Accelerating topical formulation development for inflammatory dermatoses; an ex vivo human skin culture model consistent with clinical therapeutics

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Accelerating topical formulation development for inflammatory dermatoses; an ex vivo human skin culture model consistent with clinical therapeutics

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ABSTRACT

Although animal models have been extensively used to evaluate human topical therapeutics, they exhibit marked physiological differences to human skin. Our objective was to develop a human *ex vivo* skin culture model to explore the pathophysiology of inflammatory dermatoses and for preclinical testing of potential therapeutic treatments. *Ex vivo* skin barrier integrity and metabolic activity was retained for 5 days and stimulation of T-helper cells (Th1), which produce proinflammatory cytokines, provided inflammatory responses similar to those reported from *in vivo* biopsy. Tissue responses to established therapies of pimecrolimus (Elidel) and clobetasol propionate (Dermovate) were evaluated using the human *ex vivo* skin culture, assessing pharmacodynamic changes in gene expression alongside the pharmacokinetics of drug penetration with both products showing time dependent efficacies. The translational utility of the human *ex vivo* skin culture model of inflammatory dermatoses was demonstrated through comparison with an *in vivo* clinical study, with similar reductions in inflammatory gene expression recorded for both drug treatments. Thus, this model can reduce, replace or refine animal testing and also mitigate the risk of failure in costly and time-consuming clinical trials associated with novel topical therapeutic development.

Keywords: human ex-vivo skin; skin model, Th1, dermatitis, eczema, preclinical dermatology

1. INTRODUCTION

Animal models have been widely used to help understand human skin disorders and to estimate the pharmacokinetics and pharmacodynamics of existing and potential new topical therapeutics prior to clinical trials. Through genetic manipulations, animal models can present pathologies that mimic human disease, however significant physiological and anatomical differences create challenges when using such models to evaluate novel therapeutics. For example, the NC/Nga mouse model presents with itching, erythema, scaling and alopecia at two months of age (Matsuda, 1997). A decrease in barrier function and water retention capability coupled with decreased levels of ceramide reflect human atopic dermatitis (AD) pathology (Aioi et al., 2001). However, this model, like most animal AD models, is based on a Th2 immune response more closely associated with human acute or contact AD, rather than later stage Th1-mediated chronic AD in humans (Yagi et al., 2002). An alternative murine AD model (Kitagaki et al., 1995) generates a hapten-induced disease state of contact hypersensitivity which resembles contact dermatitis and requires hypersensitization prior to any pathological response. One common sensitization reagent is 2,4,6-trinitrochlorobenzene (TNCB), which results in epidermal hyperplasia, accumulation of mast cells and elevated serum IgE levels. Gene expression analysis following TNCB treatment shows an initial acute Th1 immune response followed by a shift to a chronic Th2 inflammatory state, the opposite of the typical shift from acute Th2 to chronic Th1 immune response in the human AD response (Kitagaki et al., 1997). Oxazolone, an alternative chemical sensitization hapten, elicits a different immune response in mice where a combined Th1/Th2 response is achieved with acute oxazolone treatment, shifting to a Th2 prominent response with repeated applications, again differing from the common human immune cell switch (Shiohara et al., 2004). Such differences in immune response are highlighted by the immune cell dysregulation between mice and humans. Differential neutrophil defensin

expression, interleukin class switching, and cluster of differentiation expression are some of the ways that human and mice immune system differences are observed (Risso, 2000)(Snapper and Finkelman, 1997)(Gordon et al., 2001).

As Janus Kinase 3 (JAK3) is the major signal transducer for common gamma chain, the above lack of concordance between human and murine immunology can have a profound effect on efficacy testing for new JAK-targeting therapeutics for inflammatory dermatoses, such as Abrocitinib (Pfizer), Ruxolitinib (Incyte), Cerdulatinib (Dermavant) and Upadacitinib (Abbvie) which are currently approved or in clinical trials (Ballard, 2021) (Mestas and Hughes, 2004). The alternate skewing of T cell populations during inflammation also delineates mouse models from humans. In the human response to viral infection, IFNα is secreted by several cell types as a protective measure to induce Th1 differentiation via STAT4 activation, but in mice IFNα does not activate STAT4 nor induce Th1 differentiation, altering the inflammatory response compared to humans (Farrar et al., 2000). Further dissimilarities include the JAK-STAT signalling IL-10 anti-inflammatory cytokine (del Prete et al., 1993), endothelial P-selectin (Pan et al., 1998), MHC class II molecules on activated T cells (Barnaba et al., 1994), calcium flux mediated by a potassium channel (Koo et al., 1997), and chemokine expression disparities between mice and humans (Olson and Ley, 2002)(Zlotnik and Yoshie, 2000). Clearly these physiological dissimilarities between human and mouse models present challenges when seeking to evaluate and progress topical therapies for inflammatory skin disorders from the laboratory to clinic.

An alternative animal model for atopic dermatitis is dogs. The disease occurs in about 10% of all canine breeds and is the most commonly diagnosed atopic disease (Hillier and Griffin, 2001). While atopic dermatitis in dogs is an allergen-based reaction, similar to contact or acute dermatitis, it has been shown to be familial with a genetic predisposition as with human AD. When considering secondary infections, both dogs and humans often present with

Staphylococcus bacterial infection (Marsella and Olivry, 2003). House dust mite sensitization is commonly used to elicit an allergen-induced canine AD disease state. Typically, Beagles with inherently high levels of IgE are exposed to house dust mites to elicit an allergic reaction presenting with erythema, edema, epidermal hyperplasia and lymphocyte cell infiltration, similar to human AD pathology (Olivry et al., 2006) and with an inherent barrier dysfunction represented by increased transepidermal water loss as seen with AD-presenting human infants in the first year of life (Marsella and Girolomoni, 2009). It has been reported that canine AD has a 96% concordance rate with human AD when evaluating puritis, morphology and distribution, and chronic relapse (Terada et al., 2011) . These phenotypic similarities between canine and human AD make dogs a superior animal model for the developmental characterization of novel AD therapeutics when compared to mice.

Beyond the physiological differences between animal models and human skin, anatomical differences are axiomatic. Follicular density, skin thickness (and different thicknesses of the stratum corneum in particular) vary markedly between human and animal skins, and there are differences in the lipid composition of the tissues and hence the inherent permeability of the tissue. For example, the stratum spinosum is comprised of only 1 or 2 layers in the hairy regions of mice and dogs, however is composed of up to 4 layers in humans (Souci and Denesvre, 2021). Murine epidermis is thinner than human, approximately 25 µm vs ~100 µm respectively. Human skin exhibits rete ridges and eccrine sweat glands which are absent in mice (Zomer and Trentin, 2018). Numerous studies have highlighted the limitations of using animal skin to predict the penetration of topical therapeutics through human skin *in vivo,* and indeed *in vitro* penetration testing using human skin is recommended by regulatory authorities including the US FDA and EU MCA. There are thus compelling scientific and regulatory drivers to focus on *ex vivo* human skin for pre-clinical evaluation of topical drug

delivery to risk mitigate costly investment and time into clinical trials (Brown and Williams, 2019).

The human *ex vivo* skin culture (HESC) model provides a 3-dimensional skin tissue culture from intrinsic cell types, with antigen presentation, stratum corneum barrier, and immune variation derived from the tissue donor (Neil et al., 2020). This clearly avoids the immunological and anatomical differences seen when using animal models to evaluate potential topical therapeutics. However, due to the abbreviated time in culture, the HESC does not show the phenotypic changes commonly seen in AD such as stratum corneum barrier disruption and does not allow vascular immune cell infiltration (Hofmann et al., 2021). Here, the correlation of gene expression profiles elicited in an *ex vivo* skin model with those known in inflammatory skin conditions has been explored and suggests that a robust *ex vivo* model to assess the potential efficacy of novel therapeutics prior to clinical investigation can be achieved (Jardet et al., 2020). To this end, two established topical therapeutics, Elidel cream (1% pimecrolimus) and Dermovate cream (0.0525% clobetasol propionate) were selected for evaluation in our *ex vivo* inflammatory skin system since the effects of these products on *in vivo* gene expression profiles in patients with AD have been reported (Guttman-Yassky et al., 2017).

2. MATERIALS AND METHODS

2.1 Human ex vivo skin culture

Human tissue was obtained via elective abdominoplasty with donor consent under Pearl IRB approval in accordance with FDA 21 CFR 56.104 and DHHS 45 CFR 46.101 regulations (Pearl Pathways. Exemption Determination Submission. IRB Study Number: 15-MEDP-101). Written informed consent was obtained from all subjects or, if subjects were under 18, from a parent and/or legal guardian and all experiments were performed in accordance with

relevant guidelines and regulations. Donors were healthy and not currently taking systemic corticosteroid treatment. No identifying information beyond ethnicity and age was provided. Tissue was maintained in humidified incubators at 37°C and 5% CO_2 in 9 mm diameter Franz cells (~0.63 cm^2 tissue surface area). Tissue was chilled until processing for culture and used within 24 h of surgery. Tissue was defatted and dermatomed (Integra Padgett Slimline SB) to a thickness of 750 µm; tissue exhibiting abnormalities such as oedema, abrasion, or heavy striation was discarded. The well was then filled with modified DMEM/Hams F12 Cornification media resulting in an air-liquid interface *ex vivo* skin culture. Media was changed every 48 hours with *ex vivo* skin culture maintained in the modified Cornification media (Neil et al., 2020)(Smith et al., 2016)(Vostálová et al., 2018). The Th1 stimulation cocktail was applied basally to the tissue in the Cornification media.

2.2 RNA Isolation and RT-qPCR

Human *ex vivo* skin culture was stored in 1 mL RNALater (Invitrogen) to allow permeation overnight at 4°C. Following tissue mincing and homogenization with an Omni BeadRuptor 24, RNA was isolated per Qiagen RNeasy Mini instructions. RNA was normalized using RNase-free water to a concentration of 25 ng/µL. Reverse transcription used High-Capacity cDNA kits (Applied Biosystems). RT-qPCR was run on an Applied Bioscience QuantStudio 6 Flex Real-Time PCR System. All primers were commercially available and purchased from Invitrogen Life Technologies.

RT-qPCR values were calculated using the relative quantification approach (Livak and Schmittgen, 2001). Briefly, fold change was calculated as the ratio of the power of the gene of interest divided by the average power of the untreated samples. Percent activity was calculated as the percent increase or decrease in stimulation compared to the stimulated samples set at 100% maximum activity.

2.3 Pharmacodynamic translation

Human skin *ex vivo* skin culture was processed as described previously and cut into ~2 cm^2 pieces then clamped into Franz cells to avoid lateral migration of apically applied formulation; the receiver chamber was filled with ~2 mL Cornification media without stirring. Tissue was allowed to equilibrate un-occluded in a 37°C humidified CO_2 incubator prior to treatment. As drug content was not quantified from the receiver fluid, sink conditions were not considered due to inherent drug solubility and media conditions. At 40 h pre-stimulation, 10 µL (~18 µL/cm^2) of each formulation was applied topically using a displacement pipet and sterile syringe plunger tip for application. Vehicle (Glaxal Base Cream Moisturizer; WellSpring Pharma, lot #%P6247); Elidel (1% Pimecrolimus ; Valeant Pharma; lot # 322841); Dermovate (0.05% Clobetasol Propionate ; Taro Pharma; lot # F8757) was used. The next day, 16 h prior to stimulation, the existing formulation was removed with a sterile q-tip and fresh formulation added to both the 40 h and 16 h samples. At the time of treatment, the existing formulation was removed with a sterile q-tip and fresh formulation added topically to all treatment samples. Stimulation cocktail (Th1-mediated as described in Neil et al, 2022 was applied to basolateral compartment for 24 h stimulation. The next day the tissue was harvested by wiping residual formulation with a clean Kim-wipe, then tape-stripped 5 times (CuDerm D-squame sampling disk #D100). The tissue was then cut in half; one half placed in RNAlater for qRT-PCR; the other half heat separated (2 min at 60°C) and the epidermis and dermis placed into pre-weighed metal bead tubes for drug extraction. After weighing, the tubes were stored at -80°C until drug extraction using 90:10 v/v ethanol:water and homogenization. The clobetasol propionate and pimecrolimus were then analysed by LC-MS/MS for drug in the epidermis and dermis.

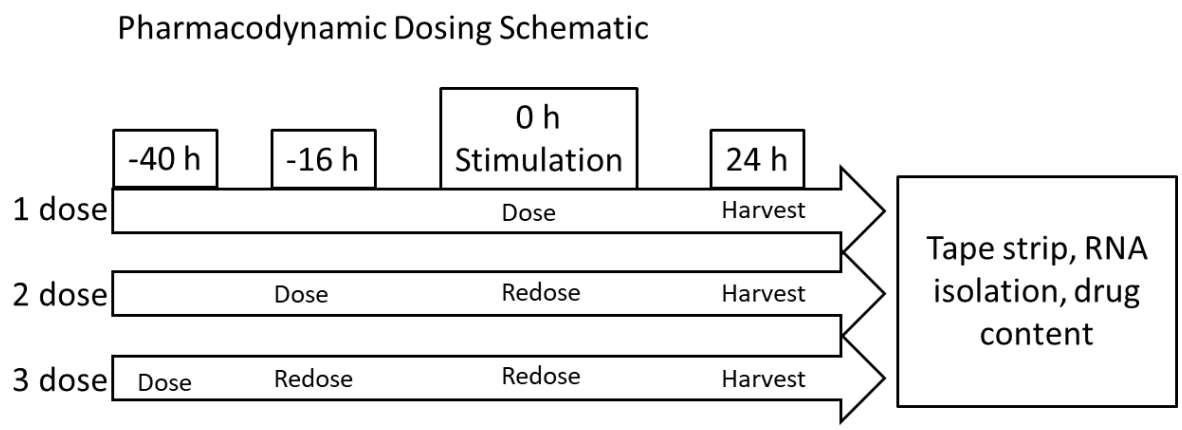

Figure 1. **Pharmacodynamic Dosing Schematic**. The dosing schedule for both active and vehicle formulations on human *ex vivo* skin culture. At time of stimulation; 1 dose; -16 h prior to stimulation and at time of stimulation; 2 dose; -40 h prior to stimulation, -16 h prior to stimulation and at time of stimulation; 3 dose.

Given the natural variability in gene expression, data was analysed in terms of fold-change compared to control (unmedicated vehicle) treatment for both our *ex vivo* model and from clinical biopsies. Percent reduction was based on calculations from Microchem Laboratory ("Log and Percent Reductions in Microbiology and Antimicrobial Testing," 2018). Briefly, values were estimated from a published graph (Guttman-Yassky et al., 2017). The values of fold change (normalized to non-lesional/untreated) were then converted to log reduction values (L) by the equation L=log10(veh)-log10(trx). In the event that the treated samples gave a negative percent reduction value, the equation summed the two values to depict the cumulative reduction to vehicle. The log reduction to vehicle values were then converted to percent reduction to vehicle (P) by the equation P=(1-10^-L)*100. Negative numbers denote an increase to vehicle.

2.4 Pharmacokinetic translation

Samples reserved to test drug uptake were cleaned and heat-separated into the epidermis and dermis skin strata. Each section was weighed and then drug extracted and quantified using an internally verified LC-MS/MS method (data not shown). Percent uptake of the applied dose was calculated as drug recovered multiplied by 2 (to normalize for skin samples being halved) divided by the drug amount applied to the skin.

2.5 Analytical method for drug quantitation in tissue

Concentrations of clobetasol propionate and pimecrolimus were determined with a qualified UPLC-MS/MS method. The instrumentation consisted of a Waters Acquity I-Class Ultra Performance Liquid Chromatograph (UPLC) interfaced with a Waters TQ-XS triple quadrupole mass spectrometer (MS/MS). Chromatographic separation was performed using a

Waters HSS T3 column (2.1 x 50 mm, 1.8 µm particles) held at 30°C with mobile phases of 0.01% formic acid in water (A) and neat methanol (B). Compounds were eluted with a linear gradient of A in B starting at 2%B at 0.1 min and ramping to 98%B at 3.5 min at a flow rate of 0.4 mL/min; the total run time was 4.5 min. The MS was operated in positive ion mode with an electrospray ion source. Precursor to fragment ions monitored for clobetasol propionate, pimecrolimus, and betamethasone valerate (internal standard) were 467.2/355.0, 832.6/604.5, and 477.3/355.3, respectively. The quantitation range for each analyte was typically 0.1 – 1000 ng/mL.

3. RESULTS AND DISCUSSION

3.1 Comparison of pre-dosing HESC with Th1 stimulation with Elidel and Dermovate

To explore the pharmacokinetic/pharmacodynamic correlation of two different topical anti-inflammatory drugs in the HESC model to clinical application, Th1 stimulation was chosen due to the abundance of Th1 T-cells intrinsically present in the skin (der Veen et al., 2011), and the Th1 relationship to chronic atopic dermatitis (Oyoshi et al., 2009). Elidel cream containing 1% pimecrolimus (Pim 1%) and Dermovate cream containing 0.05% clobetasol propionate (CP 0.05%) were used to correlate potency, penetration, and dosing to clinical AD biopsies with the same formulations. The vehicle in this assay, Glaxal Base Cream Moisturizer, was chosen to directly correlate to the vehicle utilized in the clinical trial by Guttman-Yassky et al (Guttman-Yassky et al., 2017). In clinical application, the topical treatment was applied daily over a two-week period, whereas the *ex vivo* skin cultures were dosed at ~18.0 µL/cm^2 to allow for accurate and reproducible dispensing and full coverage of the formulation, either as a single dose at the time of stimulation (cotreatment), two doses at

16 h prior to stimulation and cotreatment, or three doses at ~40 h and 16 h prior to stimulation and cotreatment to discern the optimal treatment paradigms with the therapeutic prior to stimulation for comparison to clinical AD gene expression. Tissue was harvested 24 h post-stimulation and analysed by RT-qPCR for gene expression with changes compared to vehicle treatment (Figure 2). In general, anti-inflammatory activity increased with repetitive dosing of both Elidel and Dermovate, with 3-day treatment most closely correlating with the gene expression values reported after 2 weeks treatment taken from *in vivo* biopsies. For example, IFNg gene expression was reduced 36% (P=0.0671) following a single application of pimecrolimus, 37% (P=0.0170) following two applications, and 69% (P=<0.0001) after three applications to the *ex vivo* skin culture. The 3-day pimecrolimus treatment resulted in a 22% reduction (P=0.4610) compared to vehicle alone in *ex vivo* skin culture, similar to the c.a. 20% reduction seen with clinical treatment (Figure 4). Likewise, *ex vivo* skin culture treatment with clobetasol reduced MMP12 gene expression incrementally by 92%, 94%, and 96% (P=<0.0001) with increasing doses compared to stimulated alone, with 3-day treatment resulting in 96% reduction (P=0.0012) to vehicle *ex vivo* compared to c.a. 100% reduction *in vivo*. As illustrated by the inability of pimecrolimus to reduce inflammation of IFNg with either 1 or 2 doses, the same trend was observed in multiple biomarkers, exhibiting the necessity of at least 3 doses for drug efficacy in the HESC model for this drug. Thus, on average, the 3 day pre-dosing schedule with topical therapeutics most closely mimicked the results of 2 week dosing employed in the clinical trial with regard to inhibition of inflammatory gene expression and so this treatment regimen was employed for subsequent studies to determine the effects on gene expression without compromising the *ex vivo* skin culture integrity.

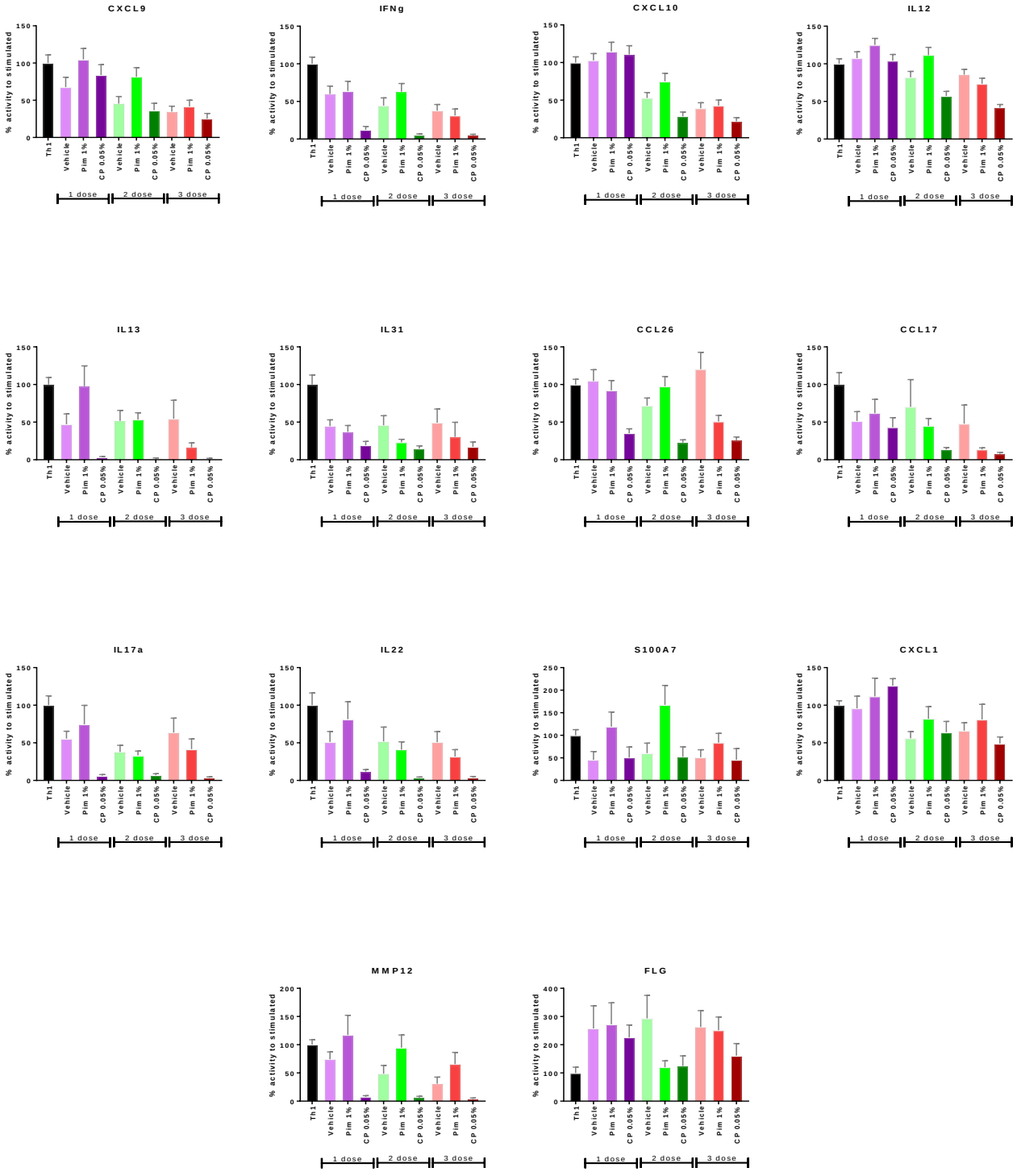

Figure 2. **Percent activity of gene expression with topical application of Elidel and Dermovate compared to Th1-mediated stimulation with one, two and three daily doses.** Average fold change of four combined donors stimulated by Th1 cytokine cocktail. N=4 samples per treatment per donor. Error bars represented as standard error of mean (SEM). Pim 1% is Elidel cream; CP 0.05% is Dermovate cream. Statistical analysis performed using GraphPad Prism v9 software; unpaired non-parametric T-test with Mann-Whitney test.

3.2 Pharmacokinetic evaluation of Elidel and Dermovate tissue penetration

Similar to the *in vivo* situation, pharmacokinetic analysis of these treatments in our *ex vivo* skin culture showed a time and formulation dependent penetration profile. Application of Elidel 1% resulted in 87-295 µg/g drug recovery from the epidermis, supporting the data from Gschwing et al who observed variable steady state absorption as early as 0.5hr after application (Gschwind et al., 2008) similar to the early saturation seen in the *ex vivo* skin culture. Elidel 1% formulation resulted in a ca. 0.05-0.11% applied dose recovery from the dermis independent of the number of applications suggesting early tissue saturation; 3.45 µg/g of pimecrolimus was recovered from the tissue after 16 h pretreatment (ca. 40 h drug application), similar to the penetration study run by Meingassner et al showing 4.4 µg/g after 48 h application (Meingassner et al., 2005). Dermovate 0.05% dosing resulted in an increase from 27 to 46 µg/g drug recovery from the epidermis and 2 to 10 µg/g drug recovery from the dermis, (ca. 1.3% to 6.2% of the applied dose) with increasing applications (Figure 3). An *in vitro* penetration test assessing topical application of clobetasol propionate found similar recovery from Temovate emollient cream of 4.3% of the applied dose in the receptor solution 48 h post-application (Lehman and Franz, 2014).

While the mass of pimecrolimus delivered to the dermis was approximately 1.5 times that of clobetasol from a single dose, due to differences in molecular weight between pimecrolimus (810 Da) and clobetasol (467 Da), the molar equivalency was nearly identical; $4.26x10^{-6}$ and $4.28x10^{-6}$ respectively. However with repeat dosing, pimecrolimus penetration did not increase over time, as discussed below, while clobetasol continued to increase to a concentration of $2.14x10^{-5}$ moles after 3 applications (40 hr and 16hr pretreat and cotreat). It is well known that the physico-chemical properties of a permeant affect its transport through human skin. In very broad terms, permeation is relatively high for molecules with a molecular weight of <500 Da and which are relatively (but not highly) lipophilic – typically

with a log $P_{octanol/water}$ value between 1 and 4 (Brown and Williams, 2019). From the molecular weight data above, it is clear that pimecrolimus (810 Da) is beyond the ideal molecular weight for delivery whereas clobetasol is within the optimal range and this may partially explain the lack of increasing delivery with increased pimecrolimus dosing. In terms of lipophilicity, clobetasol propionate has a logP of 3.5, within the optimal range whereas pimecrolimus is more highly lipophilic with an experimentally determined logP of 4.4. Whilst this increased lipophilicity will be advantageous in pimecrolimus permeating through the lipophilic stratum corneum barrier, clearance from the stratum corneum into the essentially aqueous environment of the epidermis could be limited, and allow a reservoir of the drug to accumulate in the stratum corneum (Brown and Williams, 2019). As a consequence, it seems likely that pimecrolimus approximately "saturated" the stratum corneum and could not clear into the epidermis and hence repeat dosing caused no further increase in drug delivery whereas the less lipophilic steroid could enter the epidermis and so further dosing delivered greater levels of the drug to the tissue. Further, it should be noted that non-sink conditions can influence the permeation of a drug. For our study, to maintain tissue integrity, we were required to use Cornification media as the receiver fluid in a static (i.e. not flow through) system. In a static system, it is feasible that the drug could accumulate to a point where the receiver solution no longer provides sink conditions and thus decreases the thermodynamic driving force for permeation from the outer surface to the receiver medium. Whilst pimecrolimus is very poorly water soluble (<1mg/mL), the Cornification media contains serum albumin which improves drug solubility through protein binding, media were changed every 48 hours to mitigate loss of sink conditions and the amount of drug applied and which could transport to the receiver fluid was low.

From this data, a correlation between drug penetration and biological activity can be seen, for example, between dermal drug levels and anti-inflammatory gene expression. Considering

IFNγ gene expression, Dermovate delivered ~2 ug/g clobetasol to the dermis after a single application resulting in ca. 88% reduction in activity. Elidel delivered similar levels (3.45 µg/g) of pimecrolimus after two applications and resulted in ~ 37% reduction in IFNγ gene expression. This suggests clobetasol, a corticosteroid known to directly regulate gene transcription of up to 100 genes through glucocorticoid receptor signalling (Barnes, 2006), possesses 2-fold greater potency compared to pimecrolimus which specifically inhibits calcineurin inhibition in human skin, indirectly affecting IFNγ gene expression. Further, these data correspond to published clinical Total Sign Scores (TSS) assessing erythema, edema/papulation, oozing/crusting, excoriation, lichenification, and dryness using the same formulations (Guttman-Yassky et al., 2017); Dermovate 0.05% resulted in 75.5% TSS reduction on day 15 compared to Elidel 1% with a TSS reduction of 39.6%, similar to the IFNγ gene expression effects seen above.

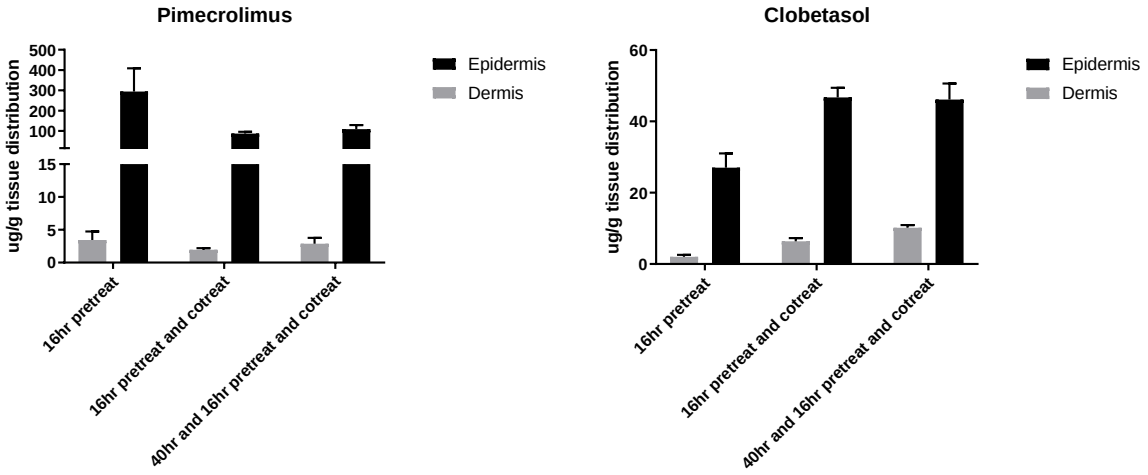

Figure 3. **Average tissue distribution of Elidel (Pimecrolimus) and Dermovate (Clobeatsol) (ug/g tissue) with topical application of one, two and three daily doses.** Tissue distribution presented as ug/g tissue N=4 donors, 4 samples per treatment. Error bars represented as standard error of mean (SEM).

3.3 Correlation of *in vivo* clinical biopsies with Elidel and Dermovate treatment to HESC model with Th1 stimulation

A recent study compared daily application of 2.0 mg/cm^2 of either Elidel cream containing 1% pimecrolimus (Pim 1%) or Dermovate cream containing 0.05% clobetasol propionate (CP 0.05%) (Guttman-Yassky et al., 2017). Thirty adult patients were recruited with mild-to-moderate atopic dermatitis to compare the anti-inflammatory profiles of these two prescription therapeutics against a non-medicated cream vehicle. On day 15, biopsies were taken and gene expression quantified and converted to percent reduction compared to vehicle treated skin sites ("Log and Percent Reductions in Microbiology and Antimicrobial Testing," 2018). This data was used to correlate our HESC inflammatory model findings with *in vivo* efficacy as a direct measure of biological target engagement as opposed to the common practice of simply quantifying drug levels within and traversing the tissue.

Figure 4 compares the changes in gene expression of inflammatory mediators from the *in vivo* study with atopic dermatitis patients (values extrapolated from the published graphs) with those determined following treatment of our Th1-mediated model; Table 1 provides the numerical values used.

The Th1-associated biomarkers, CXCL9, IFNg, CXCL10 and IL12, showed similar changes in gene expression with treatment between the *in vivo* clinical study and the Th1-mediated HESC disease model for both drugs. For clarity, the data in Figure 4 and Table 1 show the mean values for changes to gene expression. Both the derived clinical data and that from these HESC studies show significant variability due to the inherent variability of human immune response and hence statistical comparisons of the data are difficult. Notwithstanding, expression of the Th1-associated biomarkers were modified by both pimecrolimus and clobetasol propionate in a similar manner in both clinical and HESC studies with, for example, IFNg expression reduced by 20% *in vivo* and 22% in the HESC system by pimecrolimus and by 95% *in vivo* and 97% in HESC by clobetasol propionate; again illustrating the difference in potency of these two agents. The analysis compares drug

treatments with the vehicle control to show decreases or increases in expression due to the drugs. However, the authors of the clinical study noted that "Overall, vehicle and pimecrolimus show a similar pattern and still maintain upregulation of inflammatory markers, whereas these are significantly reduced with steroids".

The Th2-associated biomarkers, IL13, CCL26 and CCL17, corresponded with relative reductions in gene expression both *in vivo* and *ex vivo*; for example, with pimecrolimus treatment, IL13 (c.a 70% and 68% respectively), CCL26 (c.a. 3% and 57%, respectively) and CCL17 (c.a. 16% and 46%, respectively) expression reduced in both our model system and in the *in vivo* study. However, with pimecrolimus treatment IL31 expression increased *in vivo* but reduced in the *ex vivo* skin culture. This discrepancy may reflect that IL31 is more closely associated to a Th2-driven inflammatory state, while Th1-specific induction was employed in this study

Primary T-cell derived cytokines IL17a and IL22 are Th17-associated biomarkers and again showed good correlation between the *in vivo* biopsies and the *ex vivo* skin culture with both Elidel and Dermovate treatments. Both formulations *in vivo* resulted in c.a. 75-100% reduction in gene expression, compared to *ex vivo* reductions of c.a. 50-95% with treatment. For S100A7 and CXCL1, clobetasol treatment reduced gene expression in both scenarios (S100A7 ~100% *in vivo*; 41% *ex vivo*; CXCL1 ~ 87% *in vivo*; 31% *ex vivo*). With pimecrolimus treatment, these two biomarkers were inhibited following clinical *in vivo* dosing, but increased expression in the HESC, potentially due to the strong association of S100A7 and CXCL1 to Th17-derived inflammation.

Table 1 demonstrates that clobetasol treatment showed excellent agreement on regulating gene expression between the *in vivo* and *ex vivo* skin culture studies with 13 of the 14 genes showing similar reductions in expression. The exception was for the terminal differentiation

biomarker, FLG, where clobetasol propionate increased expression by c.a. 71% *in vivo* but decreased gene expression 42% *ex vivo*. This discrepancy may relate to the time constraints inherent with the *ex vivo* skin culture, which only allows for abbreviated tissue stimulation since FLG is engaged at a later stage in the stimulatory pathway elicited by the cocktail. As described above, the corticosteroid directly regulates gene transcription of up to 100 genes with its therapeutic efficacy demonstrated by the significant reductions seen in gene expression *in vivo*. In contrast, pimecrolimus is less effective clinically and specifically inhibits calcineurin in human skin. In addition to the concordance between *in vivo* and *ex vivo* regulation of Th1 associated biomarkers, and lack of agreement for Th2 and Th17 associated biomarkers, expression of the matrix metalloproteinase biomarker MMP12 and the barrier function marker filaggrin (FLG) also differed following pimecrolimus treatment. These results demonstrate that the *ex vivo* skin model can be used to discriminate the molecular mechanism of action of putative therapeutic agents that may be obscured in *in vivo* studies.

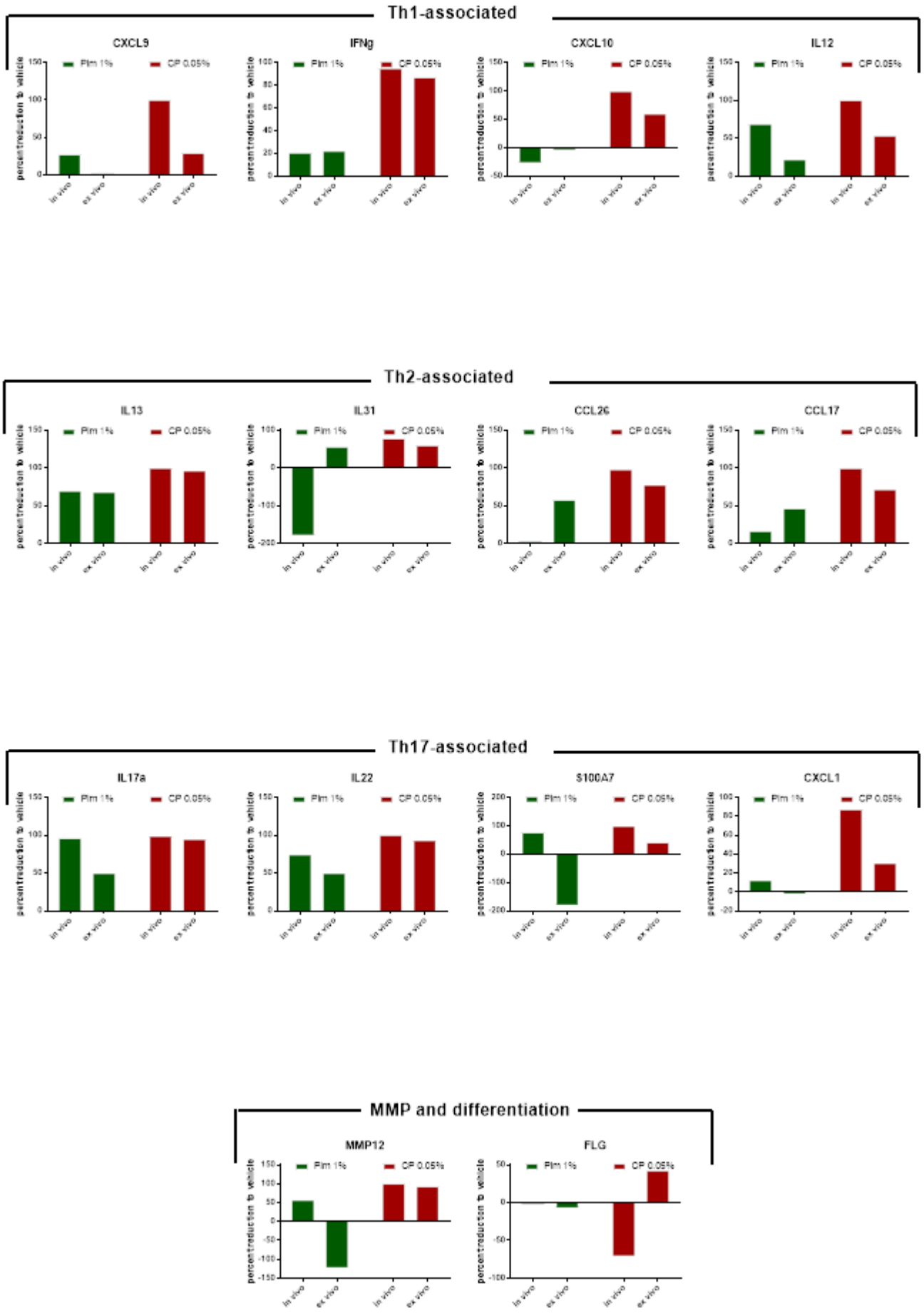
Th1-associated
CXCL9
IFNg
CXCL10
IL12
Th2-associated
IL13
IL31
CCL26
CCL17
Th17-associated
IL17a
IL22
S100A7
CXCL1
MMP and differentiation
MMP12
FLG
Plm 1%
CP 0.05%
percentreduction to vehicle
in vivo
ex vivo

Figure 4. **Percent reduction *in vivo* and *ex vivo* in gene expression calculated from fold change with 3 daily doses of Elidel and Dermovate compared to Th1-mediated stimulation**. In Vivo (Guttman); n=30 donor clinical trial, single replicate; cream applied daily for 14 days. Approximate values based on graph depiction. See Materials and Methods for calculation algorithms. Ex Vivo (HESC); n=4 donors (CXCL9 and CCL17; 3 donors), 4 replicates each; cream applied daily for 3 days with Th1 stimulation on the third day. Pim 1% is Elidel cream; CP 0.05% is Dermovate cream. All percent reductions calculated to respective vehicle.

Table 1. **Percent reduction *in vivo* and *ex vivo* in gene expression with 3 daily doses of Elidel and Dermovate compared to Th1-mediated stimulation.**

Gene of interest	Pimecrolimus		Clobetasol	
	In Vivo*	Ex vivo	In vivo*	Ex vivo
CXCL9	27	2	100	29
IFNg	20	22	95	97
CXCL10	-27	-5	99	60
IL12	69	22	100	53
IL13	70	68	100	97
CCL26	3	57	98	78
CCL17	16	46	99	72
IL17a	96	50	99	95
IL22	75	50	100	93
IL31	-179	55	77	59
S100A7	76	-182	100	41
CXCL1	13	-2	87	31
MMP12	58	-124	100	93
FLG	2	-7	-71	42

*approximate value based on graph depiction.

Table 1. **Percent reduction *in vivo* and *ex vivo* in gene expression of fold change with 3 daily doses of Elidel and Dermovate compared to Th1-mediated stimulation**. Guttman (*in vivo*) n=30 donor clinical trial, single replicate; cream applied daily for 14 days. Approximate values based on graph depiction. See Materials and Methods for calculation algorithms. HESC (*ex vivo*) n=4 donors (CXCL9 and CCL17 3 donors), 4 replicates each; cream applied daily for 3 days with Th1 stimulation on the third day. Pim 1% is Elidel cream; CP 0.05% is Dermovate cream. All percent reductions calculated to respective vehicle. Values in grey show a lack of parallel change in gene expression.

4. CONCLUSION

Pre-clinical testing of novel active ingredients or modified formulations for atopic dermatitis can assess drug delivery using *in vitro* permeation and penetration tests but currently require animal models with well-known limitations to estimate biological activity. Here, we have shown an *ex vivo* skin culture can be maintained in culture to allow rapid assessment of drug delivery to, and biological activity within, skin stimulated to mimic atopic dermatitis. The HESC model with Th1 stimulation showed a time and formulation dependent gene expression profile of inflammatory biomarkers associated with atopic dermatitis which allows characterisation and measurement of the effects of single and multiple doses with topical therapeutics in preclinical development. Penetration profiles in conjunction with gene expression data allow correlation of tissue concentrations and drug efficacy prior to clinical application. The correlation observed between clinical AD biopsy data and the *ex vivo* Th1 model shows the translation capability of this model for early development of novel topical therapeutics that is lacking in available animal models. The translation of the HESC model to *in vivo* clinical data justifies the use of human *ex vivo* skin culture in an inflammatory state in early development and the characterization and comparison of novel drugs and formulations with those already marketed has the potential of de-risking costly and time-consuming clinical trials. While clobetasol is used for a wide variety of inflammatory dermatoses given the broad range of steroidal anti-inflammatory activity, the calcineurin inhibitor pimecrolimus is mainly used topically for mild to moderate eczema. Due to the known side effects of steroids such as skin thinning, stinging, and folliculitis, steroids are not recommended for long term topical use, but may be more readily prescribed over target specific inhibitors for more extreme cases of inflammation. Additional models to represent not only Th1-mediated inflammatory dermatoses, but also Th2 and Th17 prevalent skin

disease, are required and are currently in development to add further support to these findings and provide broader potential applications (Neil et al 2022).

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