 different sites for 14 days according to the fingertip unit method.[27] One nonapplied lesion was used as a control. After 2 weeks of topical treatment, the psoriatic lesions were improved, depending on the ointments, in each patient. Notably, CB-treated lesions showed the best improvement in all patients, whereas the control sites were not substantially changed. Representative clinical pictures of cases 1 to 5 from 10 cases are shown in Fig 1. The clinical efficacy was assessed by SUM score, which includes the severity (on a 0- to 4-point scale) of erythema, induration, and scaling (see Fig E1 in this article's Online Repository at www.jacionline.org). CB and Cal treatment significantly improved the SUM score, whereas no statistically significant difference was observed between Cal and Bet. The efficacy of Cal and Bet single treatment varied in individual patients. Cal was more effective than Bet in cases 4, 5, 6, and 10, and Bet was more effective in cases 2, 3, 8, and 9. In cases 1 and 7 the 2 agents showed similar efficacy.

Combination of Cal and Bet synergistically improves psoriatic histologic changes

Skin biopsy specimens were obtained from each treated or nontreated lesion and subjected to histopathologic investigation. The whole stained skin tissue sections were captured as digital images and analyzed. Representative pictures were shown in Fig 2, *A*. The thickness of the epidermis and stratum corneum and the length of parakeratosis were measured to assess the psoriatic changes. In all cases CB and Bet, but not Cal for case 3, diminished the thickness of the epidermis. These results indicate that all treatments are capable of improving acanthosis; however, the effect of Bet was stronger than that of Cal (Fig 2, *B*, top). The keratinization status was assessed based on the thickness of

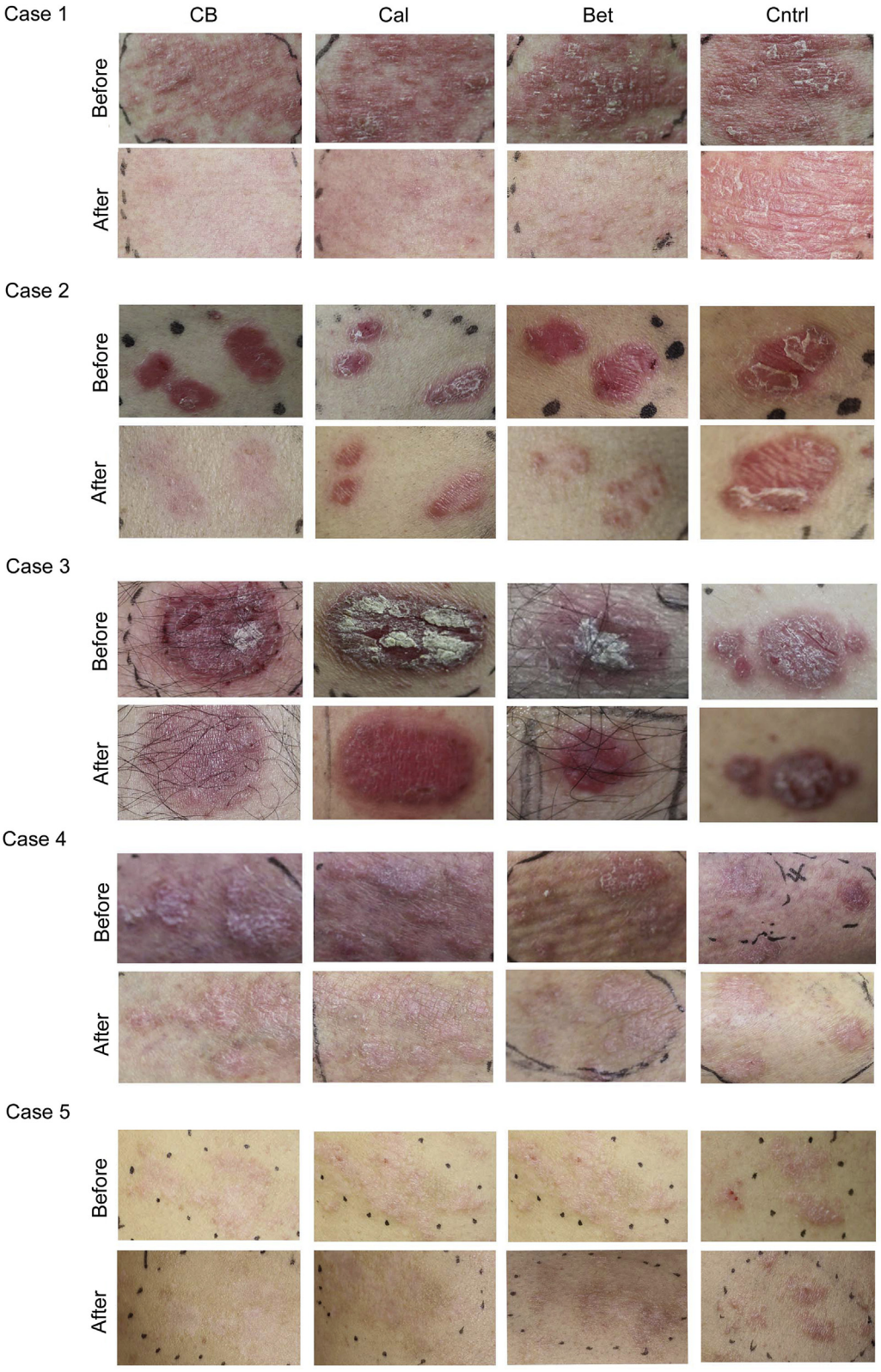

FIG 1. Clinical pictures of psoriatic plaques before and 2 weeks after topical treatment in 5 patients. *Cntrl,* Control.

the stratum corneum (Fig 2, *B*, middle) and the percentage length of parakeratosis (Fig 2, *B*, bottom). Consistent with its effect on epidermal thickness, Bet, but not Cal, has a significant amending effect on epidermal keratinization (Fig 2, *B*). These findings suggest that Cal and Bet synergistically or additionally alleviate the epidermal changes caused by psoriasis.

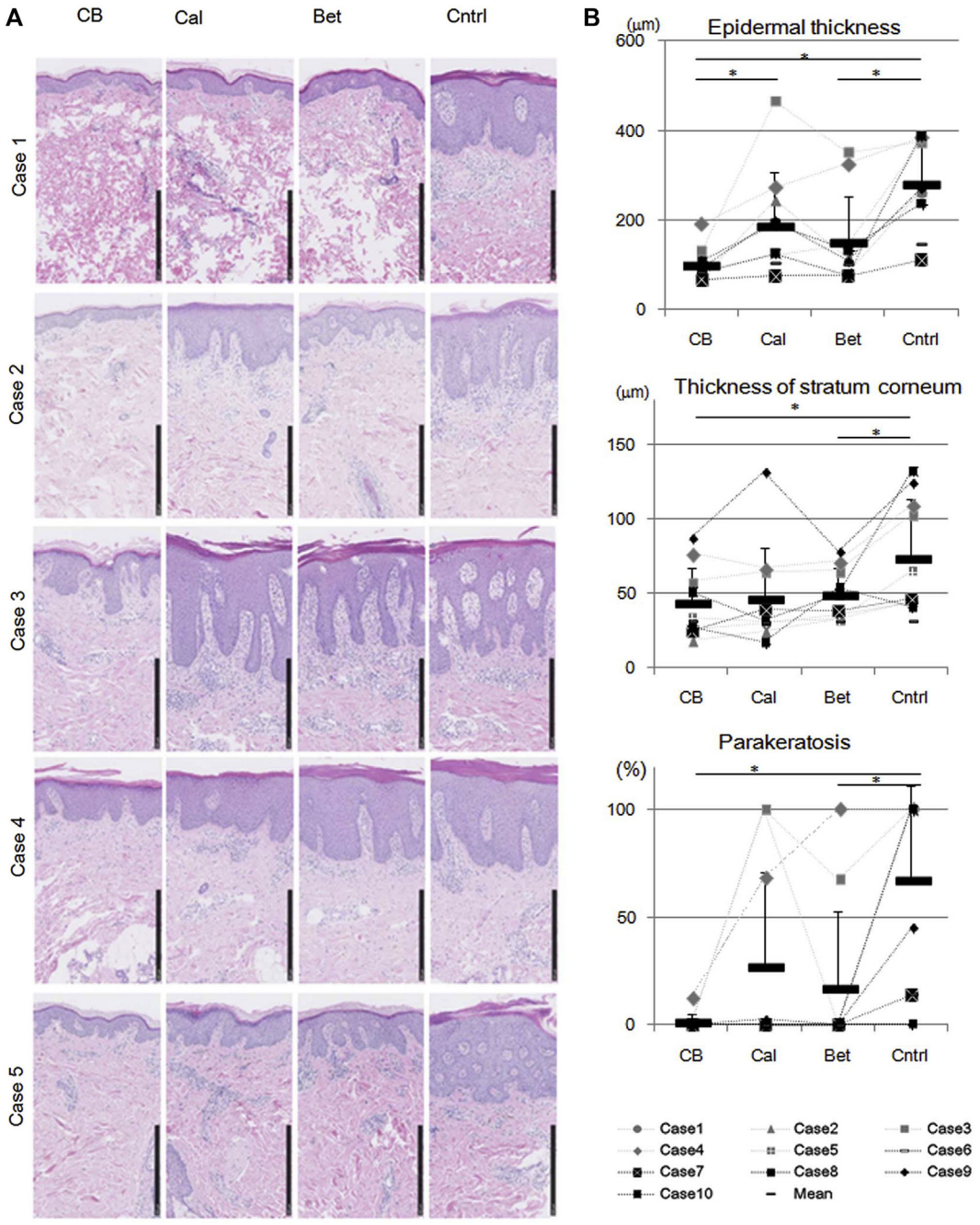

FIG 2. Histopathologic changes in skin biopsy specimens from psoriatic lesions after a 2-week topical treatment. **A,** Hematoxylin and eosin staining of specimens. *Black bar* = 250 μm. **B,** Thickness of the epidermis and stratum corneum and percentage of parakeratosis length analyzed by using digital image analyzer software. *$P < .05$. *Cntrl,* Control.

Combination of calcipotriol and corticosteroid reduces infiltration of IL-17A$^+$ cells in psoriatic lesions

We performed immunohistochemical and immunofluorescent staining of CD3 and IL-17A, respectively, and whole sections were captured as digital images to analyze the number of infiltrating cells. As shown in representative images, the topical application of Cal, Bet, and CB significantly decreased T-cell infiltration along with a reduction in epidermal thickness (Fig 3, *A*, upper panel). Numbers of IL-17A$^+$ cells tended to decrease in response to Cal or Bet single therapy, and more notably, CB significantly decreased IL-17A$^+$ cell infiltration in the 10 patients (Fig 3, *A*, lower panel, and Fig 3, *B*).

Numbers of expanded T cells from Bet- and CB-treated skin are lower than those in control skin

Because the immunofluorescent staining results are not greatly convincing and IL-17A$^+$ cells include cells other than T_H17 cells,

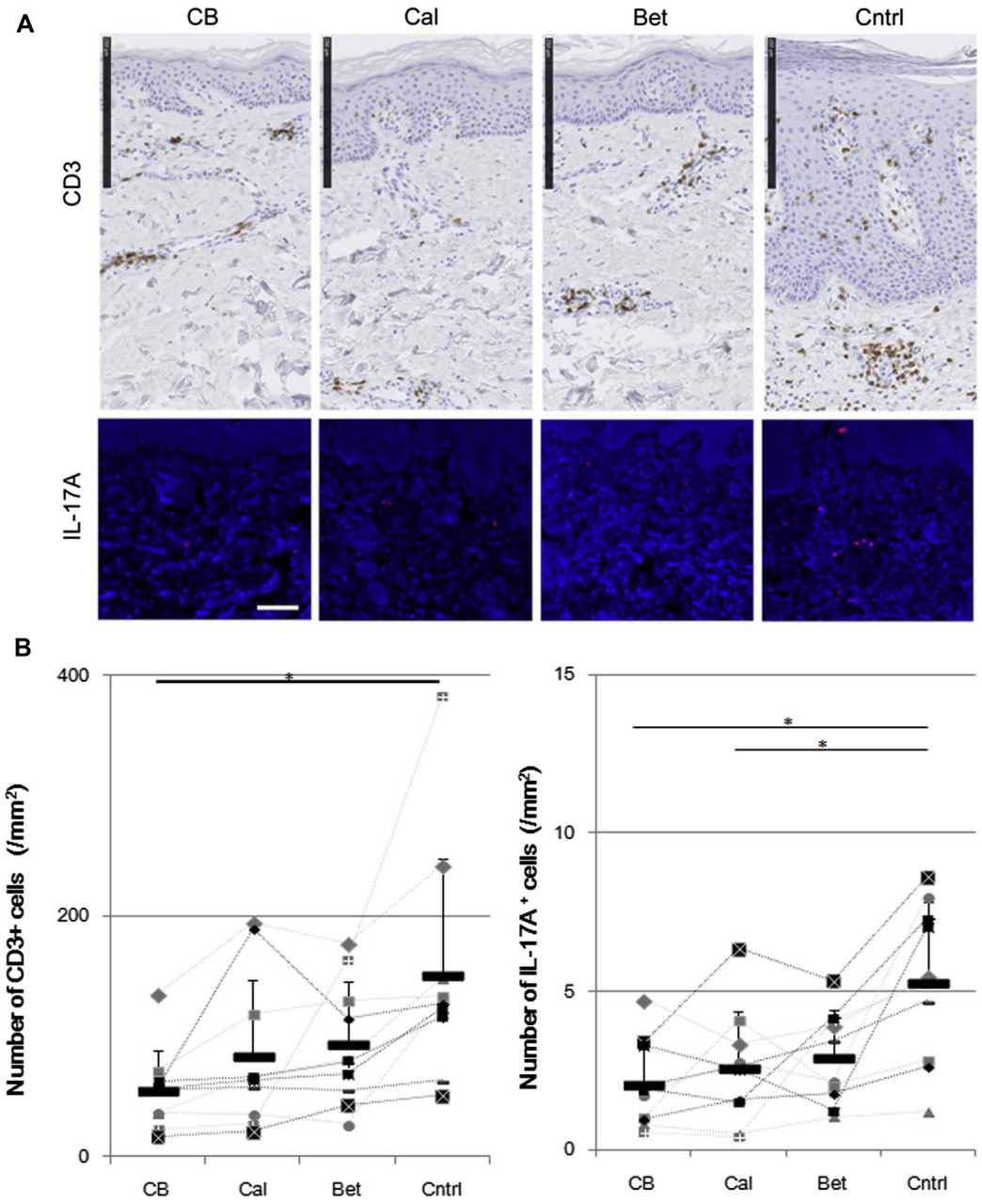

FIG 3. Numbers of $CD3^+$ and IL-17^+ cells analyzed by using digital image analyzer software. **A**, Representative immunohistochemical staining for CD3 (case 1) and immunofluorescence staining for IL-17A (case 2). *Black bar* = 250 μm; *white bar*, 100 μm. **B**, Positive cell numbers in the 10 patients and means ± SDs. **P* < .05. *Cntrl*, Control.

we performed a T-cell expansion study using the biopsied skin samples. Skin-infiltrating T cells were expanded with anti-CD3/CD28 antibody–coated microbeads and IL-2 for 2 weeks. Numbers of expanded T cells were decreased by topical application of Bet and CB to psoriatic lesions, and CB exhibited the most suppressive result in the mean of 10 patients (Fig 4, *A*), supporting the histopathologic finding. The effect of each treatment on CD4 and CD8 frequency varied in individual patients, and there were no common tendencies (Fig 4, *B* and *C*).

To verify the assumption that the expanded cells can represent the original T-cell population when using this method, 10^4 $CD4^+$ T cells from 5 healthy donors were expanded by using this method, and the cytokine profiles of the $CD4^+$ cells and $CD4^+CD45RO^+$ memory T cells before and after the expansion process were compared by using intracellular cytokine staining. In the $CD4^+$ cells the frequency of T_H17 cells was unaffected, and the frequencies of T_H1 and T_H2 cells were slightly increased after expansion, although without statistical significance. This tendency was also observed in $CD4^+CD45RO^+$ memory cells (see Fig E2 in this article's Online Repository at www.jacionline.org). Thus our expansion method reflects the original T-cell population.

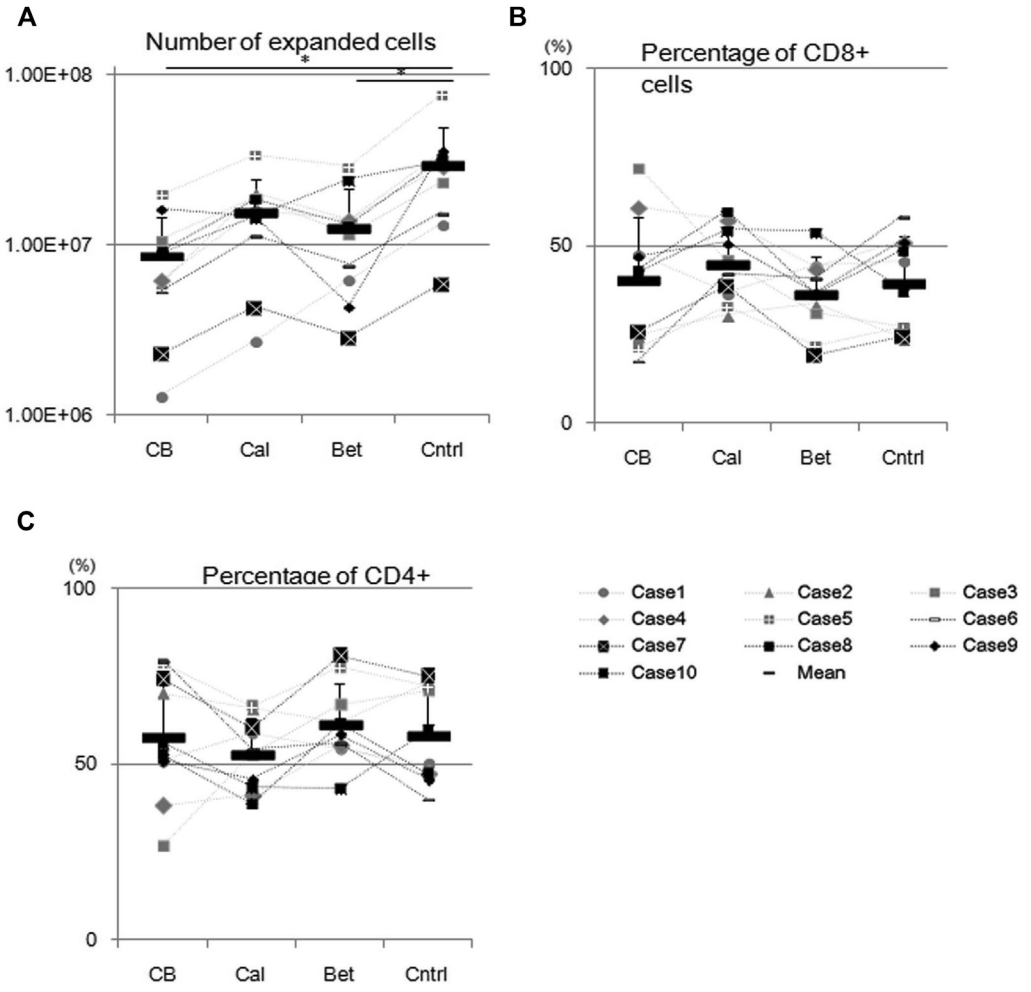

FIG 4. **A–C,** Number and frequency of CD4 and CD8 cells among expanded cells. T cells were expanded with anti-CD3/CD28 mAbs and IL-2 from biopsied skin topically treated with CB, Cal, or Bet or left untreated. **P* < .05. *Cntrl,* Control.

Cal and CB preferentially reduce T_H17 cell frequency in expanded cells

Expanded skin-infiltrating T cells were stimulated with PMA and calcium ionophore and subjected to intracellular cytokine staining. Numbers and frequencies of the IL-4^+, IFN-γ^+, IL-17^+, and IL-22^+ cells were analyzed by using flow cytometric analysis (Fig 5). Because CD4 expression is downregulated during treatment with PMA and calcium ionophore, we estimated $CD4^+$ T cells as the $CD8^-$ T-cell population. Numbers and frequencies of T_H17 cells ($CD3^+CD8^-$IL-17^+ cells) were reduced by CB and Cal treatment (Fig 5, *A*), suggesting that Cal has the capacity to preferentially suppress T_H17 cells. Numbers of T_H1, T_H2, and T_H22 cells tended to be decreased by all treatments, although the results were not statistically significant (Fig 5, *B-D*). All subsets of $CD8^+$ T cells were significantly reduced in number by CB treatment (Fig 5, *E-H*).

Cytokines produced by the expanded T cells were also measured. We stimulated the expanded cells with anti-CD3 antibodies and measured cytokine levels in the culture supernatants. IL-17A production by T cells from Cal-treated skin was significantly lower than that from control skin (see Fig E3 in this article's Online Repository at www.jacionline.org), supporting the decreased frequency of T_H17 cells after Cal treatment. There were no significant changes in levels of the other cytokines, including IFN-γ, TNF-α, IL-10, IL-4, and IL-2.

Cal downregulates T_H1/T_H17 cytokine production, whereas Bet downregulates $T_H1/T_H2/T_H17$ cytokine levels

The stocked T cells from control samples were stimulated with anti-CD3 antibodies in the presence of 1 μmol/L Bet, 100 nmol/L Cal, or both, and 6 cytokines in the culture supernatant were quantified. Although Bet inhibited the production of T_H1, T_H2, and T_H17 cytokines, Cal tended to upregulate IL-4 production and significantly downregulated IL-17A (Fig 6). These results indicate that the Cal effect is biased because it inhibits T_H17 cells and tends to upregulate T_H2 cells.

Bet, but not Cal, induces apoptosis

To clarify the differences between Cal and Bet in terms of inhibitory action on T cells, we investigated the direct apoptotic effects of Cal and Bet on skin-infiltrating T cells. Expanded T cells derived from control skin were cultured at 10^5 cells/well

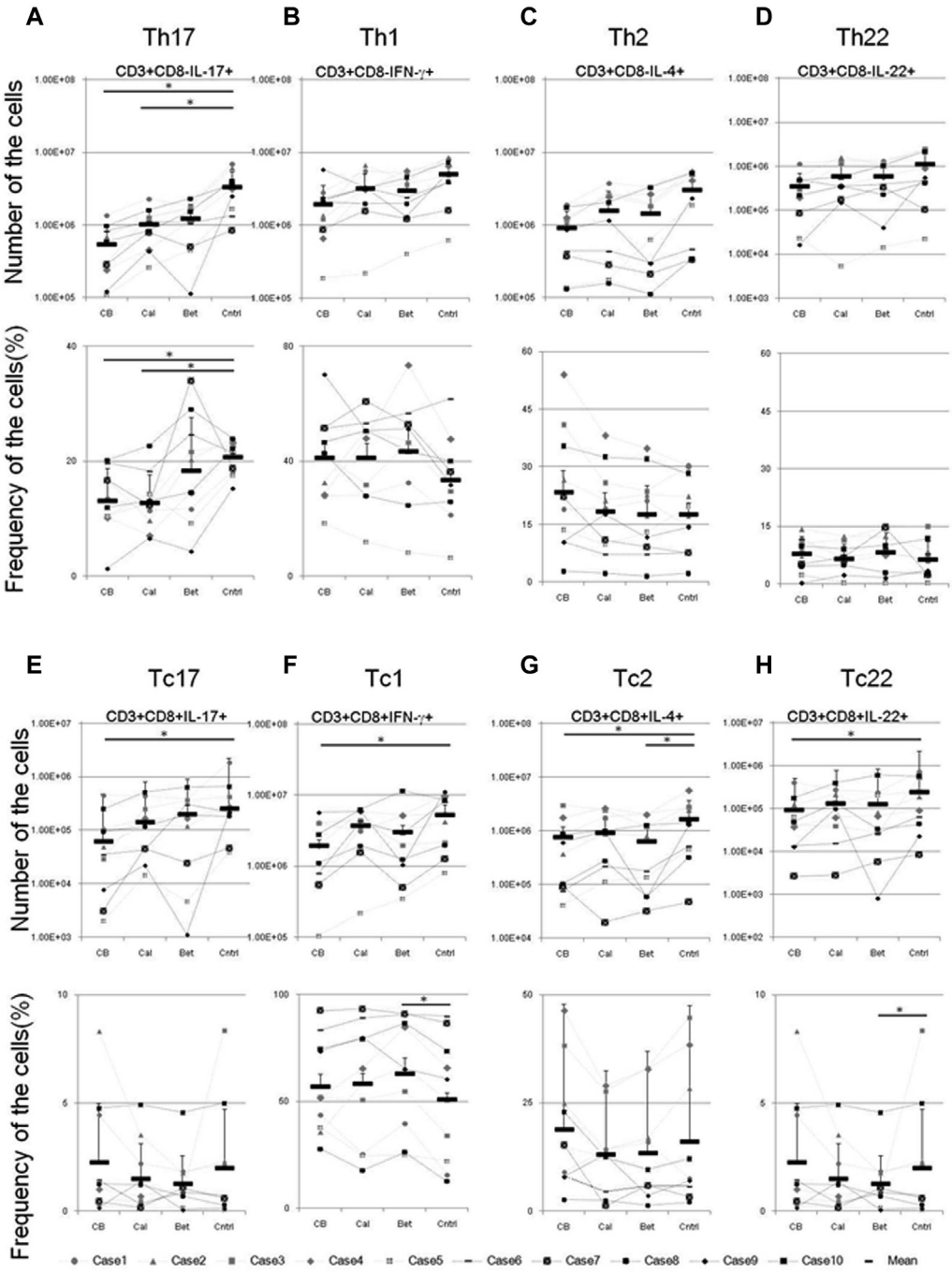

FIG 5. **A–H**, Number and frequency of expanded cells. T cells were expanded with anti-CD3/CD28 mAbs and IL-2 from biopsied skin topically treated with CB, Cal, or Bet or left untreated. They were analyzed in the surface phenotype and intracellular cytokines by using flow cytometric analysis. *$P < .05$. *Cntrl*, Control.

with or without 1 μmol/L Bet, 100 nmol/L Cal, or both for 96 hours. Then the numbers of live and dead cells were counted (Fig 7, *A*) and analyzed for apoptosis by using Annexin V. The frequency of the live cells was significantly decreased by the addition of Bet or Bet plus Cal, whereas Cal did not affect the viability of the cells (Fig 7, *B*). The frequency of Annexin V^+ 7-aminoactinomycin D $(7AAD)^-$ cells was increased by Bet or Bet plus Cal, suggesting that Bet, but not Cal, induces T-cell apoptosis (Fig 7, *C*).

To examine whether Cal or Bet is capable of inducing apoptosis specifically to certain T-cell subpopulations, we isolated naive T cells from 3 healthy donors and stimulated them to differentiate into T_H1, T_H2, and T_H17 cells *in vitro*. The differentiated cells were treated with Cal, Bet, or both and subjected to the apoptosis assay. The induction of apoptosis did not depend on the cell type but on the agents added (Fig 7, *D*). The limitation of this assay was that the frequencies of IL-4–producing T_H2 cells and IL-17A–producing T_H17 cells were less than 10%.

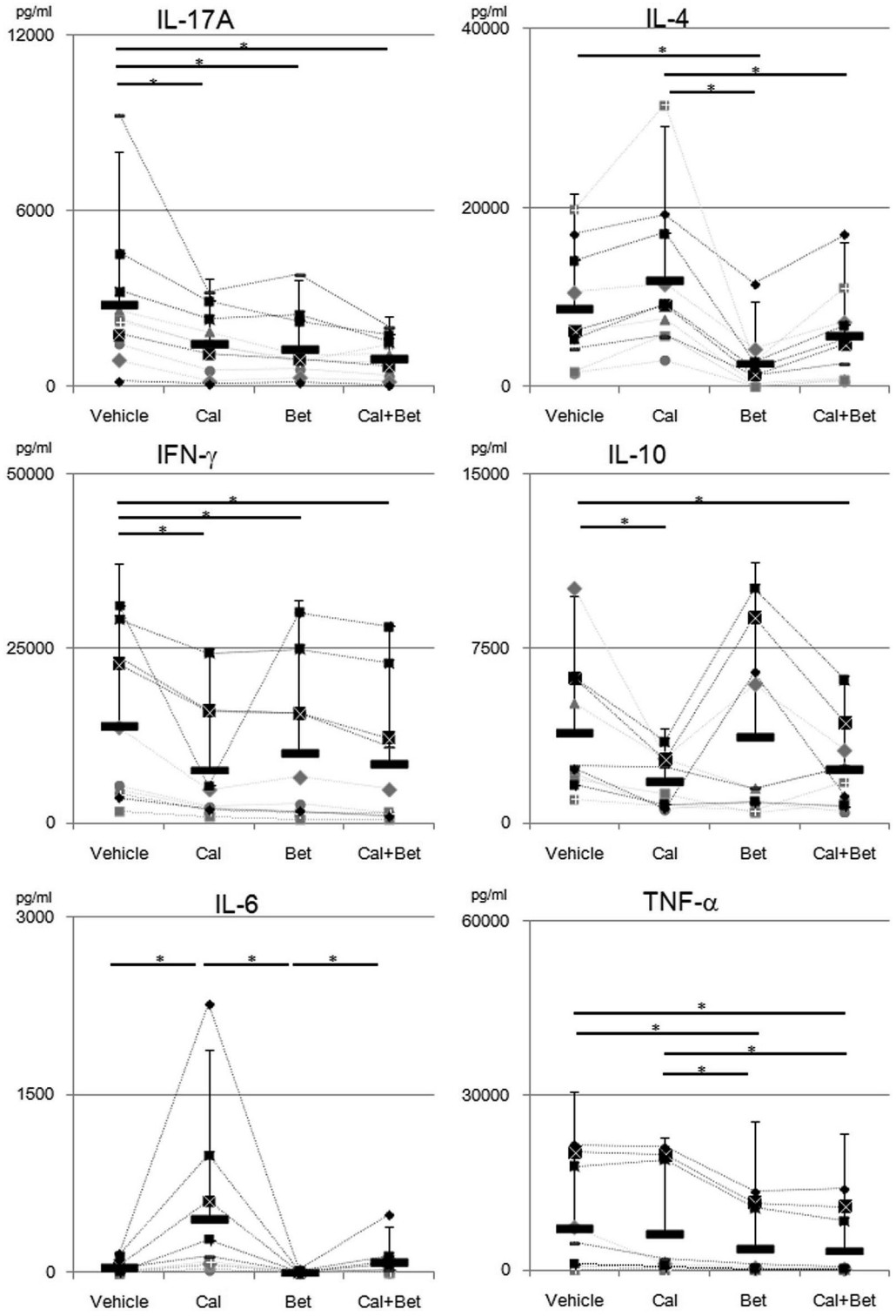

FIG 6. Cytokine production by T cells from control *(Cntrl)* skin in response to anti-CD3 antibody stimulation under the conditions of 100 nmol/L Cal, 1 μmol/L Bet, or both (Cal+Bet). **P* < .05.

Cal downregulates *IL23A* and *CCL20* expression in lesional skin and retinoic acid receptor–related orphan receptor C and α expression in expanded lesional T cells

To address the mechanism underlying T_H17 inhibition, we performed quantitative PCR of skin samples taken from the 3 patients (cases 6, 8, and 10) who responded well to Cal. We found that *CCL20* (chemoattracting T_H17 cells) and *IL23A* (maintaining T_H17 cells) expression was downregulated by Cal application along with *IL17A* reduction (Fig 8, *A*). Furthermore, expanded T cells from nontreated psoriatic skin in cases 1 to 5 were stimulated with anti-CD3 mAb and incubated with Cal. Expression of

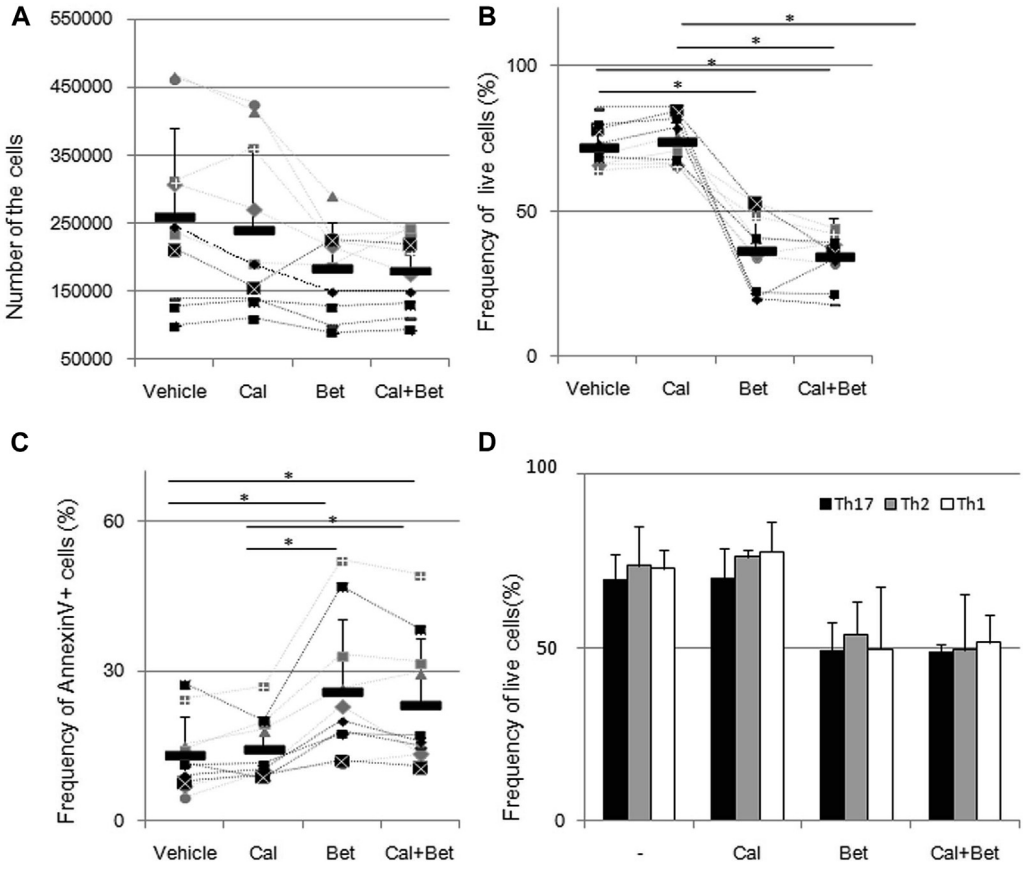

FIG 7. A–D, Total cell number and frequency of live cells and frequency of Annexin V^+ cells among $7AAD^-$ cells. Cells were cultured for 96 hours under conditions of 1 μmol/L Bet, 100 nmol/L Cal, or both (Cal+Bet). *$P < .05$. *Cntrl*, Control.

retinoic acid receptor–related orphan receptor (*ROR*) *C* and *RORα* was assessed by using real-time PCR. The mRNA expression of *RORC* was downmodulated by Cal in all cases, and that of *RORα* was decreased in 3 of 5 cases (Fig 8, *B*). These results suggest that vitamin D decreases the production of T_H17-attracting chemokines and T_H17-maintaining cytokine levels in the skin and also directly suppresses master gene expression of T_H17 cells. Therefore both direct and indirect mechanisms might exist in T_H17 inhibition by Cal.

DISCUSSION

There have been a considerable number of clinical studies on the combination efficacy of topical corticosteroids and vitamin D3 analogues.[29-34] In most reports it was concluded that the combination therapy is greater than a single agent in terms of therapeutic effectiveness or anti-inflammatory response. In this study we selected 4 untreated psoriatic plaques with similar severities in the individual patients and treated each of the lesions with either Cal or Bet ointment or their combination for 2 weeks. One lesion was left untreated as a control. Because of the technical difficulty, the majority of previous reports have shown only clinical and histologic findings, and alteration of pathogenic skin-infiltrating T_H17 cells has not been fully investigated. Here we used the skin-derived T cell–expanding *ex vivo* system. Even though the expansion step might slightly increase both T_H1 and T_H2 cell numbers, the frequency of T_H17 cells was minimally affected, and the expanded T cells reflect the original T-cell population.[28] We demonstrated that the number of skin-infiltrating T cells was decreased by both treatments with a single corticosteroid and vitamin D3 analogue. It is noted that the combination more strongly decreased skin infiltration of T cells than single-agent treatments, especially T_H17 cells. Cal preferentially inhibited T_H17 cells and upmodulated T_H2 cells without induction of apoptosis, whereas Bet uniformly downmodulated all cytokines at least partly by exerting apoptosis. It was reported that topical application of the vitamin D3 analogue reduced the number of T cells and the frequencies of skin-infiltrating $CD8^+IL\text{-}17A^+$ T cells and $CD45^+IL\text{-}17A^+$ cells but not $CD4^+IL\text{-}17A^+$ cells.[35] Our results indicate that not only $CD8^+IL\text{-}17A^+$ cells but also $CD4^+IL\text{-}17A^+$ cells, namely T_H17 cells, were decreased in frequency. The cytokine production assay further supported the decrease in numbers of IL-17A–producing T cells in Cal- or CB-treated skin. Other studies also have suggested that vitamin D3 reduces the frequency or function of T_H17 cells in mice[36] and human subjects.[37]

Although the detailed mechanism underlying the preferential inhibition of T_H17 cells by vitamin D3 is not fully understood, both direct and indirect mechanisms can be put forward. The direct mechanism is that T cells express vitamin D3 receptors and that signaling through the receptor blocks binding of the transcription factor nuclear factor of activated T cells 1 to the promotor of the IL-17 gene.[38] In addition to nuclear factor of activated T cells 1,[39,40] RORα and RORγt are also essential for

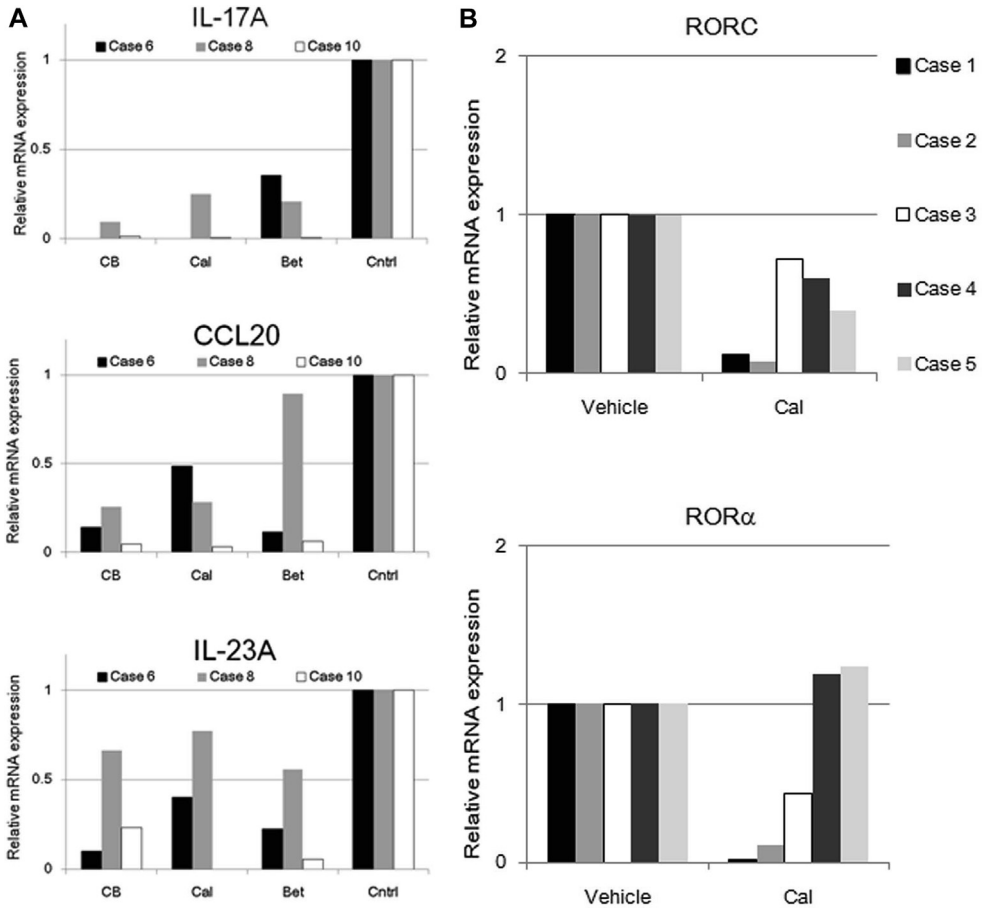

FIG 8. T_H17-related gene expression in lesional skin and expanded T cells. **A,** Relative mRNA expression for *IL17A*, *CCL20*, and *IL23A* in skin samples from 3 representative patients (cases 6, 8, and 10) topically treated with CB, Cal, or Bet or left untreated *(Cntrl)*. Whole skin samples were homogenized to extract mRNA and subjected to quantitative RT-PCR. **B,** Relative mRNA expression for *RORC* and *RORα* in expanded T cells from nontreated or Cal-treated skin specimens of 5 patients (cases 1-5).

T_H17 differentiation and generation.[41,42] Vitamin D3 also has the capacity to downregulate *RORα* and *RORC*[37,43] in T cells or PBMCs, which is presumably consistent with dose-dependent inhibition of T_H17 cells by vitamin D3.[37] In our *in vitro* study *RORC* gene expression was suppressed by Cal in all 5 investigated cases, and *RORα* expression was also strongly suppressed in some cases.

On the other hand, there are several potential mechanisms of indirect inhibition of T_H17 cells by vitamin D3. One of the major targets of vitamin D3 in patients with psoriasis is considered the keratinocyte. Keratinocytes produce CCL20, a ligand for CCR6 and a chemoattractant to T_H17 cells, thereby inducing $CCR6^+$ T_H17 cell–associated inflammation in the skin. Moreover, CCL20 expression is upregulated by T_H17 cytokines[44] to form a positive feedback loop. The mRNA expression of *CCL20* was also strongly downregulated by Cal and CB treatment, and this can be one of the major mechanisms of suppression of T_H17 cell infiltration.

In addition to the CCL20-dependent mechanism, *IL23A* gene expression was also downregulated. IL-23 is produced by macrophages and DCs and enhances the T_H17 cell proliferation,[45] and Cal and CB have been shown to have inhibitory effect of IL-23 secretion by DCs.[34] Thus downregulation of this pathway can also be involved in the decreased number of T_H17 cells in our T-cell expansion study.

Keratinocytes also expresses antimicrobial peptides or alarmins, such as psoriasin, koebnerisin, and cathelicidin LL37, expression of which is upregulated by IL-17A. These alarmins can prime epidermal keratinocytes for production of immunotropic cytokines that further amplify the inflammatory response.[46] Because Cal suppresses antimicrobial peptide expression,[46,47] this might provide an alternative indirect mechanism.

In our *in vitro* culture of T cells derived from the patients' untreated skin lesions, Cal increased the production of IL-4 in all cases. Thus Cal can directly and indirectly lead to T_H2 polarization. The type 2 cytokine environment can decrease T_H1-preponderant inflammation, resulting in alleviation of psoriasis.

In contrast to Cal, Bet exerted nonskewing suppressive effects on T cells because topical treatment of Bet suppressed *ex vivo* expansion of T_H17 cells, as well as other T-cell populations. In general, corticosteroids have a strong inhibitory effect on T-cell function. Our histologic and *ex vivo* data also demonstrated that Bet reduced T-cell infiltration in psoriatic skin lesions, and the *in vitro* data showed that Bet induced apoptosis of psoriatic skin-infiltrating T cells and uniformly inhibited production of T-cell cytokines, including IL-17A. However, recent investigation has suggested that there are glucocorticoid-resistant T_H17 cells called proinflammatory T_H17 cells[48,49] and that vitamin D3

inhibits IL-17A production in a glucocorticoid-independent manner in patients with severe asthma.[50] Proinflammatory T_H17 cells in human subjects are $CCR6^+CXCR3^{high+}CCR4^{low}CCR10^-CD161^+$ and produce both IL-17 and IFN-γ.[49] Given the presence of proinflammatory T_H17 cells in psoriatic lesions, a certain subset of T_H17 cells can survive even after Bet treatment. Further application of Cal might eradicate this T_H17 population. Such proinflammatory T_H17 cells, identified as $IL\text{-}17A^+IFN\text{-}\gamma^+$ cells, were observed in our expanded cells, and their frequency was higher in T cells from Bet-treated skin than in T cells from Cal-treated skin (Bet vs Cal was 5.89% ± 4.59% vs 2.9% ± 1.59% [mean ± SD]; $P = .013$, Wilcoxon signed-rank test), suggesting that proinflammatory T_H17 cells are resistant to Bet but susceptible to Cal.

In summary, Cal and Bet normalize psoriatic changes through different means, with decreased T_H17 cell expansion in the skin lesions. It is likely that the different actions of the 2 drugs on T cells and even subpopulations of T_H17 cells yield a superior antipsoriatic effect in relation to therapeutic effectiveness.

Clinical implications: Our *ex vivo* T-cell expansion study showed that topical treatments of Cal and Bet downmodulated psoriatic skin-infiltrating T-cell populations in different ways and that combination therapy is superior to monotreatment.

REFERENCES

1. Osamu N, Hirotaka N, Koji S, Kenji T. Clinical pharmacology of the anti-IL-17 receptor antibody brodalumab (KHK4827) in Japanese normal healthy volunteers and Japanese subjects with moderate to severe psoriasis: a randomized, dose-escalation, placebo-controlled study. J Dermatol Sci 2014;75:201-4.
2. Langley RG, Elewski BE, Lebwohl M, Reich K, Griffiths CE, Papp K, et al. Secukinumab in plaque psoriasis—results of two phase 3 trials. N Engl J Med 2014; 371:326-38.
3. Leonardi C, Matheson R, Zachariae C, Cameron G, Li L, Edson-Heredia E, et al. Anti-interleukin-17 monoclonal antibody ixekizumab in chronic plaque psoriasis. N Engl J Med 2012;366:1190-9.
4. Papp KA, Leonardi C, Menter A, Ortonne JP, Krueger JG, Kricorian G, et al. Brodalumab, an anti-interleukin-17-receptor antibody for psoriasis. N Engl J Med 2012;366:1181-9.
5. Mirandola P, Gobbi G, Micheloni C, Vaccarezza M, Di Marcantonio D, Ruscitti F, et al. Hydrogen sulfide inhibits IL-8 expression in human keratinocytes via MAP kinase signaling. Lab Invest 2011;91:1188-94.
6. Koga C, Kabashima K, Shiraishi N, Kobayashi M, Tokura Y. Possible pathogenic role of Th17 cells for atopic dermatitis. J Invest Dermatol 2008;128:2625-30.
7. Aggarwal S, Ghilardi N, Xie MH, de Sauvage FJ, Gurney AL. Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. J Biol Chem 2003;278:1910-4.
8. Zaba LC, Fuentes-Duculan J, Eungdamrong NJ, Abello MV, Novitskaya I, Pierson KC, et al. Psoriasis is characterized by accumulation of immunostimulatory and Th1/Th17 cell-polarizing myeloid dendritic cells. J Invest Dermatol 2009; 129:79-88.
9. Yoshiki R, Kabashima K, Honda T, Nakamizo S, Sawada Y, Sugita K, et al. IL-23 from Langerhans cells is required for the development of imiquimod-induced psoriasis-like dermatitis by induction of IL-17A-producing gammadelta T cells. J Invest Dermatol 2014;134:1912-21.
10. Nestle FO, Conrad C, Tun-Kyi A, Homey B, Gombert M, Boyman O, et al. Plasmacytoid predendritic cells initiate psoriasis through interferon-alpha production. J Exp Med 2005;202:135-43.
11. Suzuki T, Hirakawa S, Shimauchi T, Ito T, Sakabe J, Detmar M, et al. VEGF-A promotes IL-17A-producing gammadelta T cell accumulation in mouse skin and serves as a chemotactic factor for plasmacytoid dendritic cells. J Dermatol Sci 2014;74:116-24.
12. Midorikawa K, Sayama K, Shirakata Y, Hanakawa Y, Sun L, Hashimoto K. Expression of vitamin D receptor in cultured human keratinocytes and fibroblasts is not altered by corticosteroids. J Dermatol Sci 1999;21:8-12.
13. Binderup L, Bramm E. Effects of a novel vitamin D analogue MC903 on cell proliferation and differentiation in vitro and on calcium metabolism in vivo. Biochem Pharmacol 1988;37:889-95.
14. Hashimoto K, Matsumoto K, Higashiyama M, Nishida Y, Yoshikawa K. Growth-inhibitory effects of 1,25-dihydroxyvitamin D3 on normal and psoriatic keratinocytes. Br J Dermatol 1990;123:93-8.
15. Takahashi H, Ibe M, Kinouchi M, Ishida-Yamamoto A, Hashimoto Y, Iizuka H. Similarly potent action of 1,25-dihydroxyvitamin D3 and its analogues, tacalcitol, calcipotriol, and maxacalcitol on normal human keratinocyte proliferation and differentiation. J Dermatol Sci 2003;31:21-8.
16. Michel G, Mirmohammadsadegh A, Olasz E, Jarzebska-Deussen B, Muschen A, Kemeny L, et al. Demonstration and functional analysis of IL-10 receptors in human epidermal cells: decreased expression in psoriatic skin, down-modulation by IL-8, and up-regulation by an antipsoriatic glucocorticosteroid in normal cultured keratinocytes. J Immunol 1997;159:6291-7.
17. Milde P, Hauser U, Simon T, Mall G, Ernst V, Haussler MR, et al. Expression of 1,25-dihydroxyvitamin D3 receptors in normal and psoriatic skin. J Invest Dermatol 1991;97:230-9.
18. Berth-Jones J, Fletcher A, Hutchinson PE. Epidermal cytokeratin and immunocyte responses during treatment of psoriasis with calcipotriol and betamethasone valerate. Br J Dermatol 1992;126:356-61.
19. Boumpas DT, Chrousos GP, Wilder RL, Cupps TR, Balow JE. Glucocorticoid therapy for immune-mediated diseases: basic and clinical correlates. Ann Intern Med 1993;119:1198-208.
20. Wyllie AH. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature 1980;284:555-6.
21. Liddicoat DR, Kyparissoudis K, Berzins SP, Cole TJ, Godfrey DI. The glucocorticoid receptor 1A3 promoter correlates with high sensitivity to glucocorticoid-induced apoptosis in human lymphocytes. Immunol Cell Biol 2014;92:825-36.
22. Ferreira GB, Vanherwegen AS, Eelen G, Gutierrez AC, Van Lommel L, Marchal K, et al. Vitamin D3 induces tolerance in human dendritic cells by activation of intracellular metabolic pathways. Cell Rep 2015 [Epub ahead of print].
23. Chu CC, Ali N, Karagiannis P, Di Meglio P, Skowera A, Napolitano L, et al. Resident CD141 (BDCA3)+ dendritic cells in human skin produce IL-10 and induce regulatory T cells that suppress skin inflammation. J Exp Med 2012; 209:935-45.
24. Prietl B, Treiber G, Mader JK, Hoeller E, Wolf M, Pilz S, et al. High-dose cholecalciferol supplementation significantly increases peripheral CD4(+) Tregs in healthy adults without negatively affecting the frequency of other immune cells. Eur J Nutr 2014;53:751-9.
25. Bakdash G, Schneider LP, van Capel TM, Kapsenberg ML, Teunissen MB, de Jong EC. Intradermal application of vitamin D3 increases migration of CD14+ dermal dendritic cells and promotes the development of Foxp3+ regulatory T cells. Hum Vaccin Immunother 2013;9:250-8.
26. Boonstra A, Barrat FJ, Crain C, Heath VL, Savelkoul HF, O'Garra A. 1alpha,25-Dihydroxyvitamin d3 has a direct effect on naive CD4(+) T cells to enhance the development of Th2 cells. J Immunol 2001;167:4974-80.
27. Long CC, Finlay AY. The finger-tip unit—a new practical measure. Clin Exp Dermatol 1991;16:444-7.
28. Hashizume H, Hansen A, Poulsen LK, Thomsen AR, Takigawa M, Thestrup-Pedersen K. In vitro propagation and dynamics of T cells from skin biopsies by methods using interleukins-2 and -4 or anti-CD3/CD28 antibody-coated microbeads. Acta Derm Venereol 2010;90:468-73.
29. Kragballe K, Barnes L, Hamberg KJ, Hutchinson P, Murphy F, Moller S, et al. Calcipotriol cream with or without concurrent topical corticosteroid in psoriasis: tolerability and efficacy. Br J Dermatol 1998;139:649-54.
30. Papp KA, Guenther L, Boyden B, Larsen FG, Harvima RJ, Guilhou JJ, et al. Early onset of action and efficacy of a combination of calcipotriene and betamethasone dipropionate in the treatment of psoriasis. J Am Acad Dermatol 2003;48:48-54.
31. van der Velden HM, Pasch MC, van Erp PE, van Lingen RG, Otero ME, de Boer-van Huizen RT, et al. Treatment of plaque psoriasis with the two-compound product calcipotriol/betamethasone dipropionate versus both monotherapies: an immunohistochemical study. J Dermatolog Treat 2010;21:13-22.
32. Vissers WH, Berends M, Muys L, van Erp PE, de Jong EM, van de Kerkhof PC. The effect of the combination of calcipotriol and betamethasone dipropionate versus both monotherapies on epidermal proliferation, keratinization and T-cell subsets in chronic plaque psoriasis. Exp Dermatol 2004;13:106-12.
33. Hino R, Kabashima R, Kawakami C, Sugita K, Nakamura M, Tokura Y. Circulating Th17 cell fluctuation in psoriatic patients treated with topical calcipotriol and betamethasone butyrate propionate. J Eur Acad Dermatol Venereol 2011;25:242-4.
34. Lovato P, Norsgaard H, Tokura Y, Ropke MA. Calcipotriol and betamethasone dipropionate exert additive inhibitory effects on the cytokine expression of inflammatory dendritic cell-Th17 cell axis in psoriasis. J Dermatol Sci 2016; 81:153-64.

35. Dyring-Andersen B, Bonefeld CM, Bzorek M, Lovendorf MB, Lauritsen JP, Skov L, et al. The vitamin D analogue calcipotriol reduces the frequency of CD8+ IL-17+ T cells in psoriasis lesions. Scand J Immunol 2015;82:84-91.
36. Bruce D, Yu S, Ooi JH, Cantorna MT. Converging pathways lead to overproduction of IL-17 in the absence of vitamin D signaling. Int Immunol 2011;23:519-28.
37. Ikeda U, Wakita D, Ohkuri T, Chamoto K, Kitamura H, Iwakura Y, et al. 1alpha,25-Dihydroxyvitamin D3 and all-trans retinoic acid synergistically inhibit the differentiation and expansion of Th17 cells. Immunol Lett 2010;134:7-16.
38. Joshi S, Pantalena LC, Liu XK, Gaffen SL, Liu H, Rohowsky-Kochan C, et al. 1,25-dihydroxyvitamin D(3) ameliorates Th17 autoimmunity via transcriptional modulation of interleukin-17A. Mol Cell Biol 2011;31:3653-69.
39. Chatterjee M, Hedrich CM, Rauen T, Ioannidis C, Terhorst C, Tsokos GC. CD3-T cell receptor co-stimulation through SLAMF3 and SLAMF6 receptors enhances RORgammat recruitment to the IL17A promoter in human T lymphocytes. J Biol Chem 2012;287:38168-77.
40. Kaminuma O, Kitamura N, Mori A, Tatsumi H, Nemoto S, Hiroi T. NFAT1 and NFAT2 differentially regulate IL-17A expression in human T cells. Int Arch Allergy Immunol 2012;158(suppl 1):30-4.
41. Chang JH, Cha HR, Lee DS, Seo KY, Kweon MN. 1,25-Dihydroxyvitamin D3 inhibits the differentiation and migration of T(H)17 cells to protect against experimental autoimmune encephalomyelitis. PLoS One 2010;5:e12925.
42. Solt LA, Kumar N, Nuhant P, Wang Y, Lauer JL, Liu J, et al. Suppression of TH17 differentiation and autoimmunity by a synthetic ROR ligand. Nature 2011;472:491-4.
43. Agak GW, Qin M, Nobe J, Kim MH, Krutzik SR, Tristan GR, et al. Propionibacterium acnes induces an IL-17 response in acne vulgaris that is regulated by vitamin A and vitamin D. J Invest Dermatol 2014;134:366-73.
44. Harper EG, Guo C, Rizzo H, Lillis JV, Kurtz SE, Skorcheva I, et al. Th17 cytokines stimulate CCL20 expression in keratinocytes in vitro and in vivo: implications for psoriasis pathogenesis. J Invest Dermatol 2009;129:2175-83.
45. Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, Stockinger B. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. Immunity 2006;24:179-89.
46. Hegyi Z, Zwicker S, Bureik D, Peric M, Koglin S, Batycka-Baran A, et al. Vitamin D analog calcipotriol suppresses the Th17 cytokine-induced proinflammatory S100 "alarmins" psoriasin (S100A7) and koebnerisin (S100A15) in psoriasis. J Invest Dermatol 2012;132:1416-24.
47. Sakabe J, Umayahara T, Hiroike M, Shimauchi T, Ito T, Tokura Y. Calcipotriol increases hCAP18 mRNA expression but inhibits extracellular LL37 peptide production in IL-17/IL-22-stimulated normal human epidermal keratinocytes. Acta Derm Venereol 2014;94:512-6.
48. Schewitz-Bowers LP, Lait PJ, Copland DA, Chen P, Wu W, Dhanda AD, et al. Glucocorticoid-resistant Th17 cells are selectively attenuated by cyclosporine A. Proc Natl Acad Sci U S A 2015;112:4080-5.
49. Ramesh R, Kozhaya L, McKevitt K, Djuretic IM, Carlson TJ, Quintero MA, et al. Pro-inflammatory human Th17 cells selectively express P-glycoprotein and are refractory to glucocorticoids. J Exp Med 2014;211:89-104.
50. Nanzer AM, Chambers ES, Ryanna K, Richards DF, Black C, Timms PM, et al. Enhanced production of IL-17A in patients with severe asthma is inhibited by 1alpha,25-dihydroxyvitamin D3 in a glucocorticoid-independent fashion. J Allergy Clin Immunol 2013;132:297-304.e3.

METHODS

Reagents, antibodies, and culture medium

Fluorescein isothiocyanate–, phycoerythrin (PE)–, peridinin-chlorophyll-protein complex–, or allophycocyanin-conjugated mAbs against CD3, CD4, and CD8 were purchased from BD PharMingen. Fluorescein isothiocyanate– or PE-conjugated mAbs against IL-4, IL-17, IL-22, and IFN-γ were purchased from eBioscience (San Diego, Calif). Cells were cultured in RPMI 1640 medium (Life Technologies, Carlsbad, Calif) supplemented with L-glutamine, sodium pyruvate, 2-mercaptoethanol, nonessential amino acids (Life Technologies), and 10% heat-inactivated FCS or pooled human AB serum (cRPMI), as previously described.[E1]

Induction of $T_H1/T_H2/T_H17$ cells

PBMCs from 3 healthy donors were isolated by using the Ficoll-Hypaque method, and naive $CD4^+$ T cells were isolated by using the Naive T-cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's protocol. For induction of T_H1, T_H2, and T_H17 cells, cRPMI supplemented with 10 μg/mL anti-CD28 mAbs (Miltenyi Biotec), 10 ng/mL rhIL-12 (R&D Systems), and 10 μg/mL anti–IL-4 antibody; 20 ng/mL rhIL-2 (R&D Systems), 10 μg/mL anti-CD28 mAbs, 20 ng/mL rhIL-4 (R&D Systems), and 10 mg/mL anti–IL-12 mAb (Miltenyi Biotec); or 10 μg/mL anti-CD28 mAbs, 10 ng/mL TGF-β (R&D Systems), 10 ng/mL IL-6 (R&D Systems), and 20 ng/mL IL-23 (R&D Systems) was used for differentiation medium of T_H1, T_H2, and T_H17, respectively. Naive $CD4^+$ T cells (10^5 cells/mL) were cultured in an anti-CD3 mAb (Miltenyi Biotec)–coated 24-well dish for 4 days, and differentiation medium without anti-CD28 mAbs was added from day 5 to 7.

Cell survival assay

Stocked cells expanded from nontreated skin were suspended in cRPMI supplemented with 10 U/mL human recombinant IL-2 (2×10^5/well). One micromole per liter of Bet and/or 0.1 μmol/L Cal or vehicle (0.01% dimethyl sulfoxide and ethanol) was added and cultivated for 4 days. All cells were collected, and the numbers of live and dead cells were counted manually by staining with 0.4% wt/vol Trypan Blue Solution (Wako, Osaka, Japan). The rest of the cells were washed twice with PBS and stained with Annexin V and 7AAD and analyzed by using fluorescence-activated cell sorting with the PE Annexin V Apoptosis Detection Kit 1 (BD), according to the manufacturer's protocol.

Real-time PCR

Some part of the fresh skin samples for pathologic investigation was homogenized and used for RNA extraction in cases 6, 8, and 10. *Ex vivo* expanded skin-infiltrating T cells from nontreated skin were stimulated with anti-CD3 mAb with or without Cal and/or Bet treatment for 48 hours, and mRNA was extracted by using the RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. cDNA synthesis was performed with TaqMan Reverse transcription regents (Applied Biosystems, Foster City, Calif). cDNA was amplified by using quantitative real-time PCR with TaqMan Gene Expression Assays (*IL4*, Hs00174122_m1; *IL17A*, Hs00174383_m1; *IL23*, Hs00900828_g1; *IFN-γ*, Hs00989291_m1; *RORC*, Hs01076112_m1; *RORA*, Hs00536545_m1; *CCL20*, Hs00355476_m1; and glyceraldehyde-3-phosphate dehydrogenase *[GAPDH]*, Hs99999905_m1) with qPCR Mastermix Plus (Eurogenetec, Seraing, Belgium) and the 7000 Sequence Detection System (Applied Biosystems, Foster City, Calif).

PBMC expansion

To verify the method, PBMCs from 5 healthy donors were isolated by using the Ficoll-Hypaque method, and $CD4^+$ T cells were isolated by using the T-cell Isolation Kit (Miltenyi Biotec), according to the manufacturer's protocol. Isolated T cells (10^4/sample) were cultivated under the same conditions as the skin-infiltrating T cells from patients with psoriasis. Numbers of the cells in the culture well were kept at less than 10^6 cells/well in the 6-well plate.

REFERENCE

E1. Hashizume H, Takigawa M, Tokura Y. Characterization of drug-specific T cells in phenobarbital-induced eruption. J Immunol 2002;168:5359-68.

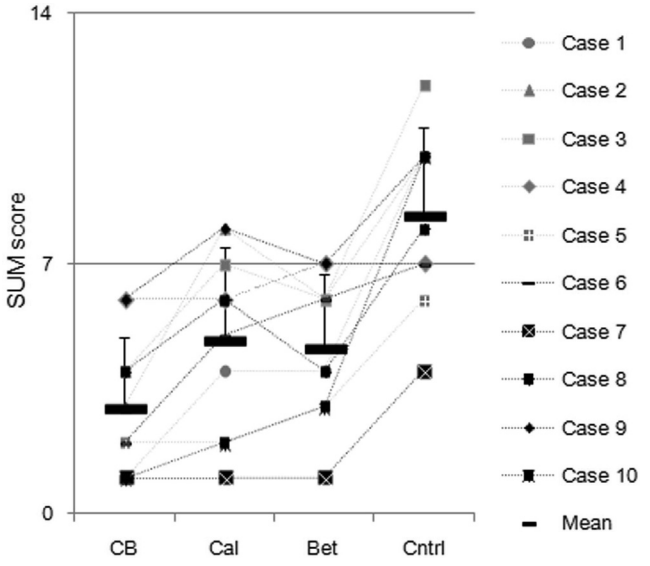

FIG E1. SUM score, which includes the severity (0- to 4-point scale) of erythema, induration, and scaling of the 10 cases. *Cntrl*, Control.

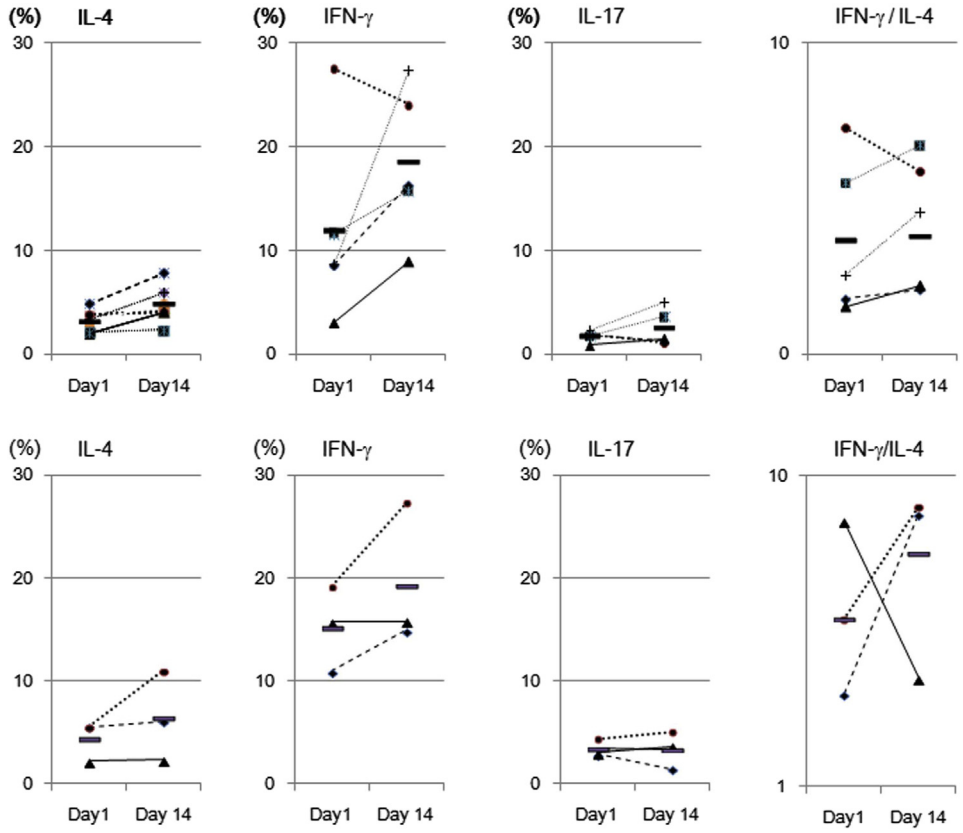

FIG E2. Cytokine profiles of $CD4^+$ *(upper panels)* and $CD4^+CD45RO^+$ (memory helper T cells; *lower panels*) cells before and after expansion. $CD4^+$ T cells were isolated from PBMCs from 5 healthy donors and expanded with IL-2 and anti-CD3/CD28 antibodies for 2 weeks.

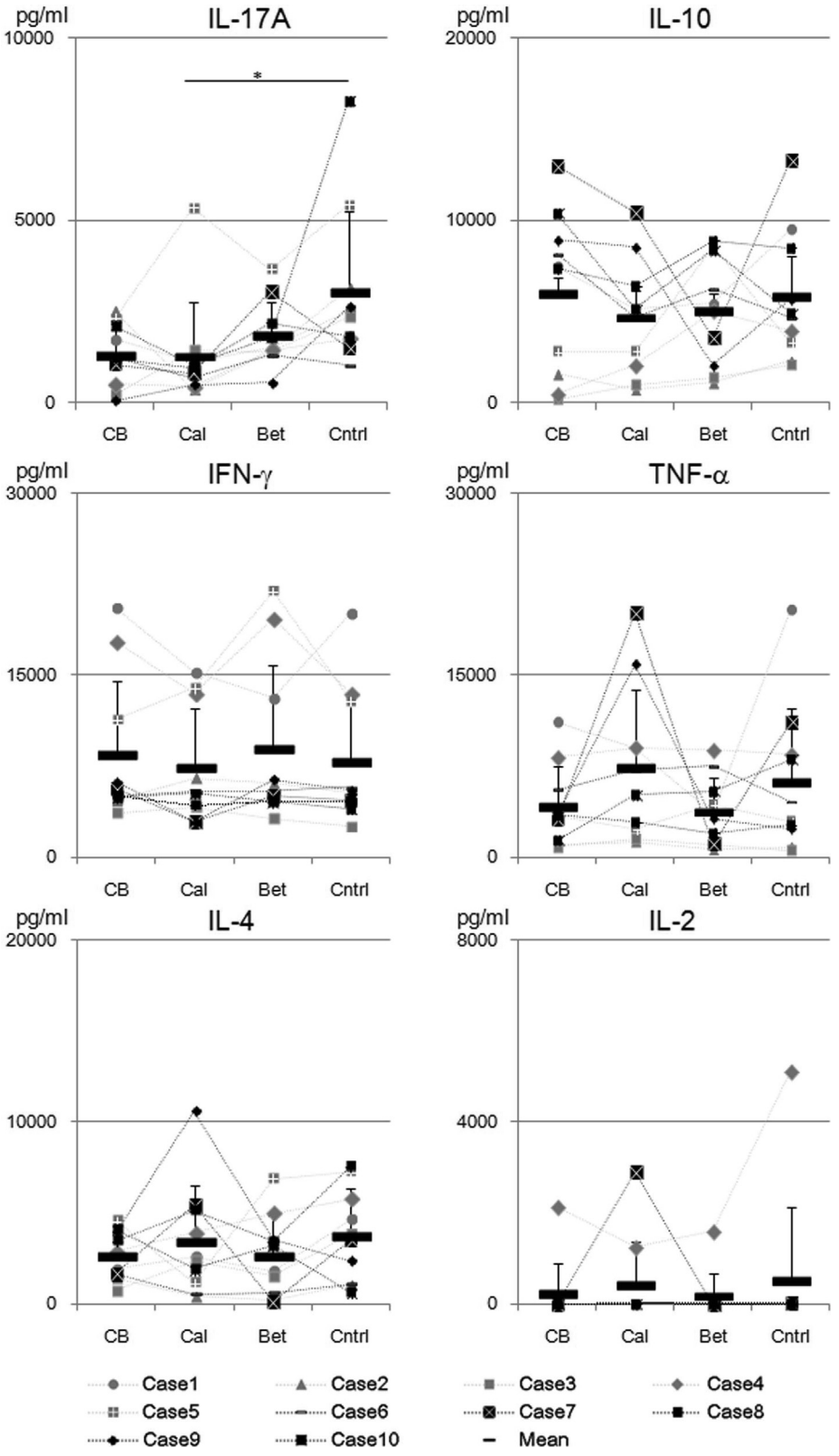

FIG E3. Cytokine concentration of culture supernatants. T cells were expanded with anti-CD3/CD28 mAbs and IL-2 from biopsied skin topically treated CB, Cal, or Bet or left untreated. Cytokine production by T cells in response to anti-CD3 antibody stimulation was assessed by means of Cytokine Bead Array measurement of cytokines in the culture supernatants. *$P < .05$. *Cntrl*, Control.

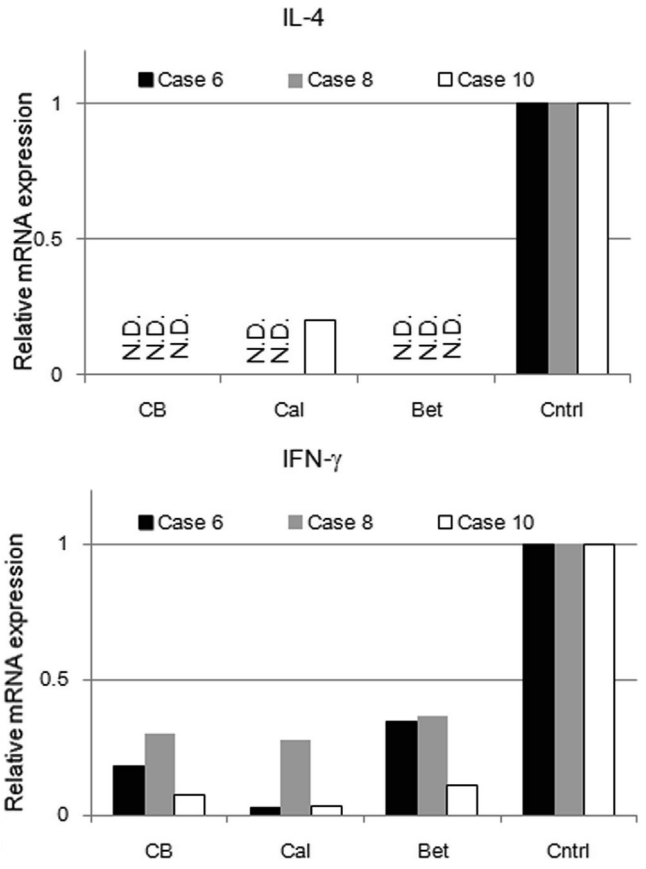

FIG E4. T_H1- and T_H2-related gene expression in lesional skin. Relative mRNA expression for *IL4* and *IFN-γ* in skin samples from 3 representative patients (cases 6, 8, and 10) topically treated with CB, Cal, or Bet or left untreated *(Cntrl)*.

double knockout of Kcnk4 and Kcnk5, however, suggested that there may be some redundancy of K^+ channels in sweat glands, and sufficient activity remains or compensates when only one is lost. As a less likely speculative alternative, different channels might function differentially in different cells, with one cell type(s) able to increase its activity to compensate for the loss of function in another type.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

This work was supported in part by Intramural Research Program of National Institute on Aging. *Kcnk5* knockout mice and *Kcnn4* knockout mice were kindly provided by Dr. Bayliss and Dr. Melvin, respectively.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jdermsci.2015.11.001.

References

[1] K. Sato, W.H. Kang, K. Saga, K.T. Sato, Biology of sweat glands and their disorders. I. Normal sweat gland function, J. Am. Acad. Dermatol. 20 (1989) 537–563.
[2] C.Y. Cui, D. Schlessinger, Eccrine sweat gland development and sweat secretion, Exp. Dermatol. 24 (2015) 644–650.
[3] F. Sato, K. Sato, Effect of periglandular ionic composition and transport inhibitors on rhesus monkey eccrine sweat gland function in vitro, J. Physiol. 393 (1987) 195–212.
[4] K. Saga, Structure and function of human sweat glands studied with histochemistry and cytochemistry, Prog. Histochem. Cytochem. 37 (2002) 323–386.
[5] M. Kunisada, C.Y. Cui, Y. Piao, M.S. Ko, D. Schlessinger, Requirement for Shh and Fox family genes at different stages in sweat gland development, Hum. Mol. Genet. 18 (2009) 1769–1778.
[6] C.P. Lu, L. Polak, A.S. Rocha, et al, Identification of stem cell populations in sweat glands and ducts reveals roles in homeostasis and wound repair, Cell 150 (2012) 136–150.
[7] C.Y. Cui, V. Childress, Y. Piao, et al., Forkhead transcription factor FoxA1 regulates sweat secretion through Bestrophin 2 anion channel and Na–K–Cl cotransporter 1, Proc. Natl. Acad. Sci. U. S. A. 109 (2012) 1199–1203.
[8] R. Warth, H. Barrière, P. Meneton, et al., Proximal renal tubular acidosis in TASK2 K+ channel-deficient mice reveals a mechanism for stabilizing bicarbonate transport, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 8215–8220.
[9] T. Begenisich, T. Nakamoto, C.E. Ovitt, et al., Physiological roles of the intermediate conductance, Ca^{2+}-activated potassium channel Kcnn4, J. Biol. Chem. 279 (2004) 47681–47687.
[10] A. Gabriel, M. Abdallah, C.S. Yost, B.D. Winegar, C.H. Kindler, Localization of the tandem pore domain K^+ channel KCNK5 (TASK-2) in the rat central nervous system, Brain Res. Mol. Brain Res. 98 (2002) 153–163.

Chang-Yi Cui*, Jian Sima
Mingzhu Yin
Marc Michel
Laboratory of Genetics, National Institute on Aging, National Institutes of Health, 251 Bayview Blvd., Suite 100, Baltimore, MD, USA

Makoto Kunisada
Division of Dermatology, Department of Internal Related, Graduate School of Medicine, Kobe University, Kobe, Japan

David Schlessinger
Laboratory of Genetics, National Institute on Aging, National Institutes of Health, 251 Bayview Blvd., Suite 100, Baltimore, MD, USA

* Corresponding author.
E-mail address: cuic@mail.nih.gov (C. Cui).

Received 14 October 2015
Received in revised form 17 November 2015
Accepted 26 November 2015

http://dx.doi.org/10.1016/j.jdermsci.2015.11.001

Letter to the Editor

Reciprocal contribution of Th17 and regulatory T cells in severe drug allergy

Keywords
$CD4^+$ T lymphocyte
Stevens–Johnson syndrome
Toxic epidermal necrolysis
Th17
Treg
Drug-induced hypersensitivity syndrome

Stevens–Johnson syndrome (SJS)/toxic epidermal necrolysis (TEN) are pathogenetically classified into the same entity characterized by epidermal necrosis with diversity of the affected area. Drug-induced hypersensitivity syndrome (DIHS), also called as drug rash with eosinophilia and systemic symptoms, is clinically distinct from SJS/TEN by a delayed onset after taking the inducing drug, severe cutaneous and extracutaneous organ involvement and the reactivation of human herpesviruses during disease course. Although cytotoxic $CD8^+$ T cells play a crucial role in the pathogenesis of both disease spectrums, the roles of $CD4^+$ T cells that collocate with $CD8^+$ T cells in skin lesions remain poorly understood. To clarify this issue, we immunologically investigated pathogenetic $CD4^+$ T cells from the skin lesions of these diseases and delineated their characteristics.

All patients (SJS/TEN, $n = 6$; DIHS, $n = 10$, supplementary Table E1) enrolled in this study were informed and agreed to participate. The skin-infiltrating T cell analysis was approved by the ethical committee of Hamamatsu Univirsity School of Medicine. We first expanded infiltrating (for SJS and DIHS) and blister-containing (for TEN) T cells from skin lesions using our previously established method [1] and generated drug-specific $CD4^+$ T cell clones (TCCs) from these cells. After stimulation with phorbol 12-myristate 13-acetate and ionomycin an intracellular interleukin (IL)-17 was stained with fluorescent-tagged antibody (R&D Systems, Minneapolis, MN, USA) in skin-infiltrating cells, and after stimulation with immobilized anti-CD3 mAb for 72 h, concentrations of IL-2, IL-4, IL5, IL-6, IL-8, IL-10, IL-17, interferon (IFN)-γ, and tissue necrosis factor (TNF)-α were measured in culture supernatants of $CD4^+$ TCCs by cytometric bead array assay (Th1/Th2/Th17 cytokine CBA and inflammation cytokine CBA kits; BD Biosciences).

Abbreviations: DIHS, drug-induced hypersensitivity syndrome; DRESS, drug rash with eosinophilia and systemic symptoms; SJS, Stevens–Johnson syndrome; TCCs, T cell clones; TEN, toxic epidermal necrolysis.

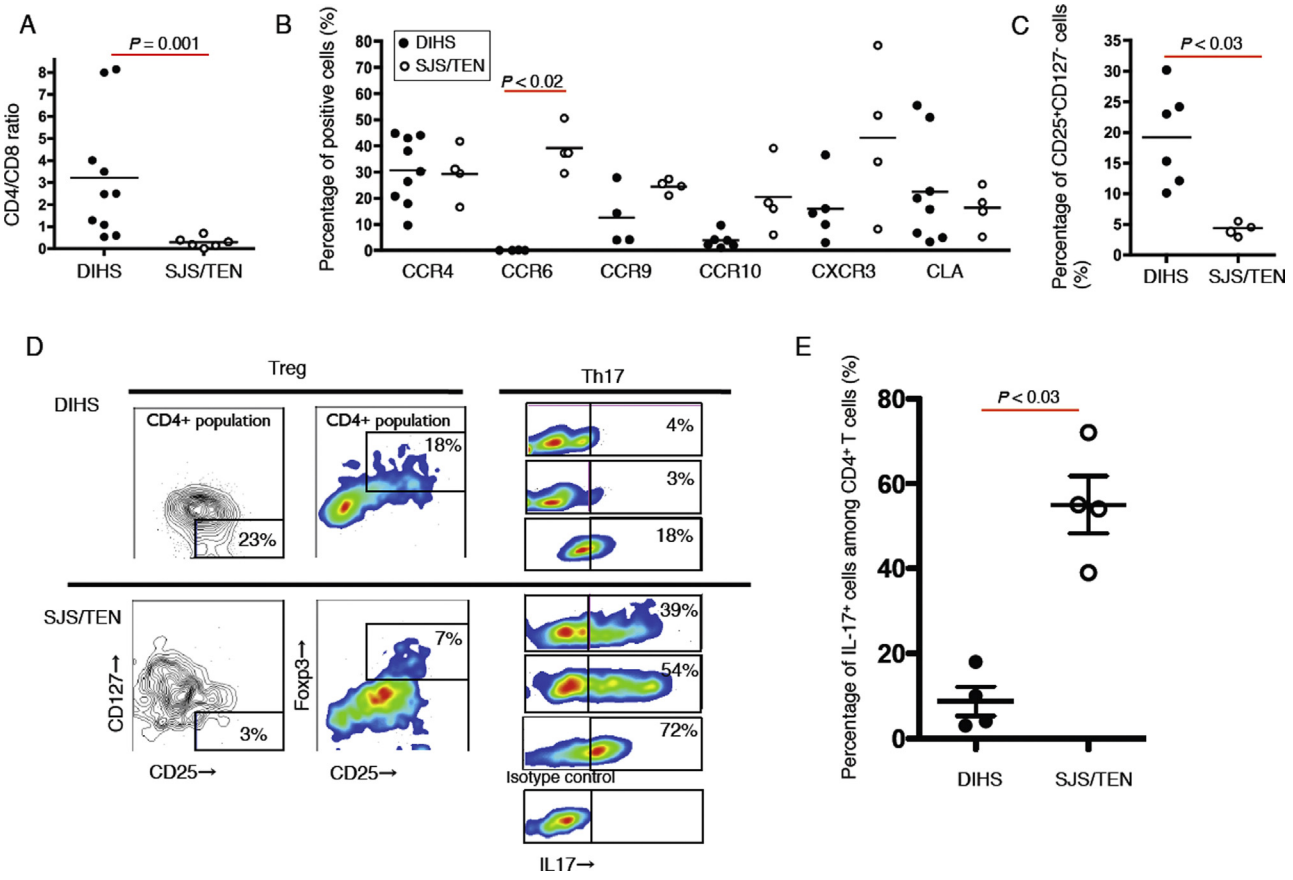

Fig. 1. Different characteristics of $CD4^+$ T cells from skin lesions between SJS/TEN and DIHS. (A) CD4/CD8 ratio. (B) Chemokine receptor expressions. (C) Percentage of T cells indicating Treg phenotype ($CD25^+CD127^-$) in $CD4^+$ cells. (D) Foxp3 and IL-17 expression. IL-17 expression was measured after stimulation with phorbol myristate acetate (10 ng/ml) for 3 h. Numbers indicate percentage of gated cells among the $CD4^+$ cells. Representative data of $CD4^+$ $CD25^+$ CD127- cells/$CD4^+$ $CD25^+$ $Foxp3^+$ cells from a DIHS and a SJS patients (left), and IL-17 expression of 3 DIHS patients (Pt#1-3) and 3 SJS/TEN patients (Pt#4-6) (right). (E) Percentage of T cells expressing IL-17 in $CD4^+$ cells.

The ratio of $CD4^+$ T cells/$CD8^+$ T cells in SJS/TEN skin lesions ($n = 6$) was significantly lower than in DIHS/DRESS skin lesions ($n = 10$) (Fig. 1A, $P < 0.001$, Mann–Whitney test). The percentage of $CCR6^+$ cells among total $CD4^+$ T cells was significantly greater in SJS/TEN ($n = 4$) than DIHS ($n = 4$) (Fig. 1B, $P < 0.02$, Mann–Whitney test). Higher levels of IL-17 production were observed in $CD4^+$ T cells from SJS/TEN skin lesions ($n = 4$) than in DIHS skin lesions ($n = 4$) after phorbol myristate acetate stimulation (Fig. 1D, right and Fig. 1E). Consistent with this, immunofluorescence analysis revealed that IL-17^+ $CD4^+$ cells infiltrated in the SJS/TEN skin lesions but not in the DIHS skin lesions (Supplementary Fig. E1). On the other hand, the percentage of $CD4^+CD25^+CD127^-$ cells, likely Treg cells, increased in T cells from DIHS skin lesions compared with those from SJS/TEN (Fig. 1C, $P < 0.03$, Mann–Whitney test, and Fig. 1D, left in Treg). We also confirm an increase of Treg cells by comparison of $CD4^+CD25^+Foxp3^+$ cells in expanded T cells between DIHS and SJS/TEN skin lesions after a more 4 days' culture with low dosage of IL-2 in several cases (Fig. 1D, right in Treg). We also found higher production of IL-17 in $CD4^+$ T cells expanded from SJS/TEN lesions than those from DIHS/DRESS lesions (Fig. 1D). These observations suggest that Th17 and Treg cells dominated in skin lesions of SJS/TEN and DIHS, respectively, although we could not convince that these cells were pathogenetic. To confirm these findings, we further investigated cytokine production of drug-reactive $CD4^+$ T cells; generated 15 drug-reactive $CD4^+$ TCCs from skin lesions including acetaminophen-induced TEN (4 clones), ibuprofen- and phenobarbital-induced SJS (3 clones and 2 clones), and carbamazepine-induced hypersensitivity syndrome (DIHS) (6 clones). TCCs of SJS/TEN released significantly higher IL-17 (mean ± SD, 15,680 pg/ml ± 13,990 pg/ml; $p = 0.026$, Mann–Whitney test) compared with DIHS, in which TCCs produced marginal levels (58.7 pg/ml ± 21.2 pg/ml) (Fig. 2A). Furthermore, IL-17 concentration had tendency to be greater in a severer clinical type, TEN than SJS. High amounts of IFN-γ and TNF-α were detected in SJS/TEN and DIHS groups, however, production levels were significantly higher in the latter than the former ($p = 0.008$, Mann–Whitney test). Interestingly, we found that two drug-reactive IL-17^+ $CD4^+$ TCCs, E11 and C5 from SJS skin lesions had significant expression of granulysin as well as a drug-reactive IL-17^-CD8^+ TCC, C6 (Fig. 2B). Furthermore, we established one TCC that expressed the molecules specific to Tregs and showed an inhibitory effect on autologous lymphocyte proliferation form DIHS skin lesions (supplementary Fig. E2) despite hyporesponsiveness of Tregs to IL-2. Surprisingly, this clone expressed the human herpes virus (HHV)-6 antigen (Fig. 2C).

Th17/Treg cells are reciprocally generated from naïve $CD4^+$ T cells depending on their surrounding cytokine milieu. IL-17-producing $CD4^+$ T cells, designated Th17, express CCR6 and high levels of transcription factor RORγt, and contribute to defense against microorganisms and enhance inflammation and regeneration. On the other hand, Treg cells are $CD4^+CD25^{high}CD127^-$ and the forkhead family transcription factor $Foxp3^+$ and have regulatory function against inflammatory responses.

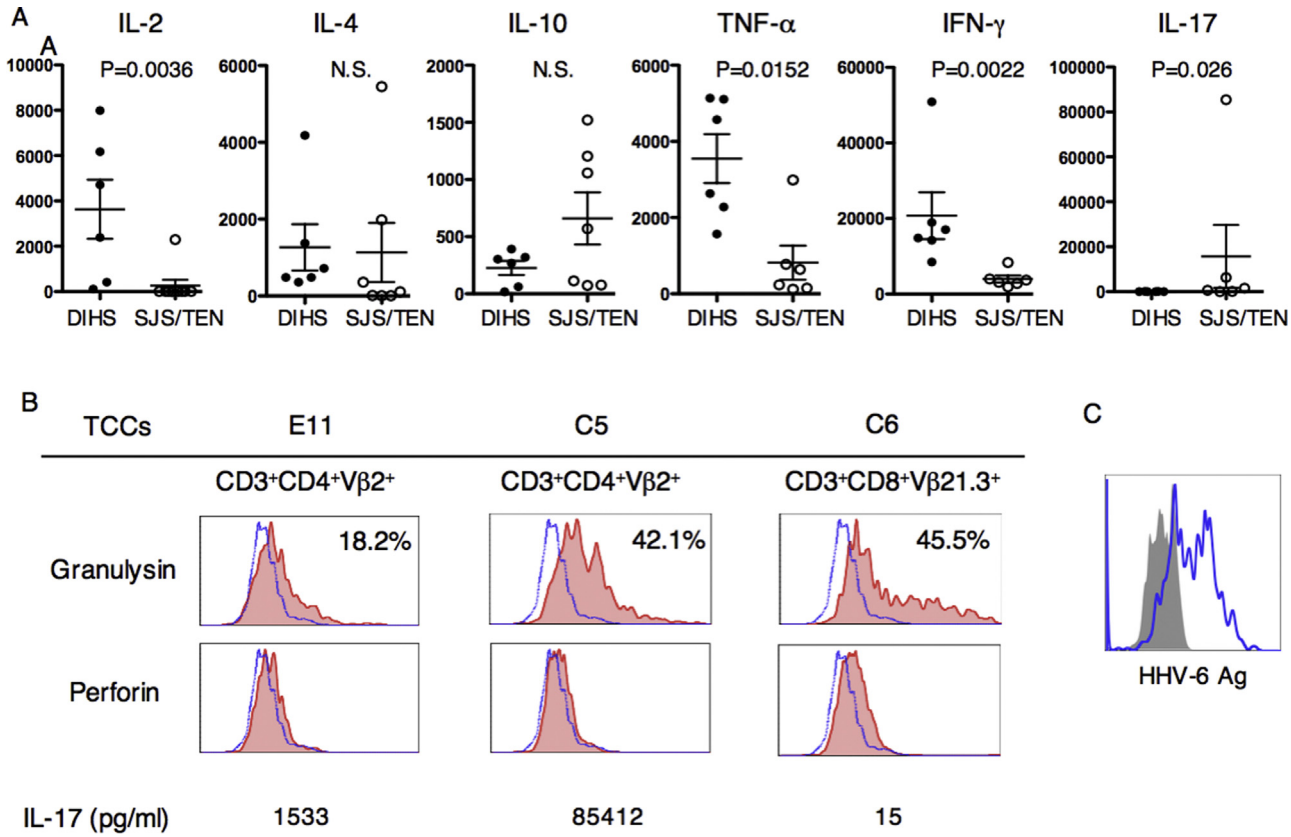

Fig. 2. Different characteristics of drug-specific TCCs from skin lesions/blood between SJS/TEN and DIHS. Drug specificity of TCCs was confirmed as previously described (Hashizume et al., J. Immunol. 2002; Hashizume et al., J. Imunnol. 2005). (A) Cytokine production (pg/ml) between SJS/TEN and DIHS. Vertical bars – mean value; horizontal bars – standard deviation. (B) Granulysin and perforin expression of 2CD4$^+$ TCCs (E11, C5) and a CD8$^+$ TCC (C6) from the skin lesions of SJS. Blue dots—isotype controls. C: HHV-6 antigen expression of a TCC from the skin lesion of DIHS. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Our observation suggests involvement of Th17 cells in the pathogenesis of SJS/TEN as reported previously [2,3] although their role remains unclear. Moreover, we firstly found that granulysin-expressing drug-reactive Th17 cells infiltrated in the skin lesions, suggesting that they originally infiltrate the skin in response to drug. Granulysin is a member of the saposin-like protein family that forms cytotoxic molecules with proinflammatory activity for the induction of keratinocyte apoptosis in SJS/TEN [4] and the activation of monocytes and dendritic cells by binding to Toll-like receptor-4/Myd88 as an alarmin [5]. On the other hand, the pathogenesis of DIHS/DRESS is complicated: CD4$^+$ T cells that respond to drug antigen initially infiltrate the skin following emergence of CD8$^+$ T cells that target virus-infected cells [6]. Emergence of great numbers of functional Treg cells in skin and blood in DIHS/DRESS [7] would modify antivirus immune response, resulting in the replication of HHV-6 [8], contrasting numerical and functional loss of Treg cells in SJS/TEN [7,9]. Furthermore, HHV-6 can infect Tregs resided in the skin and may play a special role in the pathogenesis [10]. Our observations demonstrate that the Th17/Treg axis contributes to the pathogenesis of SCARs, and may provide a perspective on therapeutic options in severe cutaneous adverse reactions by targeting the Th17/Treg axis.

Acknowledgements

This study was supported in part by a Grant-in-Aid for Scientific Research (No. 21591458) and a Health and Labor Sciences Research Grant (Research on Intractable Diseases) from the Ministry of Health, Labour and Welfare of Japan (H26-nanchi-ippan-003).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jdermsci.2015.11.002.

References

[1] H. Hashizume, A. Hansen, L.K. Poulsen, A.R. Thomsen, M. Takigawa, K. Thestrup-Pedersen, In vitro propagation and dynamics of T cells from skin biopsies by methods using interleukins-2 and -4 or anti-CD3/CD28 antibody-coated microbeads, Acta Derm. Venereol. 90 (2010) 468–473.
[2] Y. Teraki, M. Kawabe, S. Izaki, Possible role of TH17 cells in the pathogenesis of Stevens–Johnson syndrome and toxic epidermal necrolysis, J. Allergy Clin. Immunol. 131 (2013) 907–909.
[3] G. Porebski, T. Pecaric-Petkovic, M. Groux-Keller, M. Bosak, T.T. Kawabata, W.J. Pichler, In vitro drug causality assessment in Stevens–Johnson syndrome—alternatives for lymphocyte transformation test, Clin. Exp. Allergy 43 (2013) 1027–1037.
[4] W.H. Chung, S.I. Hung, J.Y. Yang, S.C. Su, S.P. Huang, C.Y. Wei, et al., Granulysin is a key mediator for disseminated keratinocyte death in Stevens–Johnson syndrome and toxic epidermal necrolysis, Nat. Med. 14 (2008) 1343–1350.
[5] P. Tewary, D. Yang, de, R. la, G. osa, Y. Li, M.W. Finn, A.M. Krensky, et al., Granulysin activates antigen-presenting cells through TLR4 and acts as an immune alarmin, Blood 116 (2010) 3465–3474.
[6] T. Shiohara, Y. Ushigome, Y. Kano, R. Takahashi. Crucial role of viral reactivation in the development of severe drug eruptions: a comprehensive review. Clin. Rev. Allergy Immunol. (in press).
[7] R. Takahashi, Y. Kano, Y. Yamazaki, M. Kimishima, Y. Mizukawa, T. Shiohara, Defective regulatory T cells in patients with severe drug eruptions: timing of the dysfunction is associated with the pathological phenotype and outcome, J. Immunol. 182 (2009) 8071–8079.
[8] H. Hashizume, T. Fujiyama, J. Kanebayashi, Y. Kito, M. Hata, H. Yagi, Skin recruitment of monomyeloid precursors involves human herpesvirus-6 reactivation in drug allergy, Allergy 68 (2013) 681–689.

[9] S. Iwai, H. Sueki, H. Watanabe, Y. Sasaki, T. Suzuki, M. Iijima, Distinguishing between erythema multiforme major and Stevens–Johnson syndrome/toxic epidermal necrolysis immunopathologically, J. Dermatol. 39 (2012) 781–786.
[10] S. Mine, K. Suzuki, Y. Sato, H. Fukumoto, M. Kataoka, N. Inoue, et al., Evidence for human herpesvirus-6B infection of regulatory T-cells in acute systemic lymphadenitis in an immunocompetent adult with the drug reaction with eosinophilia and systemic symptoms syndrome: a case report, J. Clin. Virol. 61 (2014) 448–452.

Hideo Hashizume[a,b,*]
[a]Department of Dermatology, Shimada Municipal Hospital, 1200-5 Noda, Shimada 427-8502, Japan, [b]Department of Dermatology, Hamamatsu University School of Medicine, Hamamatsu 431-3125, Japan

Toshiharu Fujiyama, Yoshiki Tokura
Department of Dermatology, Hamamatsu University School of Medicine, Hamamatsu 431-3125, Japan

* Corresponding author at: Department of Dermatology, Shimada Municipal Hospital, 1200-5 Noda, Shimada 427-8502, Japan.
Fax: +81 547 36 9155.
E-mail address: hihashiz0001@mac.com (H. Hashizume).

Received 14 October 2015
Received in revised form 17 November 2015
Accepted 26 November 2015

http://dx.doi.org/10.1016/j.jdermsci.2015.11.002

Letter to the Editor

Bi-allelic nonsense mutations in *ABHD5* underlie a mild phenotype of Dorfman-Chanarin syndrome

Keywords
Dorfman-Chanarin syndrome
ABHD5

Dear Editor,

Dorfman-Chanarin syndrome (DCS) (neutral lipid storage disease with ichthyosis) is an autosomal recessive disorder [1,2]. Nearly all cases present with skin manifestations of moderate to severe congenital ichthyosiform erythroderma (CIE). Other organs might be affected in the form of fatty liver, myopathy, hearing loss, sub-capsular cataract and mental retardation. Since 2001, ~30 mutations in *ABHD5* (also termed *CGI-58*), which encodes αβ hydrolase domain-containing 5 (ABHD5), have been found in DCS [3]. Here, we report compound heterozygous nonsense mutations in *ABHD5* (p.Arg234* and p.Arg280*) associated with particularly mild DCS that has implications for genotype–phenotype correlation.

The patient is a 9-year-old Chinese girl. She was born by Caesarean section. From the age of 2–3 months, she developed dry skin. She has no family history of consanguinity or skin disorders. On examination, she has generalized scaling with erythroderma on her face, trunk and extremities (Fig. 1a–c). She had no pruritus and no hair or nail abnormalities were observed. There was neither apparent cognitive impairment nor evidence of growth retardation. The patient had no subjective symptoms of muscle weakness, ear anomalies, hearing involvement, or cataract. We were not able to perform further investigations including peripheral blood film, lipid profile, cataract screening, electrocardiogram and echocardiogram. Routine serum biochemistry showed the following abnormal parameters: AST, 75 IU/L (normal range, 0–41); ALT, 88 IU/L (normal range, 0–45); LDH, 287 IU/L (normal range, 100–250). We checked titers of the anti-viral antibodies to rule out viral hepatitis caused by hepatitis type A, B, C, herpes simplex, cytomegalovirus and Epstein–Barr virus. All the antibodies were negative. Four months later, these mildly elevated enzymes spontaneously reverted to normal. Light microscopy of a lesional skin revealed marked hyperkeratosis with only a small number of parakeratotic cells. Intra-cytoplasmic lipid droplets within epidermal keratinocytes were not observed.

Following informed consent, and in accordance with the Declaration of Helsinki principles, genomic DNA from the patient was used for whole-exome sequencing analysis, using methodology described elsewhere [4]. In the patient, compound heterozygous *ABHD5* mutations were identified: a previously reported nonsense mutation c.700C > T (p.Arg234*) in exon 5 (Fig. 1d) and a novel nonsense mutation c.838C > T (p.Arg280*) in exon 6 (Fig. 1e) (GenBank accession no. NM_016006). Neither mutation was detected in DNA from 674 normal control individuals.

Most of the molecular pathology in DCS involves truncation mutations in *ABHD5*, although 8 missense mutations also have been reported (www.hgmd.cf.ac.uk). Of note, compound heterozygosity for two mutations c.700C > T (p.Arg234*) and c.245A > G (p.His82Arg) was previously reported in 42-year-old man with CIE, muscle weakness (including elevated creatine phosphokinase), progressive hearing loss and bilateral sub-capsular cataracts, although liver enzymes were normal [5]. With regard to the more severe phenotype in that case compared to our patient, we hypothesized that the different consequences of the second mutant allele (p.Arg280* vs p.His82Arg) might relate to the differences in clinical features. However, we cannot exclude the possibility that some features related to other organ systems onset in the later stage of the present case. Thus, in DCS cases, close monitoring for systemic involvements is recommended even if their phenotypes are mild in the childhood.

At present, genotype–phenotype correlation in DCS is uncertain. Previous reports have shown that certain homozygous missense mutations in *ABHD5* (p.Gln130Pro and p.Glu260Lys) underlie severe phenotypes of DCS [3]. It is known that Gln^{130} and Glu^{260} are important residues in the interaction with adipocyte triglyceride lipase, and these interactions are important for normal physiology of the cell [6]. In addition, amino acids 69–87 in ABHD5 form a highly hydrophobic region corresponding to the lipid-binding domain of protein [7]. The present nonsense mutations p.Arg234* and p.Arg280* cause premature termination of translation and result in truncated amino acid peptides instead of the normal protein. Thus, the reported active sites within the encoded transcript may be spared if some truncated mutant protein is synthesized. Nonsense mutations may also result in RNA decay unless sited in the last exon or distal third of the penultimate exon. Given that p.Arg280* is located within exon 6 of this 7 exon gene, we hypothesize that there may be some residual ABHD5 enzyme activity in this case, which contributed to her mild DCS phenotype.

The issue of transiently elevated liver enzymes is also difficult to explain based on the mutation findings. We previously reported a

Abbreviations: DCS, Dorfman-Chanarin syndrome; CIE, congenital ichthyosiform erythroderma; ABHD5, αβ hydrolase domain-containing 5.

JDA Japanese Dermatological Association
THE JOURNAL OF DERMATOLOGY
doi: 10.1111/1346-8138.13469
Journal of Dermatology 2016; ●●: 1–1

LETTER TO THE EDITOR

Hypocomplementemia is a diagnostic clue for parvovirus B19 infection in adults

Dear Editor,

Parvovirus B19 infection in adults manifests as a low grade fever, non-specific rash and edema of the extremities with the simultaneous emergence of polyarthralgia, mimicking diseases of other infections, drug eruptions, rheumatic arthritis and collagen disorders associated with arthralgia.[1,2] To prevent the transmission of this virus to others and more importantly to pregnant women in whom it can cause miscarriage,[3] a prompt diagnosis should be made. Detection of immunoglobulin (Ig)M antibodies to parvovirus B19 is a suitable test for diagnosis; however, this method is relatively time-consuming and costly, and in practical terms it is not covered by health insurance in Japan except for pregnant women. Of note, hypocomplementemia is frequently present in patients infected with parvovirus B19, similar to those with lupus erythematosus,[4,5] although the precise mechanism remains to be elucidated. Therefore, we considered that hypocomplementemia might provide an early diagnostic clue to parvovirus B19 infection, and investigated how many patients with suspected parvovirus B19 infection had hypocomplementemia, and validated whether they were "true" infected patients.

Fifteen adult patients with the sudden emergence of generalized rash, edema of the extremities and arthralgia, clinically suggestive of parvovirus B19 infection, were enrolled in this study after providing oral informed consent. All the patients visited our clinic within 5 days after the onset of clinical manifestations. Blood was taken i.v. from these patients to investigate serum complement levels and anti-parvovirus B19 IgM titers simultaneously. Patients with low serum levels of either CH50 or C4 had a significant association with positive anti-parvovirus B19 IgM ($P = 0.0256$ and $P = 0.0014$, respectively; Fisher's exact test; Table 1). There was no association between serum C3 levels and positive anti-parvovirus B19 IgM. Surprisingly, patients with low serum levels of either C3, C4 or CH50 (low in complements as indicated in Table 1) were exclusively positive for anti-parvovirus B19 IgM while others were negative. The duration of hypocomplementemia ranged 11–68 days (mean, 39.8; standard deviation, 23.4) in the five follow-up cases. Our results suggest that hypocomplementemia is an early diagnostic marker of parvovirus B19 infection in adults, and measurement of serum complement levels is a useful test for differential diagnosis of adult patients with suspected parvovirus B19 infection.

ACKNOWLEDGMENT: This study was supported in part by a Health and Labor Sciences Research Grant (Research on Intractable Diseases) from the Ministry of Health, Labor and Welfare of Japan (H26-nanchi-ippan-003).

CONFLICT OF INTEREST: None declared.

Hideo HASHIZUME, Reiko KAGEYAMA
Department of Dermatology, Shimada Municipal Hospital, Shimada, Japan

REFERENCES

1 Drago F, Ciccarese G, Broccolo F, Javor S, Parodi A. Atypical exanthems associated with Parvovirus B19 (B19V) infection in children and adults. *J Med Virol* 2015; **87**: 1981–1984.
2 Rezaei F, Sarshari B, Ghavami N *et al.* Prevalence and genotypic characterization of Human Parvovirus B19 in children with measles- and rubella-like illness in Iran. *J Med Virol* 2016; **88**: 947–953.
3 Nabae K, Satoh H, Nishiura H *et al.* Estimating the risk of parvovirus B19 infection in blood donors and pregnant women in Japan. *PLoS ONE* 2014; **9**: e92519.
4 Sugimoto T, Tsuda A, Uzu T, Kashiwagi A. Emerging lupus-like manifestations in acute parvovirus B19 infection. *Clin Rheumatol* 2008; **27**: 119–120.
5 Saeki T, Shibuya M, Sawada H *et al.* Human parvovirus B19 infection mimicking systemic lupus erythematosus. *Mod Rheumatol* 2001; **11**: 308–313.

Table 1. Positivity of anti-parvovirus B19 immunoglobulin M and serum complement levels

	C3*		C4**		CH50***		Complement†	
Ab	Low	Normal or high	Low	Normal or high	Low	Normal or high	Low	Normal or high
+	1	6	4	3	6	1	7	0
−	0	8	0	8	0	8	0	8

Values indicate numbers of patients. *$P = 0.467$, **$P = 0.0256$, ***$P = 0.0014$. †"Complement" denotes serum levels of C3, C4 and CH50, and "low" indicates low serum levels of either C3, C4 or CH50. $P = 0.0002$, Fisher's exact test. Ab, antibody.

Correspondence: Hideo Hashizume, M.D., Ph.D., Department of Dermatology, Shimada Municipal Hospital, 1200-5 Noda, Shimada, Shizuoka 427-8502, Japan. Email: hihashiz0001@mac.com

fact, this patient showed significantly increased number of CD3-positive cells compared with other AA patients (Fig. 1h–j). There was no significant differences in CD4/8 ratio between this patient and other AA patients. Considering that there are few case reports of AA with NF1[2,3] and that our case lacks typical trichoscopic findings as AA, it would be also possible that this is not a typical AA, but a novel case of alopecia mimicking AA associated with MC infiltration.

Further investigations are still needed to dissect the pathological connection between NF1 and alopecia including AA.

ACKNOWLEDGMENT: The authors would like to thank *Professor* Masahiko Osawa for performing excellent CD3, 4 and 8 immunohistochemistry.

CONFLICT OF INTEREST: None declared.

Yukari MIZUKAMI, Koji SUGAWARA, Daisuke TSURUTA
Department of Dermatology, Osaka City University Graduate School of Medicine, Osaka, Japan

doi: 10.1111/1346-8138.13183

REFERENCES

1 Bertolini M, Zilio F, Rossi A *et al.* Abnormal interactions between perifollicular mast cells and CD8+ T-cells may contribute to the pathogenesis of alopecia areata. *PLoS ONE* 2014; **9**: e94260.
2 Nanda A. Autoimmune diseases associated with neurofibromatosis type 1. *Pediatr Dermatol* 2008; **25**: 392–393.
3 Hubble D, Rogerson CH. Anxiety, Alopecia areata, neurofibromatosis, auricular fibrillation. *Br Med J* 1944; **1**: 486–488.
4 Yang FC, Ingram DA, Chen S *et al.* Nf1-dependent tumors require a microenvironment containing Nf1+/– and c-kit-dependent bone marrow. *Cell* 2008; **135**: 437–448.
5 Bulfone-Paus S, Bahri R *et al.* Mast Cells as Regulators of T Cell Responses. *Front Immunol* 2015; **6**: 394.

Neurotoxicity induced by the recommended acyclovir dosing in a dialysis patient with herpes zoster: A case letter

Dear Editor,

Acyclovir is highly effective and widely used in the treatment of herpes zoster (HZ) and herpes simplex. However, it is crucial for physicians to be aware of appropriate dosing, to prevent serious adverse effects.[1,2] We present the case of a dialysis patient with HZ who developed acyclovir neurotoxicity during treatment after administration of only two i.v. doses of 125 mg acyclovir at a 24-h interval. This dose was lower than that recommended by the manufacturer's data sheet for dialysis patients.

A 75-year-old man with a more than 6-year history of chronic renal failure who had been on hemodialysis for 2 years, presented with sudden appearance of erythema with multiple blisters on his right T1–T2 dermatomes. Bodyweight was 60 kg and body temperature was 38°C. The patient complained of headache and pain in the right arm. Based on the clinical features, a diagnosis of HZ was made, and he was admitted to our hospital for HZ treatment. After i.v. administration of 125 mg acyclovir 2.1 mg/kg per day, initiated on successive 2 days as recommended by the manufacturer's data sheet, the patient's fever and headache lessened. However, he suddenly complained of visual hallucinations, and dysarthria and dyskinesia of the arms and legs. On physical examination, patient blood pressure was 158/70 mmHg with heart rate 85 b.p.m. and oxygen saturation of 94% on room air. On neurological examination, he had slurred speech but the cranial nerves were intact. Brain computed tomography and magnetic resonance imaging revealed no specific findings except for small, old cerebral infarctions. Laboratory examination disclosed normal blood sugar levels and no ammonemia. Because of suspected acyclovir encephalopathy, acyclovir was immediately discontinued and the patient was restarted on hemodialysis. His neurological symptoms dramatically improved thereafter, and clinical symptoms completely disappeared 3 days later. The serum acyclovir doses during this episode were 5.96 μg/mL at 15 h, 4.73 μg/mL at 43 h and 1.49 μg/mL at 63 h after the last acyclovir administration, which were well correlated with his neurological improvement.

Because acyclovir is mainly eliminated by the kidneys, dose adjustment in patients with impaired renal function should be considered. Serum concentration levels of acyclovir above 2.5–4.5 μg/mL,[3] or its main metabolite carboxymethoxymethylguanine above 10.8 μmol/L,[4] cause neurotoxicity. i.v. acyclovir administration of 2.5–5 mg/kg at 24-h intervals is recommended for dialysis patients by the manufacturer's data sheet. Recently in Japan, recommended doses of acyclovir and oral valacyclovir for dialysis patients have been more strictly revised by the Japanese Society of Nephrology (http://jsnp.org/docs/yakuzai_dosing_26.pdf). In our case, despite keeping within this range, serum concentrations of acyclovir increased to neurotoxic levels, with simultaneous emergence of neurological symptoms. Serum carboxymethoxymethylguanine levels could not be measured. Moreover, dramatic

Correspondence: Hideo Hashizume, M.D., Ph.D., Department of Dermatology, Shimada Municipal Hospital, 1200-5 Noda, Shimada, Shizuoka 427-8502, Japan. Email: hihashiz0001@mac.com

improvement after discontinuation of acyclovir followed by hemodialysis supports our clinical impression that acyclovir directly caused the neurotoxicity. Our case shows that the dosing recommended by the manufacturer's data sheet does not consider prevention of acyclovir-induced neurotoxicity.[5] This highlights the importance of careful clinical monitoring in renal failure patients with HZ who are receiving even the recommended acyclovir dosing regimen.

CONFLICT OF INTEREST: None declared.

Reiko KAGEYAMA, Hideo HASHIZUME
Department of Dermatology, Shimada Municipal Hospital, Shimada, Japan

doi: 10.1111/1346-8138.13196

REFERENCES

1 Martinez-Diaz GJ, Hsia R. Altered mental status from acyclovir. *J Emerg Med* 2011; **41**: 55–58.
2 Haefeli WE, Schoenenberger RA, Weiss P, Ritz RF. Acyclovir-induced neurotoxicity: concentration-side effect relationship in acyclovir overdose. *Am J Med* 1993; **94**: 212–215.
3 Feldman S, Rodman J, Gregory B. Excessive serum concentrations of acyclovir and neurotoxicity. *J Infect Dis* 1988; **157**: 385–388.
4 Hellden A, Odar-Cederlof I, Diener P *et al.* High serum concentrations of the acyclovir main metabolite 9-carboxymethoxymethylguanine in renal failure patients with acyclovir-related neuropsychiatric side effects: an observational study. *Nephrol Dial Transplant* 2003; **18**: 1135–1141.
5 MacDiarmaid-Gordon AR, O'Connor M, Beaman M, Ackrill P. Neurotoxicity associated with oral acyclovir in patients undergoing dialysis. *Nephron* 1992; **62**: 280–283.

Erythema multiforme caused by triple therapy with amoxicillin, clarithromycin and vonoprazan for *Helicobacter pylori*

Dear Editor,
Erythema multiforme (EM) is an acute, self-limited skin disease associated with a hypersensitivity reaction to infections or medications.[1] Here, we report a case of EM that was triggered by triple therapy with amoxicillin, clarithromycin and vonoprazan for *Helicobacter pylori* infection.

A 69-year-old Japanese woman presented with a pruritic rush acutely occurring on the trunk. The patient had been treated with 1500 mg/day of amoxicillin, 400 mg/day of clarithromycin and 40 mg/day of vonoprazan for eradication of *H. pylori*. After the 7-day treatment, she developed erythematous macules and targetoid lesions on the trunk, which were coalesced (Fig. 1a) and rapidly spread to the face (Fig. 1b) and extremities (Fig. 1c). A biopsy specimen showed a lymphocytic infiltrate intermingled with eosinophils around small vessels in the upper dermis and lymphocytic exocytosis into the epidermis, indicating interface dermatitis (Fig. 1d). The patient had a fever reaching 39.0°C. As the skin eruption progressed, blisters and erosions arose on the face and trunk. Mucosal lesions were also observed as conjunctival congestion and painful lip erosions with less severity than Stevens–Johnson syndrome (SJS). Under the diagnosis of EM, the patient was treated with corticosteroid pulse therapy, followed by 5 g/day of i.v. immunoglobulin therapy for 3 days and 30 mg/day (0.5 mg/kg per day) of oral prednisolone. These treatments improved clinical symptoms and the dose of oral prednisolone was successfully tapered off. During the clinical course, we did not observe clinical manifestations or positive serum findings suggestive of herpes simplex virus infection or mycoplasma infection. To identify the causative drugs of EM, drug-induced lymphocyte transformation test (LTT) and patch tests were performed 3 months after the onset of the disease. Provocation test was not performed. Results of LTT showed a positive reaction to amoxicillin (stimulation index, 2.44; positive, >1.80), clarithromycin (2.20) and vonoprazan (2.39), and results of patch tests showed a positive reaction at 48 h to amoxicillin but not to clarithromycin or vonoprazan (Fig. 1e). Amoxicillin was the main culprit drug in our case. Results of LTT for clarithromycin and vonoprazan may be false positive.

The regimen most commonly recommended for the first-line therapy of *H. pylori* is triple therapy with amoxicillin, clarithromycin and a proton-pump inhibitor.[2] Amoxicillin-based triple therapy is safe in eradicating *H. pylori*, and the adverse effects are usually mild.[3] Several antibiotics, such as aminopenicillins and macrolides, are associated with significant but lower risk of severe cutaneous adverse reactions.[4] Thus, not amoxicillin only, but combination of clarithromycin and vonoprazan in addition to amoxicillin may cause severe drug eruptions. Vonoprazan, which is an orally bioavailable potassium-competitive acid blocker, has recently been approved in Japan for the treatment of acid-related diseases, including *H. pylori* eradication.[5] Currently, there are few reports about adverse drug reactions due to vonoprazan. Further case accumulation will contribute to the better understanding of drug eruptions induced by vonoprazan.

It should be kept in mind that triple therapy for *H. pylori* has a risk of severe mucocutaneous reactions such as EM and SJS.

JDA Japanese Dermatological Association
THE JOURNAL OF DERMATOLOGY
doi: 10.1111/1346-8138.13073
Journal of Dermatology 2016; **43**: 321–324

CONCISE COMMUNICATION

Toxic epidermal necrolysis caused by acetaminophen featuring almost 100% skin detachment: Acetaminophen is associated with a risk of severe cutaneous adverse reactions

Hideaki WATANABE,[1] Taisuke KAMIYAMA,[1] Shun SASAKI,[1] Kae KOBAYASHI,[1] Kenichiro FUKUDA,[2] Yasufumi MIYAKE,[2] Tohru ARUGA,[2] Hirohiko SUEKI[1]

[1]*Department of Dermatology, Showa University School of Medicine,* [2]*Department of Emergency and Critical Care Medicine, Showa University, Tokyo, Japan*

ABSTRACT

Toxic epidermal necrolysis (TEN) is an adverse reaction that can be induced by various drugs; the associated mortality rate is 20–25%. A previous report showed a weak association between TEN and acetaminophen. Recently, the US Food and Drug Administration declared that acetaminophen is associated with a risk of serious skin reactions, including TEN. Here, we describe the case of a 43-year-old Japanese woman with TEN caused by acetaminophen. She had poorly controlled ulcerative colitis and was treated with high doses of prednisolone, infliximab, acetaminophen and lansoprazole. Nine days after administrating acetaminophen, targetoid erythematous and bullous lesions appeared on the patient's trunk, palms and the soles of her feet. The skin lesions expanded rapidly; within 3 weeks, skin detachment was detected across nearly 100% of the patient's body. However, no mucosal involvement of the eyes, oral cavity or genitalia was found. We performed lymphocyte transformation tests using various drugs; however, a high stimulation index was obtained only with acetaminophen. The patient recovered following treatment with plasmapheresis, i.v. immunoglobulin therapy, topical medication and supportive therapy. Acetaminophen is included in many prescription and over-the-counter products; thus, clinicians should monitor their patients for severe drug reactions, including TEN.

Key words: acetaminophen, lymphocyte transformation test, severe cutaneous adverse reaction, toxic epidermal necrolysis.

INTRODUCTION

Toxic epidermal necrolysis (TEN) is a serious, life-threatening, cutaneous adverse drug reaction with a high mortality rate (20–25%).[1–3] The disorder is characterized by a rapidly developing blistering exanthema of purpuric macules and targetoid lesions accompanied by mucosal involvement and variable skin detachment.[4] TEN is defined as skin detachment exceeding 30%.[4] Strong associations have been reported between TEN and a number of drugs, including anticonvulsants, allopurinol, anti-infective sulfonamides and nevirapine.[2] A multivariate analysis showed a weak association with acetaminophen.[2] However, the US Food and Drug Administration recently informed the public that acetaminophen is associated with a risk of serious skin reactions, including TEN.[5] We herein describe a severe case of TEN due to acetaminophen that exhibited no mucosal involvement of the eyes, oral cavity or genitalia.

CASE REPORT

A 43-year-old Japanese woman with ulcerative colitis (UC) that was controlled with systemic prednisolone (PSL; 10 mg/day) reported an increase in symptoms. The patient's treatment regimen was changed to high doses of PSL (50 mg/day), infliximab (5 mg/kg), acetaminophen and lansoprazole, and her symptoms of UC improved. However, on 10 July 2013 (9 days after her first dose of acetaminophen), the patient noticed an erythematous eruption on her palms. Two days later, the patient visited her local hospital. Edematous erythema was detected on the patient's palms (Fig. 1a) and soles. Scattered, flat, atypical, targetoid, erythematous lesions were noted on the patient's trunk (Fig. 1b). Moreover, slight erosions and white crusts were evident on the lower lip. The initial diagnosis was hand–foot–mouth disease. Because the clinical findings of hand–foot–mouth disease sometimes mimic those of severe cutaneous adverse reactions,[6] drug eruption was raised as a differential

Correspondence: Hideaki Watanabe, M.D., Department of Dermatology, Showa University School of Medicine, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8666, Japan. Email: hwatanabe@med.showa-u.ac.jp
Received 3 June 2015; accepted 10 July 2015.

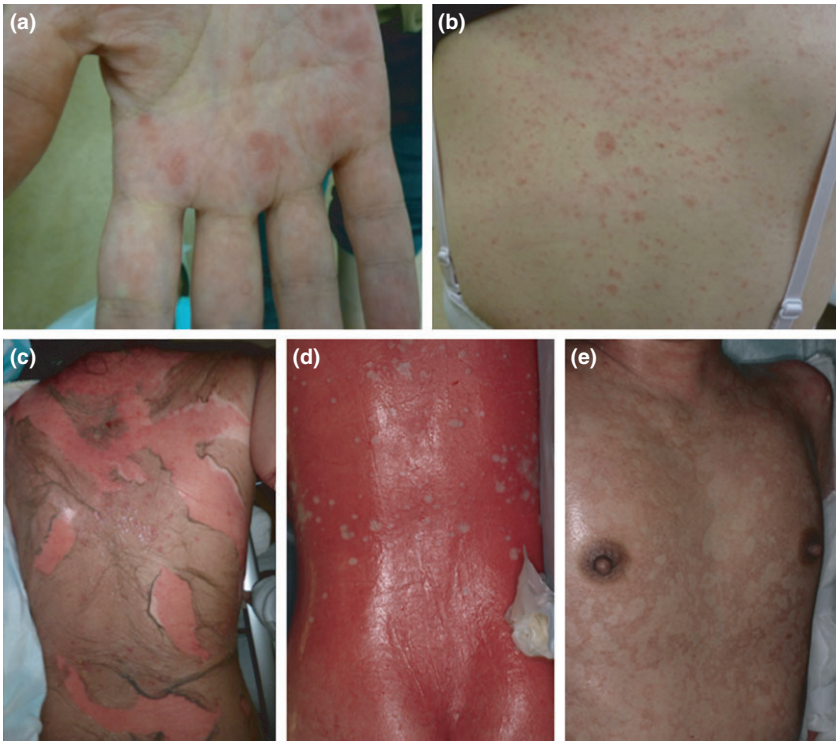

Figure 1. Clinical findings 2 days after toxic epidermal necrolysis (TEN) onset. (a) Edematous, erythematous lesions were seen on the patient's palms. (b) Papules and atypical targetoid erythematous lesions were also observed on her back. (c) Epidermal detachment was noted 12 days after the onset of TEN. (d) Skin detachment was noted across nearly 100% of the patient's body 20 days after the onset of TEN (on admission to our hospital). (e) One month after the onset of TEN, the patient's skin lesions had improved.

diagnosis. All drugs except PSL were discontinued. However, the patient's skin lesions expanded rapidly. Twelve days after the onset of TEN, epidermal detachment was detected on the patient's trunk (Fig. 1c). At that point, skin detachment was noted across 60% of the patient's body. The patient was treated with systemic PSL (50 mg/day) for TEN. She was also given i.v. immunoglobulins (3 g/day for 3 days and 10 g/day for 5 days).[7] In addition, plasmapheresis was performed twice, 12 and 14 days after the onset of TEN, respectively.[8,9] Despite these treatments, the patient's skin lesions did not improve, and methicillin-resistant *Staphylococcus aureus* (MRSA) was detected in her blood. The patient was transferred to our hospital 20 days after the onset of TEN (1 August 2013) (Fig. 2).

On admission to our hospital, epidermal detachment was noted across nearly 100% of the patient's body (Fig. 1d). However, no mucosal involvement of the eyes, oral cavity or genitalia was found.

Laboratory investigations on admission revealed the following: white blood cell count, 7.7 $\times$ 10^9/L (normal, 3.5–9); aspartate aminotransferase, 16 IU/L (10–30); alanine aminotransferase, 7 IU/L (5–25); serum creatinine, 0.38 mg/dL (0.5–0.9); total protein, 4.2 g/dL (6.7–8.4); albumen, 1.7 g/dL (4.0–5.1); and C-reactive protein, 5.20 mg/dL (<0.2). A lymphocyte transformation test (LTT) was performed with acetaminophen and lansoprazole; a positive result was obtained for

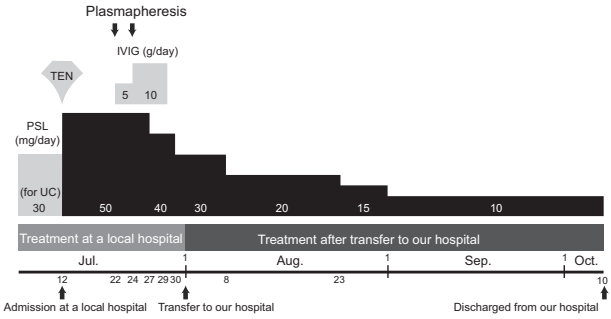

Figure 2. Treatment. A physician at the patient's local hospital treated her with systemic prednisolone (PSL) (30 mg/day) for ulcerative colitis (UC). The patient was also given intravenous immunoglobulin (IVIG) therapy (3 g/day for 3 days and 10 g/day for 5 days).[7] Moreover, plasmapheresis was performed twice.[8,9] Despite these treatments, the patient's skin lesions did not show adequate improvement. The systemic steroid dose was tapered in line with improvements in the patient's clinical symptoms. The patient was discharged without sequelae 75 days after the onset of TEN.

acetaminophen (stimulation index [SI] = 10.5; cut-off for the LTT, SI = 1.8), whereas a negative result was obtained for lansoprazole (SI = 1.1). Because the patient had severe UC, she

was given systemic PSL (30 mg/day: the same dose as on admission), which proved effective for the skin lesions. The patient was also treated with antibiotics for MRSA and methicillin-resistant coagulase-negative staphylococci sepsis. The dose of systemic PSL was tapered in line with the degree of improvement in the patient's clinical symptoms. Twelve days after being transferred to our hospital, the patient exhibited re-epithelialization across most of her skin (Fig. 1e). Given the patient's septic state, we changed the antibiotic regimen according to the results of blood cultures; antibiotic therapy was stopped when the sepsis was resolved. The PSL dose was tapered gradually; ultimately, 10 mg/day was found to effectively control the patient's UC. The patient was discharged 71 days after being transferred to our hospital. The UC relapsed 1 year after discharge and the patient was prescribed infliximab (5 mg/kg) once again. The UC is presently well-controlled with mesalazine, azathioprine and infliximab (5 mg/kg).

DISCUSSION

Here, we described a case of TEN caused by acetaminophen. Previous multivariate analyses conducted in Europe showed a weak or doubtful association of TEN with acetaminophen.[2] Recently, the US Food and Drug Administration reported that acetaminophen is associated with a risk of serious skin reactions, including TEN;[5] they also stated that Stevens–Johnson syndrome (SJS)/TEN can occur with first-time use of acetaminophen or at any time while it is being taken. Moreover, they reported that it can be difficult to determine how frequently serious skin reactions occur with acetaminophen due to the widespread use of the drug, differences in use among individuals (e.g. occasional vs long-term use) and the long period of time that the drug has been on the market.[5] Therefore, health-care professionals should be aware of this rare risk and consider acetaminophen, along with other drugs already known to have such an association, when assessing patients with suspected drug-induced skin reactions.[5] In fact, a suspected case of SJS due to acetaminophen was confirmed by a challenge test.[10]

Regional differences in acetaminophen-induced SJS/TEN were reported by Mockenhaupt *et al.*[2] Additionally, Ueta *et al.*[11] reported that cold medicine (including acetaminophen)-related SJS/TEN was associated with human leukocyte antigen (HLA)-A*02:06 and HLA-B*44:03 in Japanese patients. Furthermore, they found that HLA-B*44:03 was significantly associated with cold medicine-induced SJS/TEN with severe ocular surface involvement in Indian and Brazilian populations, but not in a Korean population, and that HLA-A*02:06 may be weakly associated with the conditions in Korean, but not Indian or Brazilian, populations.[12] Taken together, these data suggest an association between HLA genotypes and acetaminophen in patients with SJS/TEN.

Our patient had been treated with systemic steroid therapy for UC. Notably, a large case–control study conducted by an international group of experts in 1995 demonstrated a significant association between systemic corticosteroids and SJS/TEN.[1] A multinational case–control study conducted in Europe showed that a large proportion of patients taking systemic steroids developed SJS and TEN.[2] Moreover, Lee *et al.*[13] recently showed that prior use of corticosteroids prolonged the period of disease progression without influencing disease severity or mortality. In addition, when SJS/TEN is preceded by the use of a single high-risk drug, the period of latency between the time of commencement of drug intake and SJS/TEN onset may also increase. Such findings suggest that corticosteroids mildly impact the course of SJS/TEN. Our patient had been taking systemic steroids for UC; therefore, we cannot exclude the effect of systemic steroids on SJS and TEN. However, it has not been determined whether corticosteroids are a direct cause of SJS/TEN, a risk factor (by modifying the immune response) or a confounder.[2]

Cutaneous reactions are relatively common in patients treated with tumor necrosis factor (TNF)-α inhibitors. However, SJS/TEN caused by such inhibitors is quite rare. There are only two case reports of SJS attributable to adalimumab. In our patient, infliximab was excluded as the causative drug, because this had been prescribed to treat UC after resolution of TEN, and no skin rash relapse had been noted. However, several recent reports have shown that the TNF-α antagonist etanercept can be used to effectively treat TEN.[14,15] Thus, TNF-α antagonists should be prescribed with caution because of the risk of severe drug reactions. These antagonists modify the immune response in the same manner as corticosteroids. Further work is needed to clarify the roles played by TNF-α antagonists in SJS/TEN treatment.

In conclusion, we presented a case of TEN due to acetaminophen that showed almost 100% skin detachment. Although previous publications reported a weak association between acetaminophen and SJS/TEN,[2] we found that severe drug eruptions may be caused by acetaminophen. Acetaminophen is included in many prescription and over-the-counter products; thus, clinicians should monitor patients for severe drug reactions, including TEN.

ACKNOWLEDGMENTS: This work was partially supported by the Practical Research Project for Rare/Intractable Diseases of the Japan Agency for Medical Research and Development (AMED) (grant no. 15ek0109011) and by Health and Labor Sciences Research Grants from the Ministry of Health, Labor and Welfare of Japan (H26–nanchi [nan]–ippan–081).

CONFLICT OF INTEREST: None declared.

REFERENCES

1 Roujeau JC, Kelly JP, Naldi L *et al.* Medication use and the risk of Stevens-Johnson syndrome or toxic epidermal necrolysis. *N Engl J Med* 1995; **333**: 1600–1607.
2 Mockenhaupt M, Viboud C, Dunant A *et al.* Stevens-Johnson syndrome and toxic epidermal necrolysis: assessment of medication risks with emphasis on recently marketed drugs. The EuroSCAR-study. *J Invest Dermatol* 2008; **128**: 35–44.
3 Lee HY, Chung WH. Toxic epidermal necrolysis: the year in review. *Curr Opin Allergy Clin Immunol* 2013; **13**: 330–336.
4 Bastuji-Garin S, Rzany B, Stern RS, Shear NH, Naldi L, Roujeau JC. Clinical classification of cases of toxic epidermal necrolysis,

Stevens-Johnson syndrome, and erythema multiforme. *Arch Dermatol* 1993; **129**: 92–96.
5 FDA Drug Safty Communication. FDA warns of rare but serious skin reactions with the pain reliever/fever reducer acetaminophen. 8-1-2013. http://www.fda.gov/Drugs/DrugSafety/ucm363041.htm
6 Chung WH, Shih SR, Chang CF *et al.* Clinicopathologic analysis of coxsackievirus a6 new variant induced widespread mucocutaneous bullous reactions mimicking severe cutaneous adverse reactions. *J Infect Dis* 2013; **208**: 1968–1978.
7 Aihara M, Kano Y, Fujita H *et al.* The efficacy of additional intravenous immunoglobulin to steroid therapy in Stevens-Johnson syndrome and toxic epidermal necrolysis. *J Dermatol* 2015; **42**: 768–777.
8 Egan CA, Grant WJ, Morris SE, Saffle JR, Zone JJ. Plasmapheresis as an adjunct treatment in toxic epidermal necrolysis. *J Am Acad Dermatol* 1999; **40**: 458–461.
9 Narita YM, Hirahara K, Mizukawa Y, Kano Y, Shiohara T. Efficacy of plasmapheresis for the treatment of severe toxic epidermal necrolysis: is cytokine expression analysis useful in predicting its therapeutic efficacy? *J Dermatol* 2011; **38**: 236–245.
10 Trujillo C, Gago C, Ramos S. Stevens-Johnson syndrome after acetaminophen ingestion, confirmed by challenge test in an eleven-year-old patient. *Allergol Immunopathol (Madr)* 2010; **38**: 99–100.
11 Ueta M, Kaniwa N, Sotozono C *et al.* Independent strong association of HLA-A*02:06 and HLA-B*44:03 with cold medicine-related Stevens-Johnson syndrome with severe mucosal involvement. *Sci Rep* 2014; **4**: 4862.
12 Ueta M, Kannabiran C, Wakamatsu TH *et al.* Trans-ethnic study confirmed independent associations of HLA-A*02:06 and HLA-B*44:03 with cold medicine-related Stevens-Johnson syndrome with severe ocular surface complications. *Sci Rep* 2014; **4**: 5981.
13 Lee HY, Dunant A, Sekula P *et al.* The role of prior corticosteroid use on the clinical course of Stevens-Johnson syndrome and toxic epidermal necrolysis: a case-control analysis of patients selected from the multinational EuroSCAR and RegiSCAR studies. *Br J Dermatol* 2012; **167**: 555–562.
14 Paradisi A, Abeni D, Bergamo F, Ricci F, Didona D, Didona B. Etanercept therapy for toxic epidermal necrolysis. *J Am Acad Dermatol* 2014; **71**: 278–283.
15 Gubinelli E, Canzona F, Tonanzi T, Raskovic D, Didona B. Toxic epidermal necrolysis successfully treated with etanercept. *J Dermatol* 2009; **36**: 150–153.