

Molecular signatures order the potency of topically applied anti-inflammatory drugs in patients with atopic dermatitis

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Background: Atopic dermatitis (AD) presents a large unmet need for treatments with better safety and efficacy. To facilitate development of topical therapeutics, we need an efficient model for assessing different formulations and concentrations. The “plaque model” has been successfully implemented in patients with psoriasis, another common inflammatory disease, to assess the efficacy of topical treatments. This model has not been validated for AD, which has higher placebo responses and less stable lesions than psoriasis. **Objective:** We aimed to assess changes in molecular signatures of inpatient target lesions treated with topical therapeutics. **Methods:** We enrolled 30 patients with mild-to-moderate AD in a randomized, double-blind, intraindividual comparison of 3 approved agents applied blindly at the investigator site daily for 14 days: pimecrolimus, betamethasone dipropionate, clobetasol propionate, and a vehicle/emollient control. Changes in total sign scores (TSSs), transepidermal water loss, and tissue biomarkers (determined by using RT-PCR and immunohistochemistry) were evaluated. **Results:** TSSs showed improvements of 30%, 40%, 68%, and 76% at 2 weeks with vehicle, pimecrolimus, betamethasone, and

clobetasol, respectively, with parallel changes in transepidermal water loss ($P < .05$). Significant differences versus vehicle values were limited to steroids ($P < .0001$). Steroids (particularly clobetasol) restored epidermal hyperplasia and terminal differentiation versus minimal changes with vehicle or pimecrolimus ($P < .001$). Levels of cellular infiltrates and cytokines (IL-13, IL-22, and S100As) were similarly reduced only by steroids ($P < .001$). TSS improvement correlated with changes in hyperplasia, infiltrates, and differentiation markers. **Conclusion:** We detected significant clinical and tissue differences between agents, providing a novel approach to study the differential effects of topical formulations using a limited sample size. (J Allergy Clin Immunol 2017;■■■■:■■■■-■■■■.)

Key words: Atopic dermatitis, topical treatments, emollients, plaque model, topical calcineurin inhibitor, topical steroids, psoriasis, biomarkers

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Atopic dermatitis (AD) is the most common inflammatory skin disease, affecting 4% to 7% of adults and 20% of children, with a major effect on quality of life.^{1,2} Patients with AD experience itchy erythematous patches with frequent skin infections.³ Topical treatments are widely used in both patients with mild and those with moderate-to-severe AD.^{4,5}

Currently, topical treatments for AD are limited, consisting of emollients, corticosteroids, and calcineurin antagonists, which can be associated with side effects.⁶ Steroids cause skin thinning, striae, petechiae, and acne, whereas calcineurin antagonists are associated with burning and irritation. Model systems have linked calcineurin antagonists to carcinogenicity, resulting in a US Food and Drug Administration (FDA) black-box warning.^{7,8} Thus there is a large unmet need for better and safer topical treatments for patients with AD.

To facilitate the development of topical therapeutics for AD, we need an efficient model for assessing different formulations and concentrations. The “plaque model” has been successfully implemented in psoriasis, another common inflammatory skin disease, to assess the efficacy of topical treatments.⁹⁻¹² This model provides reliable dose- and time-response curves, allowing concomitant application of different topical compounds on multiple lesions in the same patient, allowing for direct comparison of treatment effect in a small number of subjects.¹² However, this model is difficult to apply to AD because it usually involves application of a self-adhesive tape to delineate the treated area, which could exacerbate or induce an irritant reaction in patients with AD. Furthermore, this approach has not been validated for AD, a disease with high placebo responses and typically less stable

Abbreviations used

AD:	Atopic dermatitis
DC:	Dendritic cell
LC:	Langerhans cell
TAA:	Target Area Assessment
TCI:	Topical calcineurin inhibitor
TEWL:	Transepidermal water loss
TSS:	Total sign score

lesions compared with psoriasis.¹³ Thus we aimed to examine a novel paradigm using inpatient comparison of 3 active topical compounds and an emollient in adults with mild-to-moderate AD. We performed a randomized, double-blind, intraindividual design trial in 30 patients with AD treated daily for 2 weeks with a calcineurin antagonist (pimecrolimus [Elidel; Valeant Pharmaceuticals, Bridgewater, NJ]), 2 different strengths of topical steroids (betamethasone dipropionate [Diprosone, Merck & Co, Kenilworth, NJ] and the highly potent clobetasol propionate [Dermovate, GlaxoSmithKline, Brentford, United Kingdom]), and the emollient Glaxal Base (WellSpring Pharmaceutical, Oakville, Ontario, Canada) as a placebo control. Clinical efficacy and biomarker changes in biopsy specimens were evaluated over 2 weeks. Although all products showed significant clinical improvements compared with baseline values, only the steroids but not pimecrolimus induced significant clinical and tissue improvements compared with vehicle (particularly clobetasol). Our study provides a valid intrapersonal method to comparatively assess topical formulations for AD that can be incorporated in future trial design with a limited number of patients.

METHODS**Patients and interventions**

Thirty adult patients (16 male and 14 female patients; mean age, 24 years; age range, 18–71 years) with mild-to-moderate AD (Investigator Global Assessment of 2 or 3) were enrolled under an institutional review board–approved protocol in a randomized, double-blind, intraindividual comparison trial (NCT02376049, Table I). Four different topical agents were applied daily for 2 weeks to each target lesion (approximately 3 cm in diameter and >2 cm apart, excluding the face and scalp; Fig 1, B), including Glaxal Base (vehicle), pimecrolimus 1% (Elidel), betamethasone dipropionate 0.05% (Diprosone), and clobetasol propionate 0.05% (Dermovate) in a 30- μ L volume per application (approximately 1.5–2.0 mg/cm²), with no occlusion. Each application area and surrounding landmarks were drawn on a transparency at baseline. Circular application areas were drawn on the skin with a marker and redrawn at subsequent visits when faded. No adhesives were used to identify the target application areas. We excluded patients with a Fitzpatrick Skin Type score greater than 5, patients treated with systemic immunosuppressants in the last 4 weeks, topical steroids/immunomodulators in the last 2 weeks, and moisturizers in the past 3 days before treatment. Patients participating in other interventional trials within 4 weeks before randomization were excluded. One patient was withdrawn after 1 week because of use of prohibited medications. Treatments were randomly assigned to target lesional areas by using Latin square randomization, so that treatments were blinded to both patients and investigators. No serious adverse events were reported (see Table E1 in this article's Online Repository at www.jacionline.org).

Clinical and tissue measures

The primary clinical measure was the total sign score (TSS),^{14–16} which includes 6 signs, erythema, edema/papulation, oozing/crusting, excoriation, lichenification, and dryness, graded according to a 4-point scale (0, absent;

1, mild; 2, moderate; and 3, severe) with a total TSS range of 0 to 18. We also evaluated the 6-point Target Area Assessment (TAA) (0 = clear to 5 = very severe). Transepidermal water loss (TEWL)¹⁷ for functional barrier assessment used a closed condenser chamber measurement system (Aquaflux AF200; Biox, London, United Kingdom). Clinical and barrier evaluations occurred at baseline/1 day, 1 week, and 2 weeks. On day 15, 4.5-mm punch biopsy specimens were taken from lesional areas and nonlesional skin for biomarker assessment. Normal biopsy specimens from healthy control subjects were not included, and assessments evaluated reversal to nonlesional skin levels.

Immunohistochemistry and real-time RT-PCR

Immunohistochemistry was performed on frozen sections by using purified mouse anti-human mAbs, as previously described.^{18,19} Antibodies are listed in Table E2 in this article's Online Repository at www.jacionline.org. Epidermal thickness and cells per millimeter were quantified with ImageJ V1.42 software (National Institutes of Health, Bethesda, Md). RNA was extracted with the miR-Neasy Mini Kit (Qiagen, Hilden, Germany). One-step quantitative PCR was performed with TaqMan Fast Virus 1-Step Master mix (Applied Biosystems, Foster City, Calif), as previously described.^{19,20} TaqMan gene expression assays are listed in Table E3 in this article's Online Repository at www.jacionline.org. Expression values were normalized to human acidified ribosomal protein. Zero on bar graphs represents nonlesional skin expression values.

Statistical analysis

Analysis of primary outcomes. A mixed-effects model approach was used to model the change of TSSs with various treatments. The model included a random intercept for each patient, the fixed categorical effects of treatment and treatment-by-visit interaction, and the fixed covariate of baseline score centered around its sample. Likelihood ratio tests and the Akaike information criterion were used to determine which covariance structure (or unstructured) provided the best fit for the data. If no significant differences were found between models fitted with both structures ($P > .05$), the covariance structure was selected; otherwise, the model with the smaller Akaike information criterion was used. Contrasts were defined in the mixed model to test for treatment differences at different time points. Final results are communicated as the percentage change from baseline calculated from the least square means of the model above.

Analysis of exploratory outcomes. For ordinal outcomes as changes in severity scores (TSSs and TAA scores), the change at 15 days was compared by using the Wilcoxon signed-rank test. A secondary analysis included ordinal logistic models that account for dependency of the repeated measures. mRNA expressions of selected genes were normalized to human acidified ribosomal protein. Values of less than the quantification level were imputed by using 20% of the smallest normalized value observed for that gene across all samples above the detection limit. Imputed data were log-transformed before analysis. Changes in expression levels across treatments were evaluated by using mixed-effects model. Similar analyses were performed for TEWL and immunohistochemistry.

Exploratory analyses. Exploratory analyses aimed to elucidate the relationship between clinical treatment effects, TEWL, and biomarkers. A histologic score was created by coupling epidermal thickness and K16 expression with multivariate U-scores (μ Scores) by using the R package.²¹ The change with treatment at 15 days was calculated for each treatment and patient. Spearman correlation coefficients were used to assess pairwise correlations and unsupervised clustering, assessing cluster structures within variables.

RESULTS**Study design**

Thirty patients with mild-to-moderate AD (16 male and 14 female patients; age, 18–71 years; mean, 29.3 years) were randomized into a double-blind, intraindividual design trial (NCT02376049, Table I). Emollient (Glaxal Base) and 3 different

TABLE I. Baseline characteristics

	No.	Mean	SD
Age (y)	30	33.9	14.9
AD duration (y)	29	29.3	16.3
Weight (kg)	30	78.6	22.1
Height (cm)	30	167.2	10.2

Parameter	No.	Percentage
Sex		
Female	14	46.7
Male	16	53.3
Race		
Asian	1	3.3
African American	4	13.3
White	25	83.3
Ethnic group		
Hispanic	0	0.0
Non-Hispanic	30	100.0

formulations (pimecrolimus 1% [Elidel], betamethasone dipropionate 0.05% [Diprosone], and clobetasol propionate [Dermovate]) were applied in a blinded fashion at the investigator site daily for 2 weeks to 4 target AD lesions comparable in severity and inflammation status, as measured based on the TSS and TAA score (approximately 3 cm in diameter, >2 cm apart). Most biopsied lesions were on the extremities (112/145), and the face and scalp were excluded from potential biopsy sites (Fig 1, B). No significant baseline differences in TSSs ($P = .34$) or TAA scores ($P = .5$) were found between inpatient area assessments. Several severity scores were determined, including TSSs (primary end point); TAA scores at baseline/1 day, 1 week, and 2 weeks; and Investigator Global Assessment (see the Methods section). Twenty-nine of 30 patients completed the study (per protocol population). TEWL, a functional measure of barrier disruption, was also measured. On day 15, 4 lesional biopsy specimens from all treatment areas and 1 nonlesional biopsy specimen were taken (see the Methods section). An overview of the study design is shown in Fig 1, A.

Topical steroids, but not pimecrolimus, showed significant clinical improvement over vehicle

There were significant differences from baseline in TSSs, TAA scores, and TEWL levels at both day 8 and day 15 among target lesions treated with vehicle, pimecrolimus, betamethasone, and clobetasol ($P < .01$ for all; Fig 2 and see Table E4 in this article's Online Repository at www.jacionline.org). TSSs and TAA scores showed comparable results at both 8 and 15 days. The largest reduction in TSS (primary end point) at day 15 was seen with clobetasol (75.5%), followed by betamethasone (66.7%), pimecrolimus (39.6%), and vehicle (29.8%; $P < .05$ for all; Fig 2, A). However, the improvement in TSSs at 15 days compared with vehicle was significant for steroids ($P < .001$) but not for pimecrolimus (although it was significant at 8 days; see Table E5 in this article's Online Repository at www.jacionline.org). At day 15, there were no significant differences between steroids ($P = .3$) or between pimecrolimus and vehicle ($P = .26$). Individual TSS subscores (erythema, edema, excoriation, dryness, lichenification, and oozing) showed a similar picture, with significant differences versus vehicle obtained only for steroids ($P < .05$), although 4/6 scores showed significant reductions with pimecrolimus compared with baseline values ($P < .05$, see Fig E1 in this article's Online Repository at www.jacionline.org). The lichenification

and oozing subscores were reduced significantly versus baseline values only with topical steroids ($P \leq .02$, see Fig E1). Changes in TAA scores mirrored changes in TSSs (Fig 2, B).

Mean TEWL values in the 4 treatment areas were comparable at baseline, ranging from 65.9 to 69.9 g/m²/h (see Table E6 in this article's Online Repository at www.jacionline.org). Significant improvements ($P < .001$) in TEWL from baseline to days 8 and 15 were observed with all 4 products. Similar to clinical data, only topical steroids showed significant improvements in TEWL compared with vehicle ($P < .0001$) but not pimecrolimus ($P = .16$). No differences were seen between clobetasol and betamethasone ($P = .78$).

Epidermal pathology and cellular infiltrates are "normalized" toward nonlesional skin levels only with steroids

We performed immunostaining and mRNA expression using RT-PCR on lesional and nonlesional biopsy specimens treated with the investigational products. Changes with treatment were represented as distance/difference from nonlesional skin to calculate improvements in individual lesions treated with various products compared with nonlesional skin. Thus the closer to nonlesional (or zero in box plots), the greater the improvement (Figs 1 and 3). At day 15, epidermal hyperplasia (measured by epidermal thickness and K16 mRNA expression) was reduced by steroids (particularly clobetasol) close to nonlesional levels; steroid-treated areas also showed resolved K16 protein expression by means of immunostaining (Fig 3, A-D). Conversely, vehicle- and pimecrolimus-treated areas did not show improvement in thickness and K16 toward nonlesional levels and maintained K16-positive staining at day 15 ($P < .001$; Fig 3, A-D). Similarly, only topical steroids (particularly clobetasol) suppressed cellular infiltrates in lesions to nonlesional levels and even beyond, including CD3⁺ T cells, CD11c⁺ myeloid dendritic cells (DCs), FcεRI⁺ DCs, and langerin⁺ Langerhans cells (LCs; Fig 3, E-L). Topical steroids showed significant differences from both vehicle and pimecrolimus ($P < .05$), whereas pimecrolimus did not show differences from vehicle for all markers except CD11c⁺ and FcεRI⁺ DCs. FcεRI⁺ DCs and LCs were normalized with steroids even beyond nonlesional levels.

Key inflammatory and barrier features of AD are reversed solely with steroids

To determine molecular effects with individual treatments, we quantified day 15 gene expression for 28 immune-, barrier-, and steroid-related genes in treatment areas using RT-PCR. Overall, all inflammatory pathways were improved toward nonlesional levels or beyond with both steroids (particularly clobetasol; Fig 4 and see Fig E2 in this article's Online Repository at www.jacionline.org). Impressive changes were seen with steroids in markers of general inflammation (matrix metalloproteinase 12) and T_H1 (CXCL9/CXCL10), T_H2 (IL-13, IL-5, and CCL17/CCL22/CCL26), and T_H17/T_H22 (IL-17A, IL-12, CXCL1, PI3, IL-22, and S100A7/S100A8/S100A12) markers, whereas pimecrolimus and vehicle maintained large differences from nonlesional skin ($P < .05$). Although neither vehicle nor pimecrolimus showed improvements in terminal differentiation markers (loricrin, periplakin, and filaggrin) compared with nonlesional skin, such an improvement was evident with both steroids,

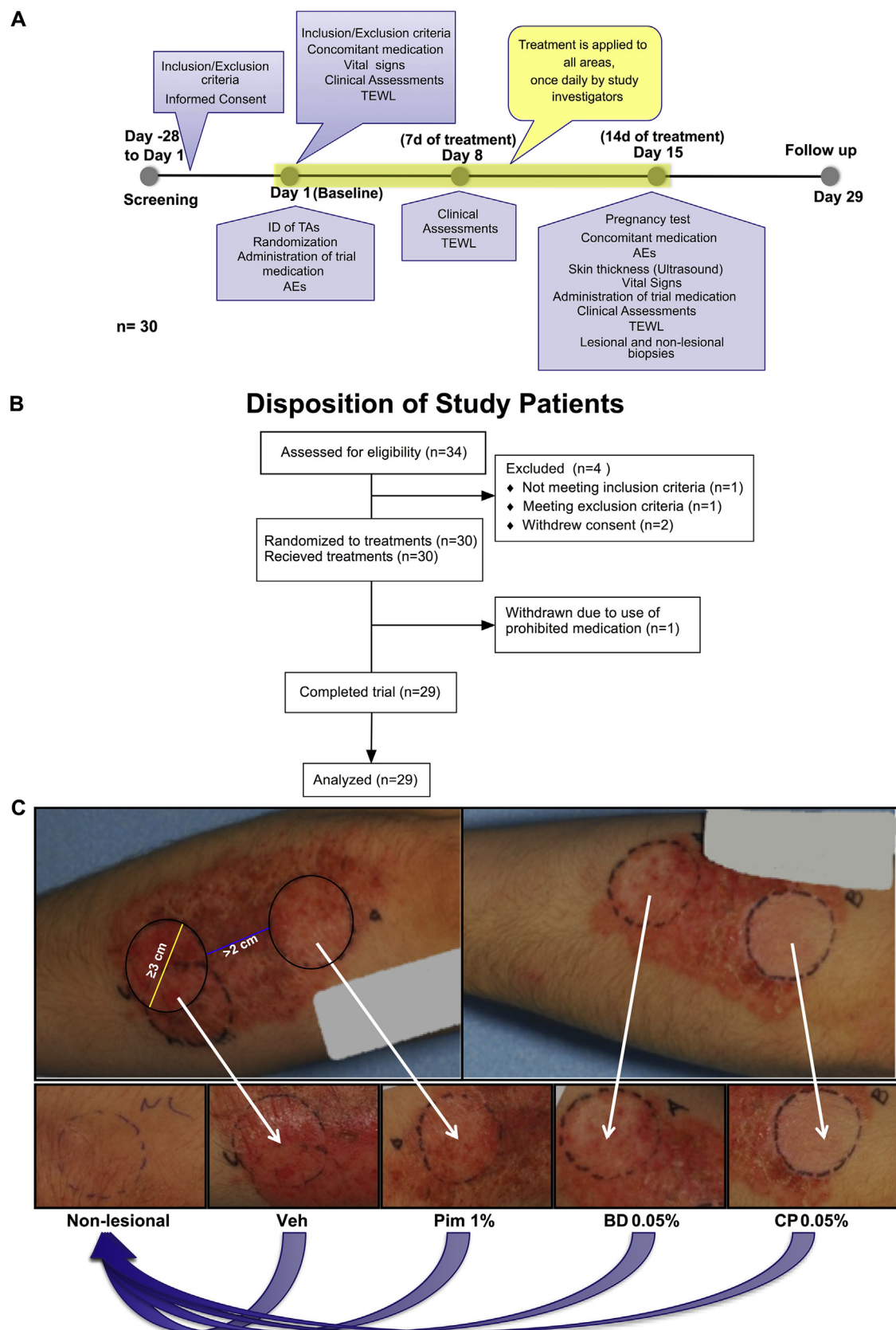
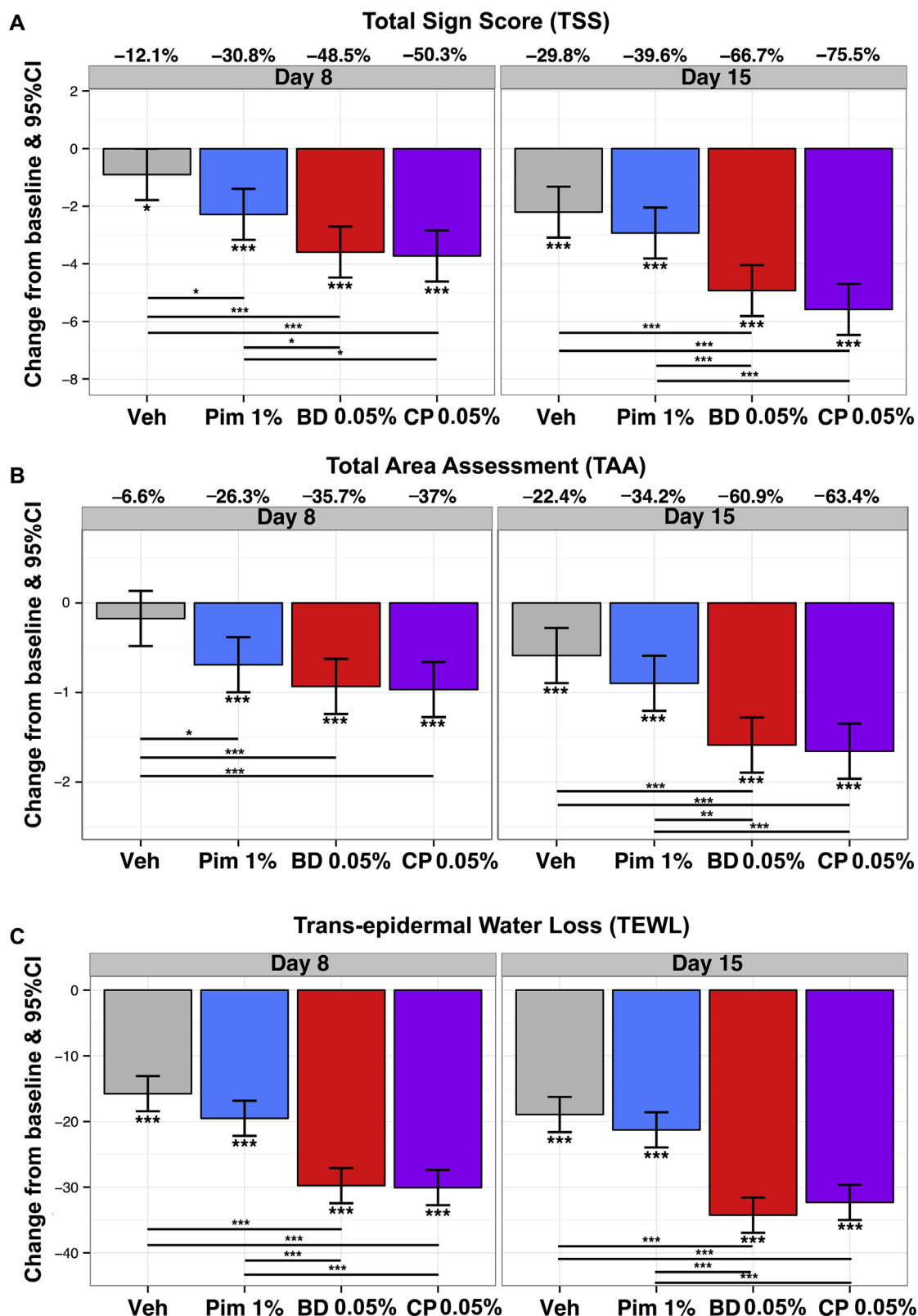


FIG 1. Schema of study design. **A**, Study design from screening to follow-up, with treatment period and data collection shown for different time points. **AEs**, Adverse events. **B**, Patient disposition. **C**, Four lesional skin areas of similar size of 3 cm or greater in diameter and separated by other lesional areas by greater than 2 cm were identified in each patient with AD. Lesional areas were treated with 4 different creams for 2 weeks, including Glaxal Base (vehicle [*Veh*]), pimecrolimus 1% (10 mg/g; *Pim* 1%), betamethasone dipropionate 0.05% (0.5 mg/g; *BD* 0.05%), and clobetasol propionate 0.05% (0.5 mg/g; *CP* 0.05%). For clinical and tissue biomarkers, each lesional skin area treated with an investigational product was compared with nonlesional skin.



*Percent Change is not presented for TEWL as this value is unknown for non-lesional skin

FIG 2. Clinical efficacy end points. Bar graphs of the change in clinical measures at days 8 and 15 versus baseline for skin lesions treated with investigational products, including TSSs (A), TAA scores (B), and TEWL (C). BD 0.05%, Betamethasone dipropionate 0.05%; CP 0.05%, clobetasol propionate 0.05%; Pim 1%, Pimecrolimus 1%; Veh, vehicle. Asterisks above black horizontal lines denote significance in change between 2 treatments; asterisks above bars denote significance in change between treatment and nonlesional skin: * $P < .05$, ** $P < .01$, and *** $P < .001$. Values are presented as means $\pm$ SEMs.

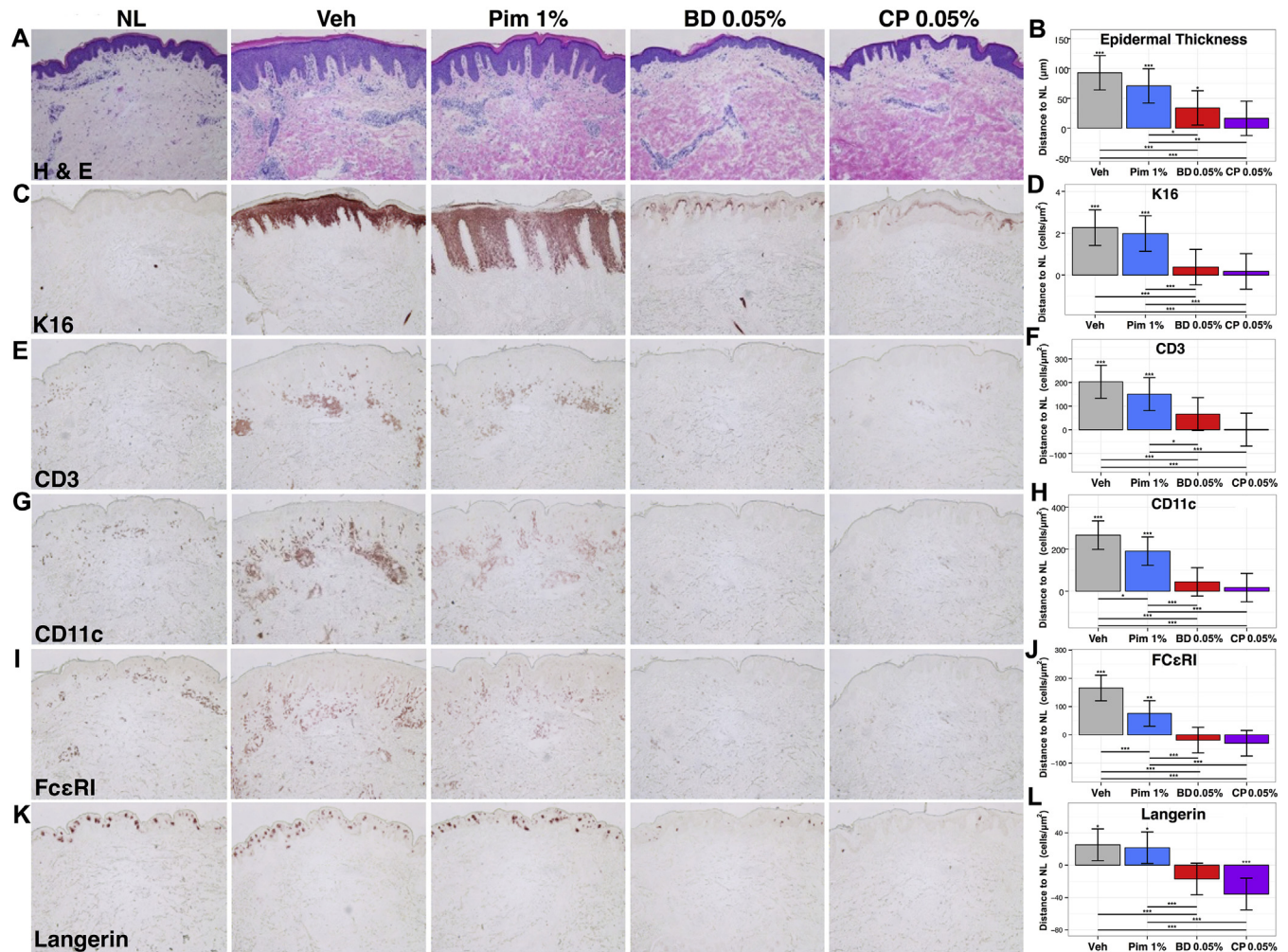


FIG 3. Representative immunohistochemical images and quantification of cellular markers. **A**, Hematoxylin and eosin (H&E) staining. **B**, Bar graphs of epidermal thickness quantified versus nonlesional skin. **C** and **D**, K16 staining (Fig 3, C) with bar graph (Fig 3, D) of K16 mRNA expression versus nonlesional skin. **E** and **F**, CD3 staining (Fig 3, E) with bar graph (Fig 3, F) of T-cell counts. **G** and **H**, CD11c staining (Fig 3, G) with bar graph (Fig 3, H) of CD11c⁺ DC counts. **I** and **J**, FcεRI staining (Fig 3, I) with bar graph (Fig 3, J) of FcεRI⁺ DC cell counts. **K** and **L**, Langerin staining (Fig 3, K) with langerin⁺ LC counts (Fig 3, L). In bar graphs zero represents nonlesional levels. Asterisks above horizontal lines denote significance in change between 2 treatments; asterisks above bars denote significance in change between treatment and nonlesional skin. **P* < .05, ***P* < .01, and ****P* < .001. BD 0.05%, Betamethasone dipropionate 0.05%; CP 0.05%, clobetasol propionate 0.05%; Pim 1%, pimecrolimus 1%; Veh, vehicle. Values are presented as means ± SEMs.

particularly with clobetasol. To determine whether responses to steroids can be differentiated by potency, we measured expression 3 steroid-response genes (FKBP5, TSC22, and TXNIP). Indeed, a dose response was observed, with higher upregulation with the more potent steroid clobetasol.

Differences in improvement toward nonlesional skin between vehicle and the 3 formulations are summarized in Fig 5, A, as a heat map (red/upregulated; blue/downregulated). Overall, vehicle and pimecrolimus show a similar pattern and still maintain upregulation of inflammatory markers, whereas these are significantly reduced with steroids. Overall, both steroids showed similar improvements, and only a few markers (including the T_H2 markers IL-5 and CCL17) showed larger reductions with clobetasol (Fig 5, A).

Clinical and tissue correlations

Clinical improvement (change in TSSs) was correlated with molecular and cellular biomarkers. Changes in epidermal thickness were also correlated with clinical and cellular/molecular measures (Fig 5, B, and see Figs E3 and E4 and Table E7 in this article's Online Repository at www.jacionline.org). The highest correlation with change in TSS was with improvement in hyperplasia (epidermal thickness and K16) and T-cell and DC markers (eg, CD3⁺ and CD11c⁺ cells). Some immune markers also showed significant correlations, including S100A7/S100A8/S100A12. Even stronger correlations were found between epidermal thickness and tissue biomarkers (Fig 5, C, and see Fig E3 and Table E7). Particularly high were correlations between epidermal thickness with DCs (CD11c⁺ and FcεRI⁺) and T-cell

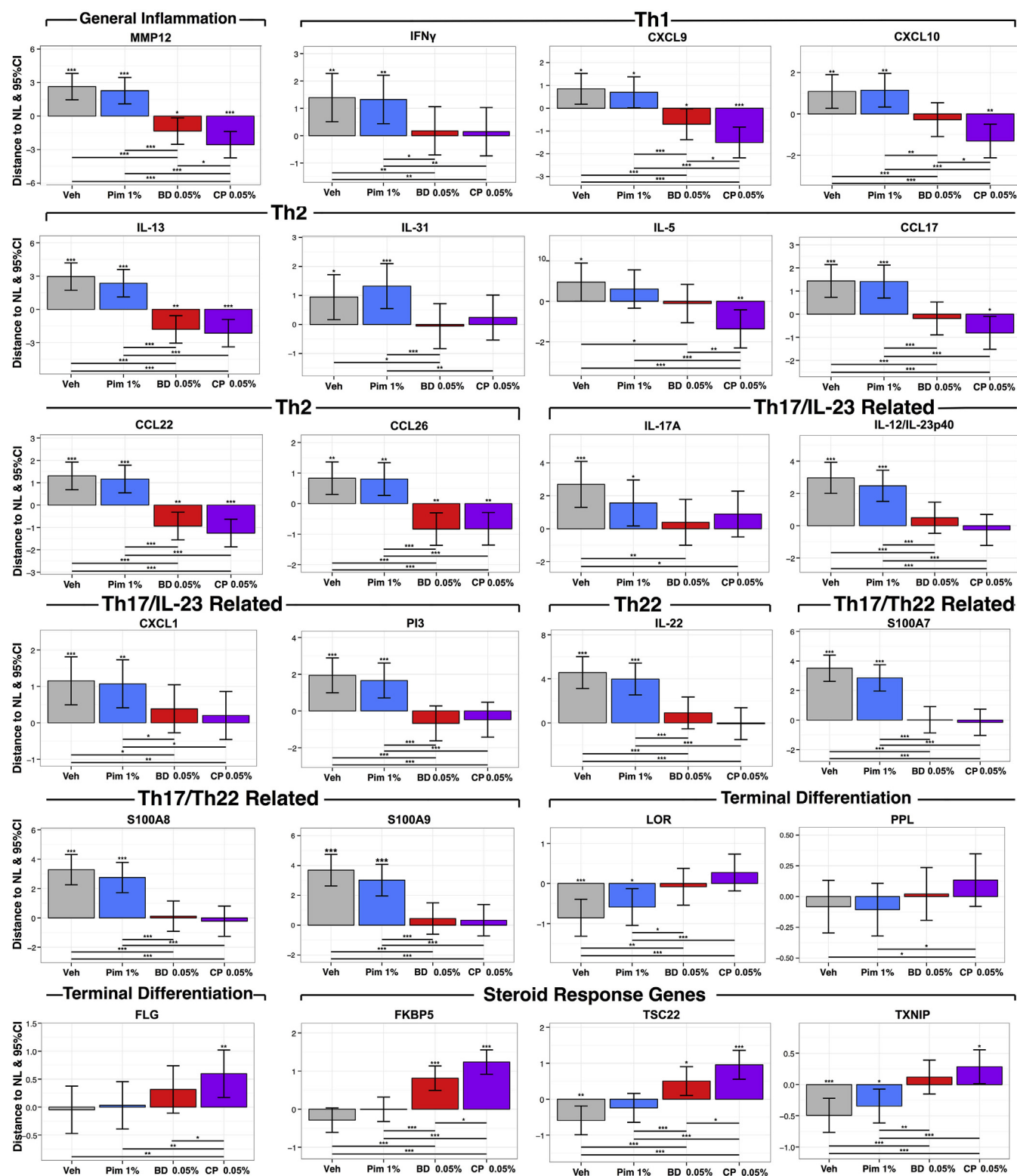


FIG 4. Immune and epidermal markers in lesional skin areas treated with each investigational product versus nonlesional skin. Bar graphs of gene expression of 28 immune response genes in lesional skin areas quantified by using RT-PCR. Zero represents nonlesional levels. Asterisks above black horizontal lines denote significance in change between two treatments; asterisks above bars denote significance in change between treatment and control values: * $P < .05$, ** $P < .01$, and *** $P < .001$. BD 0.05%, Betamethasone dipropionate 0.05%; CP 0.05%, clobetasol propionate 0.05%; Pim 1%, pimecrolimus 1%; Veh, vehicle. Values are presented as means $\pm$ SEMs.

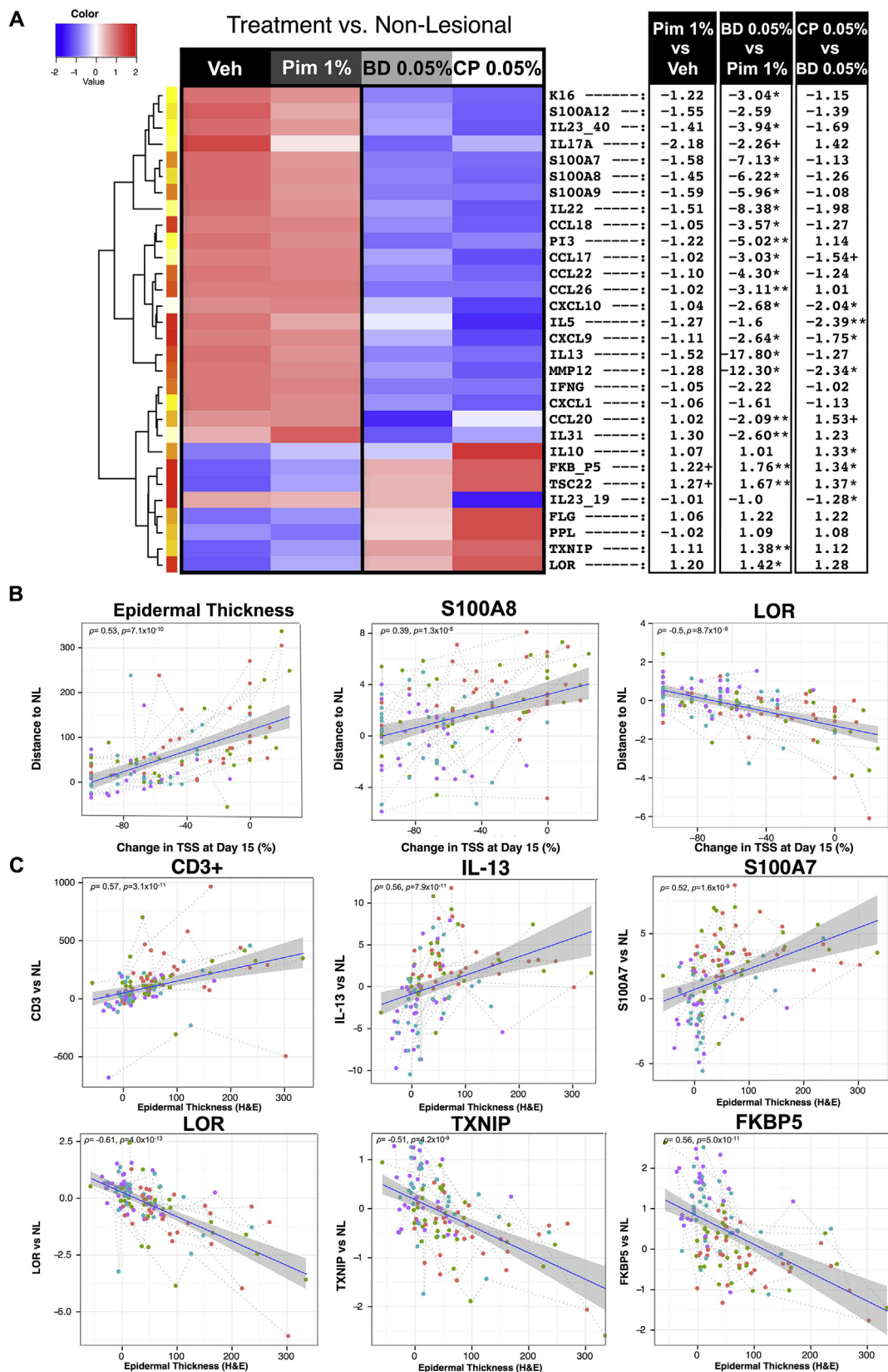


FIG 5. Select cellular and molecular biomarker mRNA expressions and correlations. **A**, Unsupervised hierarchical clustering of mRNA across lesional skin areas treated with vehicle (*Veh*), pimecrolimus 1% (*Pim 1%*), betamethasone dipropionate 0.05% (*BD 0.05%*), or clobetasol propionate 0.05% (*CP 0.05%*) visualized as a heat map. Red, Higher expression; blue, lower expression. **B**, Correlations of representative cellular and molecular biomarkers with clinical improvement (TSS reduction). **C**, Correlations of representative cellular and molecular biomarkers of clinical improvement with histologic response, as measured based on epidermal thickness. ρ = Spearman correlation coefficient with associated *P* value. Zero represents nonlesional levels.

markers (CD3⁺), as well as T_H2 (IL-13 and CCL22) and T_H17 (S100A7) markers (Fig 5, C, and see Fig E3 and Table E7).

DISCUSSION

Despite the high prevalence of AD, topical therapeutics for both adults and children with AD are currently limited. Furthermore, available treatments raise safety concerns on prolonged applications.^{22,23} Topical steroids are associated with atrophy and stria, causing a “steroid phobia,”⁶ whereas topical calcineurin antagonists have a black-box warning, limiting their use.^{24,25} The lack of topical therapeutics with safe and effective long-term profiles presents a huge unmet need for therapeutic development, leading to active testing of novel entities.²³

The present study provides a novel method of inpatient clinical and tissue evaluations of daily application of topical formulations during a 2-week period. Our approach adapts the plaque test model for investigation of topical formulations, which has been successfully implemented in patients with psoriasis, to AD without using adhesive on adjacent skin.^{9,10,12} This inpatient method reduces variability by controlling for external factors and decreases the necessary sample size to detect significant differences between treatment arms. Furthermore, it requires minimal application of investigational product, decreasing patient risk. Our study validates this method for AD, a disease with less stable lesions and higher placebo responses, providing a valid assay to assess clinical efficacy, barrier functions, and tissue biomarkers (epidermal and immune) of topical formulations. Overall, although clinical and mechanistic data were congruent, tissue analyses provided higher sensitivity and differentiation between products and vehicle, highlighting the need to incorporate these in early clinical development.

All formulations, including vehicle, showed significant improvements in clinical (TSSs and TAA scores) and barrier (TEWL) assessments after 2 weeks of daily applications. However, only topical steroids showed normalization of the aberrant immune and barrier tissue responses (epidermal hyperplasia, S100As, and terminal differentiation) in lesional skin toward and even beyond the nonlesional skin phenotype, whereas vehicle and the calcineurin antagonist pimecrolimus were still distant from nonlesional levels. Furthermore, although topical steroids (particularly clobetasol) achieved robust clinical and tissue improvements compared with vehicle, pimecrolimus largely showed no significant differences compared with vehicle. Only topical steroids, but not pimecrolimus or vehicle, reversed the epidermal hyperplasia,^{3,13,20,26} as measured based on epidermal thickness and K16 expression. Furthermore, only steroids, but largely not pimecrolimus (except for FcεRI⁺ and CD11c⁺ DCs) or vehicle, suppressed cellular infiltrates of T cells, DC subsets, and LCs to nonlesional levels and beyond. In parallel, key cytokine pathways upregulated in patients with AD, particularly T_H2 and T_H22, but also T_H1 and T_H17/IL-23 cytokine axes and associated markers (eg, IL-13, CCL17, and S100As), were significantly inhibited only with steroids. Moreover, epidermal differentiation markers were only upregulated with steroids but not with pimecrolimus (vs vehicle) to levels even higher than those in nonlesional skin, particularly with clobetasol. Three steroid-response genes showed clear dose-response differences by potency, attesting for the high sensitivity of the proposed model. Thus only the steroids normalized key immune and barrier mediators of AD pathogenesis toward nonlesional levels (and even beyond),^{27,28}

whereas pimecrolimus generally had only limited additional benefit to vehicle over a 2-week period.^{29,30}

Several meta-analyses suggested that mild-to-moderate topical steroids are more efficacious in both the short and long term than topical calcineurin inhibitors (TCIs), including pimecrolimus studied here and tacrolimus.³¹⁻³³ Some studies reported that tacrolimus (particularly 0.1%) had increased benefit over both pimecrolimus and mild steroids.³⁴⁻³⁶ Of note, large cohorts were needed to achieve significant differences between treatment arms, and some studies did not achieve significance despite their numerous patients.³⁷⁻³⁹ Only a few studies compared individual immune or barrier aspects in biopsy specimens of patients treated with topical steroids or TCIs. A study evaluating pimecrolimus and betamethasone valerate treatment for 3 weeks (without vehicle comparison) showed clinical and functional barrier improvements (TEWL and stratum corneum hydration) in both groups. Although betamethasone was superior in reducing epidermal differentiation, it improved in both treatment groups.⁴⁰ An extension study showed potent reductions in inflammatory mediators only with betamethasone, with minimal effects with pimecrolimus, similar to our observations. However, unlike our study, restoration of barrier products (filaggrin and loricrin) was observed with both treatments.⁴¹ The same group showed that topical steroids (triamcinolone acetonide and betamethasone valerate) caused higher reductions in antimicrobial agents (eg, hBD2 and psoriasin/S100A7) compared with pimecrolimus.⁴² Although both topical steroids and TCIs (particularly pimecrolimus) reduced T-cell numbers, LCs were only affected by steroids.⁴³ Despite its lesser effects, pimecrolimus showed better effects on extracellular lipids and lamellar bodies compared with topical steroids.⁴⁴ A recent clinical trial with topical steroids (lacking vehicle control) showed that even a mild-to-moderate steroid, such as triamcinolone acetonide 0.025%, is able to improve clinical indices and also restore key epidermal and immune skin abnormalities in AD lesions.²⁰

Our data expand prior evaluations of topical treatments in patients with AD,⁶ providing a more global assessment of modulation of clinical measures and tissue biomarkers with steroids of different potency, pimecrolimus, and an emollient. It provides a valid intrapersonal method to obtain unbiased correlations between individual treatment effects (vs vehicle). Although, similar to prior reports, our data showed significant improvements with pimecrolimus compared with baseline values, it highlights the important aspect (lacking in some of the above studies) of having an internal vehicle control, from which pimecrolimus did not show significant differences in our data. One must remember that emollients, which often serve as comparators in trials, have been also reported to cause improvements in clinical and barrier measures and are now tested in prevention trials in infants.⁴⁵⁻⁴⁹ Furthermore, petrolatum, a common emollient, was recently reported to improve epidermal differentiation, antimicrobial responses, and even some immune markers in biopsy specimens.⁵⁰ Overall, our inpatient study design shows significant clinical and even more pronounced tissue differences between treatment arms and vehicle with a minimal number of subjects, maximizing its utility. An important advantage of our within-subject design is that it decreases the number of patients needed for attaining significance because several treatments can be concomitantly tested in the same patients while keeping the exposure time constant.^{9,10,12} In fact, as shown in Fig E5 in this article's Online Repository at www.jacionline.org, more than

120 patients would have been required in a parallel-group design to achieve similar differences between treatment arms as those found through our inpatient design. By using this approach, if we powered a new study to detect treatment differences from baseline, a sample size of 176 would be needed for pimecrolimus, whereas only 15 and 11 subjects for betamethasone and clobetasol, respectively, would allow detection of the effect size with 80% power.

Our study presents a few limitations, including the following:

1. All topical formulations were applied once daily, whereas for maximal efficacy, these should be applied twice daily.^{4,51} However, because we wanted to make sure that applications are standardized and applied in the clinic, it was not feasible to apply more than once daily. Nevertheless, all products were used on a daily basis, allowing for proper comparisons. Future approaches should evaluate our approach by using twice-daily application.
2. Pimecrolimus might need a more prolonged treatment to reach its efficacy plateau compared with steroids.⁵² Future studies should assess our approach in a longer period of 4 weeks.
3. Lack of a baseline lesional biopsy specimen, which was intended to minimize biopsies. However, our analysis strategy focused on improvements toward the nonlesional level, allowing differentiation between products.
4. Lack of normal skin comparisons. Although many immune and barrier markers were improved with steroids to levels far beyond those in nonlesional skin (IL-13, IL-5, and filaggrin), future studies should include normal skin to assess true "normalization."
5. The vehicle used in this study is a good emollient⁵³ and might not be a true placebo, and perhaps other emollients show differential results. Still, for the majority of assessments, it failed to reverse to the nonlesional AD phenotype.
6. Although it would be desirable to also compare different concentrations of each formulation tested, it was not feasible in the current study because many more biopsy specimens would have been required in each patient.
7. *Staphylococcus aureus* colonization data of individual AD lesions are unavailable. Recent reports show that *S aureus* might be important in the induction and exacerbation of AD⁵⁴⁻⁵⁶ and should be addressed by future studies.

In sum, our inpatient approach optimizes evaluations of new topical formulations and could be incorporated in early stages of therapeutic development. Future studies should validate this novel approach across different AD phenotypes in different parts of the world,^{3,57-60} with various drug concentrations and formulations.

Clinical implications: Our inpatient model optimizes clinical and molecular evaluations of topical formulations, highlighting its benefits for early topical drug development.

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METHODS

The per-protocol population consisted of 29 subjects; 1 patient was withdrawn because of protocol violations. For an overview of the study design, see [Fig 1, A](#).

Allocation of the 4 investigational products to each of the 4 target AD lesions (A, B, C, or D) was done according to the rows in the Latin square shown in [Box E1](#).

In regard to blinding, all medicinal products were sourced by the investigational sites (4 in total). All products were blinded to all study staff performing study assessments. Consequently, all site staff performing assessments remained unaware of individual treatment assignment during the conduct of the trial. The randomization list was kept in a secure area by the designee (who was identified by the site investigator, remained unblinded, and prepared products for administration).

Box E1. Randomization of investigational products to target lesions

Treatment	Target AD lesional areas			
	A	B	C	D
IMP 1	IMP 2	IMP 3	IMP 4	IMP 1
IMP 2	IMP 1	IMP 4	IMP 3	IMP 2
IMP 3	IMP 4	IMP 1	IMP 2	IMP 3
IMP 4	IMP 3	IMP 2	IMP 1	IMP 4

Each row represents 1 specific treatment group determining the allocation of treatments to target AD lesions. Each subject was randomly assigned to a treatment group by taking the next (ascending) randomization code number available at the trial center.

IMP, Investigational medicinal product.

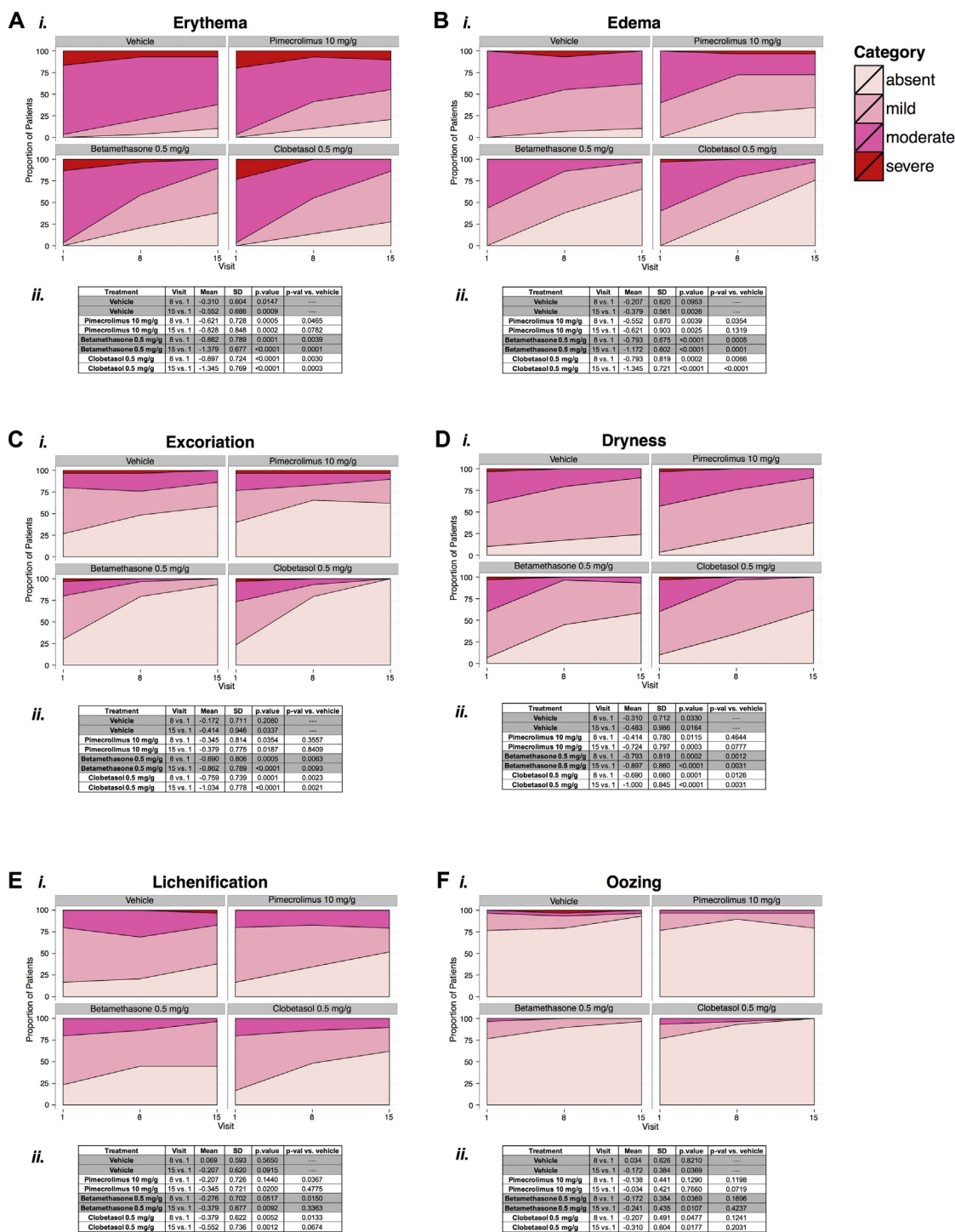


FIG E1. Proportion of patients with TSS subscores measured at days 1, 8, and 15 after treatment with investigational product. TSS subscores include oozing (A), dryness (B), edema (C), erythema (D), lichenification (E), and excoriation (F). *i*, Wilcoxon signed-rank test was performed for each subscore. *ii*, Corresponding results are shown.

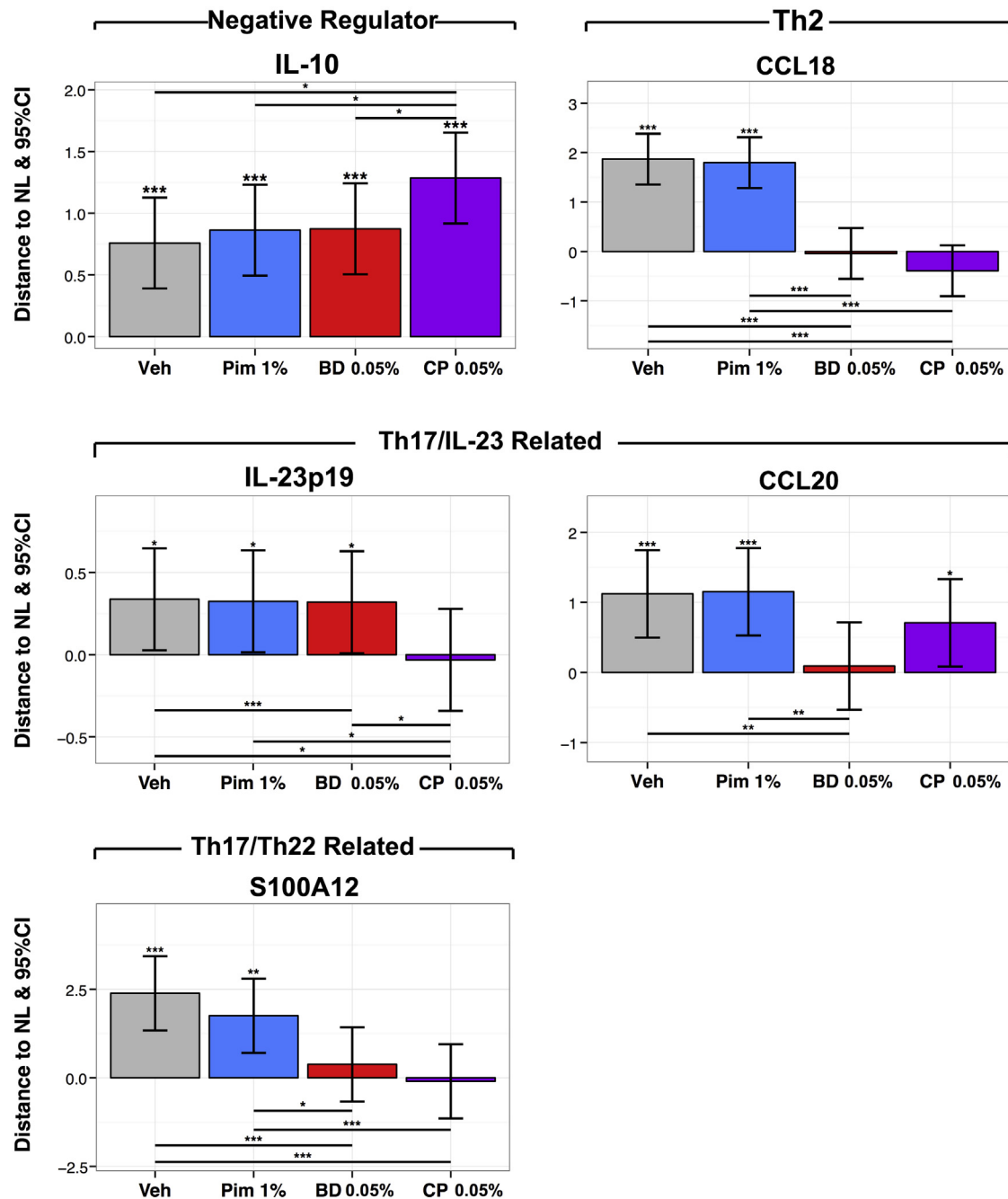


FIG E2. Bar graphs of mRNA expression in lesional skin areas treated with investigational products versus nonlesional skin quantified by using RT-PCR. Zero represents nonlesional levels. Asterisks above black horizontal lines denote significance in change between 2 treatments; asterisks above bars denote significance in change between treatment and control: * $P < .05$, ** $P < .01$, and *** $P < .001$. BD 0.05%, Betamethasone dipropionate 0.05%; CP 0.05%, clobetasol propionate 0.05%; Pim 1%, pimecrolimus 1%; Veh, vehicle. Values are presented as means $\pm$ SEMs.

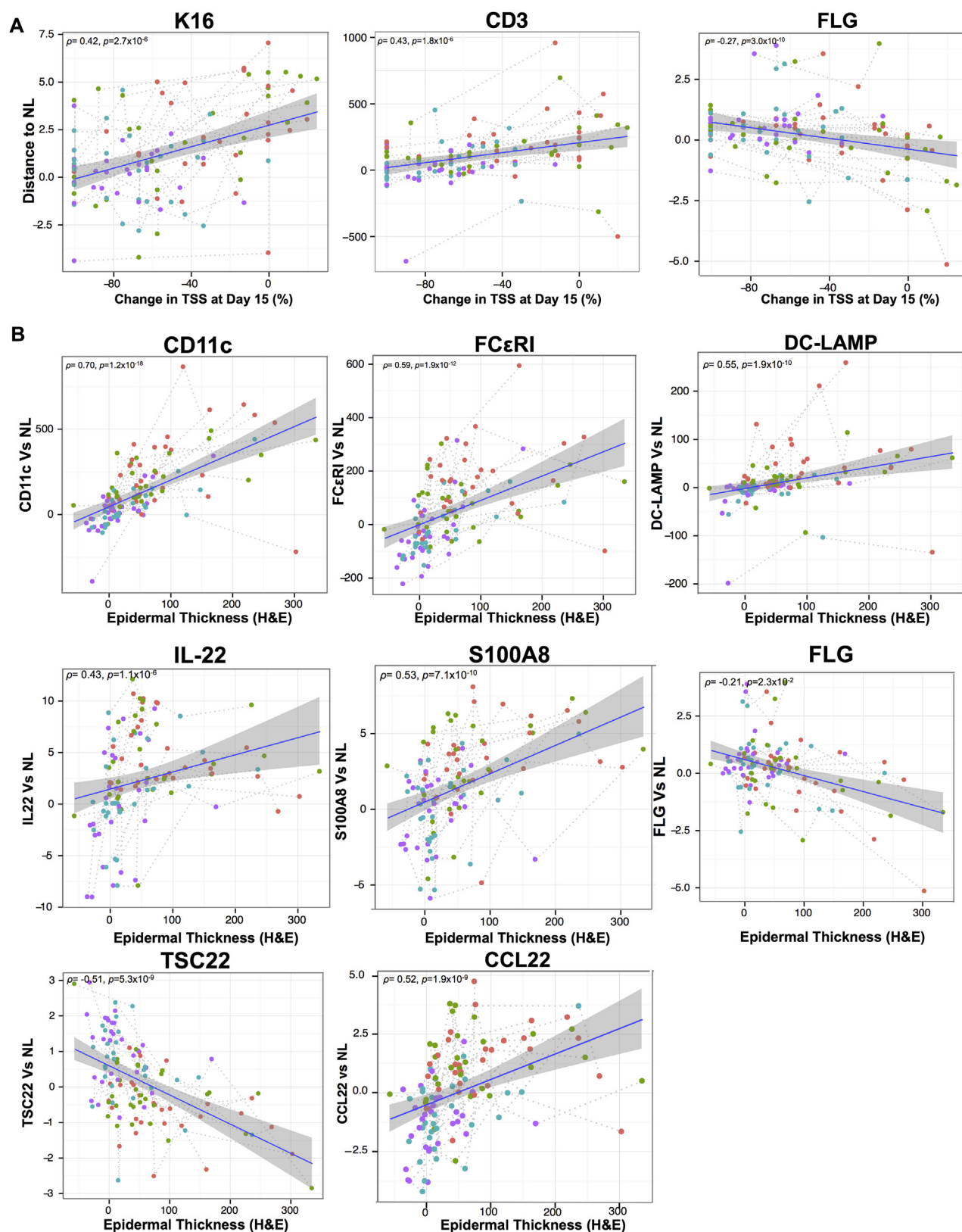


FIG E3. **A**, Correlations of representative cellular and molecular biomarkers with clinical improvement (TSS reduction). **B**, Correlations of representative cellular and molecular biomarkers of clinical improvement with histologic response, as measured based on epidermal thickness. ρ = Spearman correlation coefficient with associated P values. H&E, Hematoxylin and eosin.

Spearman Correlation of Biomarkers with Total Sign Score Improvement at Day 15

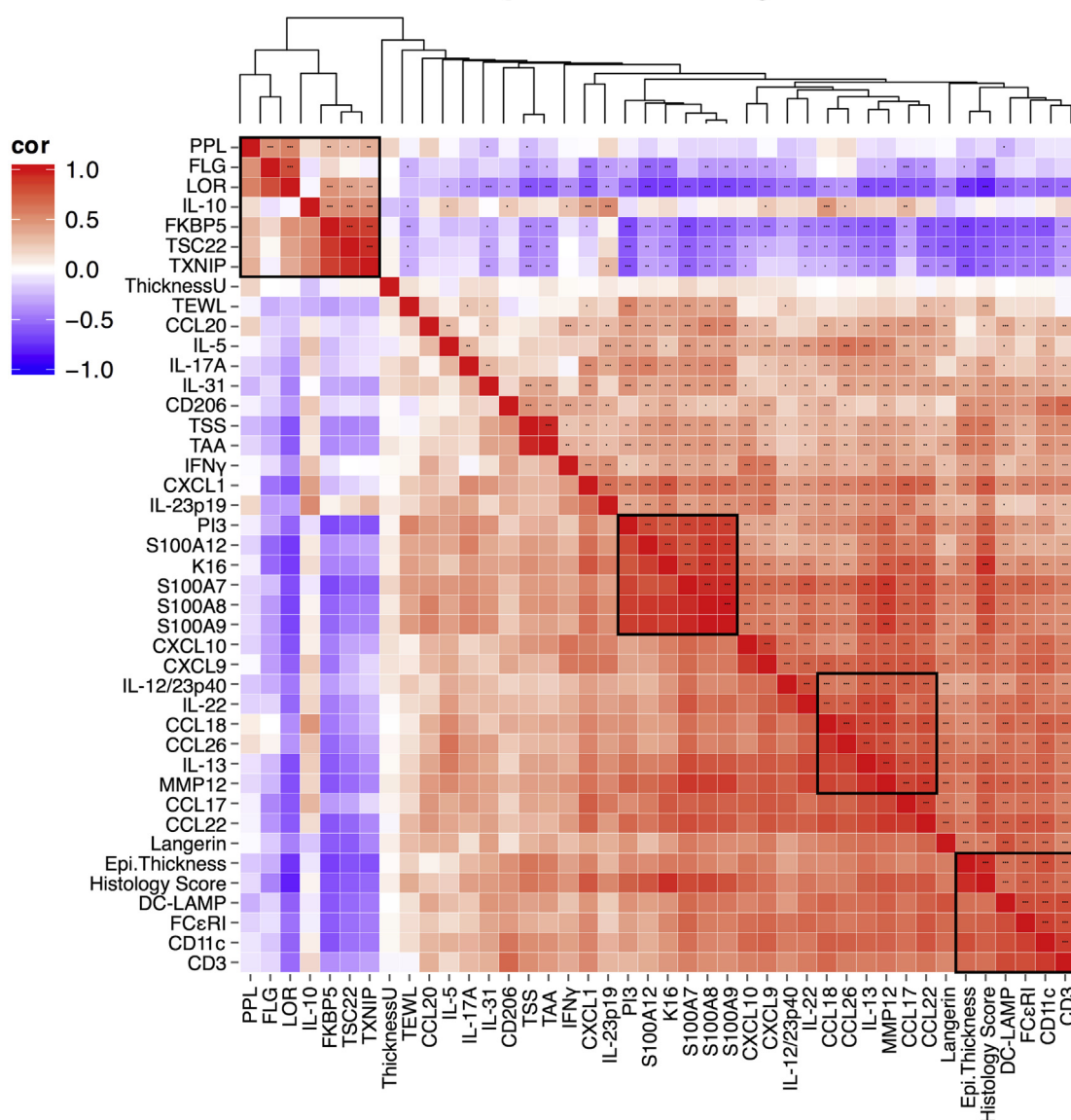


FIG E4. Spearman correlation of each biomarker and clinical measure change at day 15 visualized as a heat map. Red, Positive correlations; blue, negative correlations. *Boxes* represent biomarkers with strong Spearman correlations with reduction in TSSs on day 15. Correlations with *P* values are represented in Table E7. **P* < .05, ***P* < .01, and ****P* < .001.

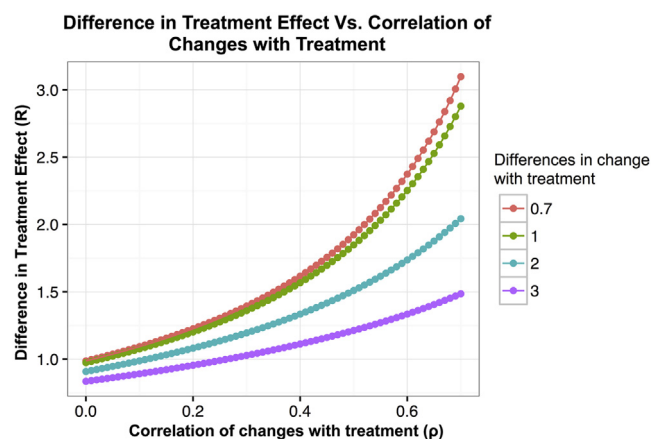


FIG E5. The within-subject design allows concomitant testing of several treatments in the same patient, decreasing the number of patients enrolled in the trial while keeping exposure time constant. If the number of patients needed for detecting a treatment effect under the inpatient study design (in which all treatments are tested in each patient) is N_i , then the number of patients needed for a parallel-group design (N_p), in which individual treatments are tested in different patient groups, can be represented as $N_p = R * K * N_i$, where K represents number of treatment groups, R is a function of the differences in treatment effects by treatment, and ρ is the correlation of treatment changes between subjects. For this trial, R is the SD of treatment changes (2.3) in TSSs observed at day 15 with all treatments. The increase in N_p is almost always higher than K times.

TABLE E1. Adverse events

No. of AEs per patient	Frequency (%)	AE	Frequency (%)
1	13 (59.09)	Allergic rhinitis	1 (4.55)
2	5 (22.73)	Common cold	2 (9.09)
3	2 (9.09)	Conjunctivitis	1 (4.55)
4	1 (4.55)	Constipation	1 (4.55)
5	1 (4.55)	Cut of the finger	1 (4.55)
Target treatment		Diarrhea	1 (4.55)
BD 0.05%*	1 (4.55)	Headache	1 (4.55)
None	21 (95.45)	Hot flush	1 (4.55)
AE, serious		Inflamed bx site*	1 (4.55)
Yes	0 (0)	Insect bite, left forearm	1 (4.55)
No	22 (100)	Intermittent Coughing	1 (4.55)
AE, related		Difficulty breathing	1 (4.55)
Related	0 (0)	Intermittent headache	1 (4.55)
Not related	22 (100)	Nausea	2 (9.09)
AE severity		Pain in legs, fibromyalgia related	1 (4.55)
Mild	20 (90.91)	Pain in right wrist	1 (4.55)
Moderate	2 (9.09)	Pharyngitis	1 (4.55)
Severe	0 (0)	Pruritus in both eyes	1 (4.55)
AE outcome		Rhinitis	1 (4.55)
Not recovered/not resolved	8 (36.36)	Worsening throat pain	1 (4.55)
Recovered/resolved	14 (63.64)		
Withdrawn because of AEs			
Yes	0 (0)		
No	22 (100)		

AE, Adverse event; *BD 0.05%*, betamethasone dipropionate 0.05%; *bx*, biopsy specimen.

*Diagnosis is not related to treatment.

TABLE E2. Antibodies used for immunohistochemical analyses

Antigen	Manufacturer	Clone*	Isotype	Dilution
CD3	BD Biosciences, San Jose, Calif	SK7	IgG ₁	1:100
CD11c	BD Biosciences	B-Iy6	IgG ₁	1:100
FcεRI	Thermo Scientific, Waltham, Mass	CRA1 (AER37)	IgG _{2b} , kappa	1:100
CD206	Genetex, Irvine, Calif	15-2	IgG ₁	1:100
DC-LAMP	Beckman Coulter, Fullerton, Calif	104.G4	IgG ₁	1:50
Langerin	Immunotec, Quebec, Canada	DCGM4	IgG ₁	1:100
K16	Sigma-Aldrich, St Louis, Mo	K8.12	IgG ₁	1:100
Filaggrin	Acris Antibodies, Herford, Germany	FLG01	IgG ₁	1:100

DC-LAMP, Dendritic cell lysosome-associated membrane protein; *FLG*, filaggrin.

*Antibodies used in this experiment were mouse mAbs, unless stated otherwise.

TABLE E3. Primers and probes used for real-time RT-PCR

Marker	Probe
K16	Hs00955082_g1
S100A12	Hs00942835_g1
MMP12	Hs00899662_m1
IL-5	Hs00174200_m1
IL-13	Hs00174379_m1
IL-31	Hs01098710_m1
IFN- γ	Hs00989291_m1
CXCL9	Hs00171065_m1
CXCL10	Hs01124251_g1
IL-17A	Hs00174383_m1
Elafin/PI3	Hs00160066_m1
CCL20	Hs01011368_m1
CXCL1	Hs00236937_m1
IL-22	Hs01574154_m1
S100A7	Hs01923188_u1
S100A8	Hs01923188_u1
S100A9	Hs00374264_g1
IL-23p19	Hs00900828_g1
IL-23p40	Hs01011518_m1
CCL17	Hs00171074_m1
CCL18	Hs00268113_m1
CCL22	Hs01574247_m1
CCL26	Hs00171146_m1
FLG	Hs00856927_g1
LOR	Hs01894962_s1
Periplakin	Hs00160312_m1
IL-10	Hs00961622_m1
FKBP5	Hs01561006_m1
TSC22D3 (GILZ)	Hs00608272_m1
TXNIP	Hs01006900_g1

FLG, Filaggrin; *LOR*, loricrin; *MMP12*, matrix metalloproteinase 12.

TABLE E4. Change from baseline (day 1) for each investigational product

Treatment	Visit	Estimate (<i>P</i> value)	CI
TSS			
Vehicle	8 vs 1	−0.897 (.047)	−1.781 to −0.012
Vehicle	15 vs 1	−2.207 (<.0001)	−3.092 to −1.322
Pimecrolimus 1%	8 vs 1	−2.276 (<.0001)	−3.161 to −1.391
Pimecrolimus 1%	15 vs 1	−2.931 (<.0001)	−3.816 to −2.046
Betamethasone 0.05%	8 vs 1	−3.586 (<.0001)	−4.471 to −2.702
Betamethasone 0.05%	15 vs 1	−4.931 (<.0001)	−5.816 to −4.046
Clobetasol 0.05%	8 vs 1	−3.724 (<.0001)	−4.609 to −2.840
Clobetasol 0.05%	15 vs 1	−5.586 (<.0001)	−6.471 to −4.702
TAA			
Vehicle	8 vs 1	−0.173 (.027)	−0.481 to 0.135
Vehicle	15 vs 1	−0.587 (.0002)	−0.895 to −0.279
Pimecrolimus 1%	8 vs 1	−0.691 (<.0001)	−0.998 to −0.383
Pimecrolimus 1%	15 vs 1	−0.897 (<.0001)	−1.205 to −0.590
Betamethasone 0.05%	8 vs 1	−0.932 (<.0001)	−1.240 to −0.625
Betamethasone 0.05%	15 vs 1	−1.588 (<.0001)	−1.895 to −1.280
Clobetasol 0.05%	8 vs 1	−0.967 (<.0001)	−1.275 to −0.659
Clobetasol 0.05%	15 vs 1	−1.656 (<.0001)	−1.964 to −1.349
TEWL			
Vehicle	8 vs 1	−15.786 (.00024)	−24.136 to −7.436
Vehicle	15 vs 1	−18.957 (<.0001)	−27.307 to −10.608
Pimecrolimus 1%	8 vs 1	−19.377 (<.0001)	−27.727 to −11.027
Pimecrolimus 1%	15 vs 1	−21.146 (<.0001)	−29.496 to −12.796
Betamethasone 0.05%	8 vs 1	−29.344 (<.0001)	−37.695 to −20.993
Betamethasone 0.05%	15 vs 1	−33.837 (<.0001)	−42.188 to −25.486
Clobetasol 0.05%	8 vs 1	−29.807 (<.0001)	−38.158 to −21.457
Clobetasol 0.05%	15 vs 1	−32.067 (<.0001)	−40.417 to −23.717

TABLE E5. Comparison of clinical scores across treatment periods for each investigational product

Treatment comparison	Visit	Estimate (P value)	SEM
TSS			
Veh/Pim 1%	8 vs 1	1.379 (.0307)	0.634
Veh/BD 0.05%	8 vs 1	2.69 (<.0001)	0.634
Veh/CP 0.05%	8 vs 1	2.828 (<.0001)	0.634
Pim 1%/BD 0.05%	8 vs 1	1.31 (.04)	0.634
Pim 1%/CP 0.05%	8 vs 1	1.448 (.0234)	0.634
BD 0.05%/CP 0.05%	8 vs 1	0.138 (.828)	0.634
Veh/Pim 1%	15 vs 1	0.724 (.255)	0.634
Veh/BD 0.05%	15 vs 1	2.724 (<.0001)	0.634
Veh/CP 0.05%	15 vs 1	3.379 (<.0001)	0.634
Pim 1%/BD 0.05%	15 vs 1	2 (.0018)	0.634
Pim 1%/CP 0.05%	15 vs 1	2.655 (<.0001)	0.634
BD 0.05%/CP 0.05%	15 vs 1	0.655 (.303)	0.634
TAA			
Veh/Pim 1%	8 vs 1	0.517 (.02)	0.221
Veh/BD 0.05%	8 vs 1	0.759 (.0007)	0.221
Veh/CP 0.05%	8 vs 1	0.793 (.0004)	0.221
Pim 1%/BD 0.05%	8 vs 1	0.242 (.275)	0.221
Pim 1%/CP 0.05%	8 vs 1	0.276 (.212)	0.221
BD 0.05%/CP 0.05%	8 vs 1	0.034 (.877)	0.221
Veh/Pim 1%	15 vs 1	0.31 (.161)	0.221
Veh/BD 0.05%	15 vs 1	1 (<.0001)	0.221
Veh/CP 0.05%	15 vs 1	1.069 (<.0001)	0.221
Pim 1%/BD 0.05%	15 vs 1	0.69 (.002)	0.221
Pim 1%/CP 0.05%	15 vs 1	0.759 (.0007)	0.221
BD 0.05%/CP 0.05%	15 vs 1	0.069 (.756)	0.221
TEWL			
Veh/Pim 1%	8 vs 1	3.591 (.549)	5.985
Veh/BD 0.05%	8 vs 1	13.558 (.0245)	5.985
Veh/CP 0.05%	8 vs 1	14.021 (.0200)	5.985
Pim 1%/BD 0.05%	8 vs 1	9.967 (.0973)	5.985
Pim 1%/CP 0.05%	8 vs 1	10.430 (.0827)	5.985
BD 0.5%/CP 0.5%	8 vs 1	0.463 (.938)	5.985
Veh/Pim 1%	15 vs 1	2.189 (.715)	5.985
Veh/BD 0.05%	15 vs 1	14.880 (.0136)	5.985
Veh/CP 0.05%	15 vs 1	13.110 (.0295)	5.985
Pim 1%/BD 0.05%	15 vs 1	12.691 (.0351)	5.985
Pim 1%/CP 0.05%	15 vs 1	10.921 (.0694)	5.985
BD 0.05%/CP 0.05%	15 vs 1	−1.770 (.768)	5.985

BD 0.05%, Betamethasone dipropionate 0.05%; CP 0.05%, clobetasol propionate 0.05%; Pim 1%, pimecrolimus 1%; Veh, vehicle.

TABLE E6. Mean TEWL values across treatment days

Treatment	Visit day	Mean TEWL value (g/m ² /h)	SE
Vehicle	1	69.3	3.221503381
Vehicle	8	53.5	3.270801541
Vehicle	15	50.3	3.270801541
Pimecrolimus 1%	1	68.8	3.221467532
Pimecrolimus 1%	8	49.5	3.270634792
Pimecrolimus 1%	15	47.7	3.270634792
Betamethasone dipropionate 0.05%	1	65.9	3.228500438
Betamethasone dipropionate 0.05%	8	36.5	3.273483274
Betamethasone dipropionate 0.05%	15	32	3.273483274
Clobetasol propionate 0.05%	1	69.9	3.221996712
Clobetasol propionate 0.05%	8	40	3.272243463
Clobetasol propionate 0.05%	15	37.7	3.272243463

TABLE E7. Spearman correlations between selected clinical, molecular, and cellular markers

Marker	ρ	<i>P</i> value
Spearman correlation with TSS reduction		
TAA	0.902	<.001
Epidermal thickness	0.533	<.001
CD11c	0.427	<.001
CD3	0.426	<.001
K16	0.42	<.001
CD206	0.403	<.001
S100A8	0.392	<.001
CXCL10	0.387	<.001
S100A7	0.365	<.001
S100A12	0.363	<.001
MMP12	0.361	<.001
DC-LAMP	0.344	<.001
IL-13	0.337	<.001
CCL17	0.331	<.001
S100A9	0.322	<.001
IL-31	0.319	<.001
FC ϵ RI	0.296	<.01
CCL18	0.293	<.01
PI3	0.279	<.01
Langerin	0.274	<.01
CCL22	0.27	<.01
CXCL1	0.263	<.01
CXCL9	0.262	<.01
CCL26	0.251	<.01
IL-22	0.245	<.01
IL-23p19	0.227	<.05
IFN- γ	0.206	<.05
IL-12/23p40	0.202	<.05
PPL	−0.187	<.05
FLG	−0.274	<.01
TXNIP	−0.308	<.01
FKBP5	−0.328	<.001
TSC22	−0.346	<.001
LOR	−0.503	<.001
Spearman correlation with epidermal thickness		
CD11c	0.704	<.001
FC ϵ RI	0.595	<.001
CD3	0.568	<.001
IL-13	0.558	<.001
DC-LAMP	0.548	<.001
TSS	0.533	<.001
CXCL10	0.53	<.001
S100A7	0.524	<.001
CCL22	0.522	<.001
K16	0.511	<.001
MMP12	0.5	<.001
TAA	0.494	<.001
Langerin	0.491	<.001
CXCL9	0.475	<.001
CXCL1	0.474	<.001
CCL26	0.471	<.001
S100A8	0.47	<.001
CCL17	0.448	<.001
PI3	0.444	<.001
IL-22	0.434	<.001
CCL18	0.431	<.001
S100A12	0.424	<.001
S100A9	0.413	<.001
CD206	0.41	<.001
IFN- γ	0.345	<.001

TABLE E7. (Continued)

Marker	ρ	<i>P</i> value
IL-12/23p40	0.333	<.001
IL-31	0.315	<.01
IL-23p19	0.261	<.01
IL-17A	0.244	<.01
FLG	−0.211	<.05
TSC22	−0.509	<.001
TXNIP	−0.512	<.001
FKBP5	−0.563	<.001
LOR	−0.609	<.001

DC-LAMP, Dendritic cell lysosome-associated membrane protein; *FLG*, filaggrin; *LOR*, loricrin; *MMP12*, matrix metalloproteinase 12; *PI3*, peptidase inhibitor 3; *PPL*, periplakin.

(Continued)