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Neil, M. S. Jessica E., Lenn, Jon D., Brown, Marc B. and Williams, Adrian C. ORCID logo ORCID: <https://orcid.org/0000-0003-3654-7916> (2022) A new ex vivo skin model for mechanistic understanding of putative anti-inflammatory topical therapeutics. *International Journal of Pharmaceutics*, 617. 121610. ISSN 0378-5173 doi: <https://doi.org/10.1016/j.ijpharm.2022.121610> Available at <https://centaur.reading.ac.uk/103845/>

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To link to this article DOI: <http://dx.doi.org/10.1016/j.ijpharm.2022.121610>

Publisher: Elsevier

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**A new *ex vivo* skin model for mechanistic understanding of putative anti-inflammatory topical therapeutics**

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## **ABSTRACT**

Several *in vitro* models have been designed as test systems for inflammatory skin conditions, commonly using cell-culture or reconstructed human epidermis approaches. However, these systems poorly recapitulate the physiology and, importantly, the metabolism and biochemical activity of skin *in vivo*, whereas *ex vivo* skin culture models can retain these features of the tissue. 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. Following exogenous stimulation, tissue integrity and ability to induce inflammatory gene expression was retained, and stimulant concentrations and duration was optimised to mimic published data from inflammatory clinical biopsies of dermatitis and psoriasis patients. The validity and utility of the model was demonstrated when challenged with 5 drugs including a corticosteroid and vitamin D3 analogue, where inflammatory biomarkers were regulated in a manner consistent with the drugs' reported *in vivo* mechanisms of action. This model retains important inflammatory gene signals observed in human inflammatory dermatoses for preclinical evaluation of novel therapeutics.

**Keywords:** human ex-vivo skin; ex vivo skin culture model, Th1, Th2, Th17, psoriasis, dermatitis, eczema, preclinical dermatology, inflammatory dermatoses

## 1. INTRODUCTION

The term “inflammatory dermatoses” encompasses a wide range of skin disorders from allergic response in contact atopic dermatitis to misdirected autoimmunity in alopecia, vitiligo, psoriasis, and rosacea (Richmond and Harris, 2014). Atopic dermatitis (AD), psoriasis and rosacea together account for over \$52 billion in medical treatment expenses (“Skin Conditions by the Numbers,” 2021). Dermatitis and psoriasis are two of the most well characterized skin diseases that can be treated with both specific target antagonists and broad range therapeutics. These two dermatoses are driven by activated T cells comprised predominantly of Th1, Th2, and/or Th17 T cell populations (Guttman-Yassky and Krueger, 2017).

Multiple animal models have been employed for research into atopic dermatitis, commonly using mice and dogs. Inbred and transgenic mouse strains such as NC/Nga and hapten-induced AD have proven utility but have the disadvantage of markedly different skin morphology and genetic manipulation to human skin (Schon, 2008). Canine models, which present spontaneous AD in about 10 % of the population and is the most commonly diagnosed atopic disease in dogs, also poorly represent human tissue (Marsella and Olivry, 2003). Beyond ethical concerns, animal models require specialist breeding and housing, there are innate differences in immune function compared to human skin, and well-reported differences in thickness and barrier properties of animal skin for topical compound treatment in humans (Shiohara et al., 2004).

Current *in vitro* human skin models also present limitations for therapeutic investigation. Notably, changes in epidermal thickness, decreases in tissue integrity, and increases in keratinocyte apoptosis are observed (Xu et al., 2012, Steinstraesser et al., 2009).

Inflammatory stimulation of reconstructed human epidermis (RHE) and human *ex vivo* skin

cultures have been explored, where differences in gene expression have focused on keratinocyte differentiation biomarkers (Rabeony et al., 2014), however the increased permeability of RHE make them inappropriate for topical application of novel therapeutics (Netzlaff et al., 2007).

*Ex vivo* skin culture models can mitigate some of the above deficiencies as the tissue contains not only differentiated keratinocytes, but also fibroblasts, Langerhans cells, dendritic cells and resident T cells (Bocheńska et al., 2017). Jarret et al (Jarret et al., 2020) recently described the development of a psoriasis-like *ex vivo* human skin model utilizing these advantages and a Th17 stimulation cocktail to stimulate resident immune cells in the human skin for therapeutic evaluation of novel anti-inflammatory agents in the laboratory. Here it is shown that, not only can human *ex vivo* skin culture (HESC) be stimulated to express immune-specific biomarkers, but also provide an inflammatory tissue environment to elicit dermatosis responses from immune cells similar to *in vivo* lesions, and responds in a predictable manner to clinically effective drugs. Trans-epithelial electrical resistance can be utilized to ensure adequate tissue barrier integrity for topical formulation application. Here we show similar inflammatory conditions can be induced for a wider range of dermatoses including not only Th17-mediated inflammation, but also for Th1 and Th2-mediated inflammatory dermatoses and that this model responds predictably to 5 different therapeutic agents.

## **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 are 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<sub>2</sub> in Costar Transwell Permeable Supports (Thermo Fisher Scientific). Briefly, tissue was chilled until processing for culture and used within 24 h of surgery. Tissue was defatted by carefully separating underlying adipose tissue from the dermal layer using a scalpel and then dermatomed (Integra Padgett Slimline SB) to a thickness of 750 µm; tissue exhibiting abnormalities such as oedema, abrasion, or heavy striation was discarded. Tissue was prepared for plating using 7 mm biopsy punches and placed into 0.33 cm<sup>2</sup> permeable supports, 0.4 µm pore size, so that the dermal tissue retains contact with the media and the stratum corneum is exposed to the air. The well was then filled with modified DMEM/Hams F12 Cornification media resulting in an air-liquid interface *ex vivo* skin culture (Vostálová et al., 2018). Media was changed every 48 h. The stimulation cocktails, Th1, Th2 or Th17, were added to fresh media and then applied basally to the tissue in the cornification media to mimic dermal stimulation of inflammation (Smith et al., 2016). For the target specific inhibition of inflammatory gene expression with Th1, Th2 or Th17 stimulation (Figure 4), HESC were treated overnight (ca. 16 h) with 1 µM compound, then stimulated for 24 h with either Th1, Th2, or Th17 stimulation cocktail at mid-level strength.

## **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 Applied Bioscience QuantStudio 6

Flex Real-Time PCR System. All primers were commercially available and purchased from Invitrogen Life Technologies (see Supplemental Data Figure 5). Fold change was calculated as the ratio of the power of the gene of interest divided by the average power of the unstimulated samples (Livak and Schmittgen, 2001) to normalize the unstimulated samples to a value of 1. For comparison of multiple skin donors, stimulated samples were normalised to 100 % maximum activity. Percent activity was calculated as the percent increase or decrease in stimulation compared to the stimulated samples set at 100 % maximum activity.

### **2.3 Inhibitor specificity**

Human *ex vivo* skin culture, processed as above, was placed in Transwells. Target specific inhibitors were applied basolaterally, each at 1  $\mu$ M for overnight treatment (ca ~16 h) prior to stimulation (Clobetasol propionate, Selleck Chemical # S2584, Batch No 02; Calcitriol, Cayman Chemical #71820, Batch No 0495381-30; Pimecrolimus, Selleck Chemical #S5004, Batch No 03; Crisaborole, MedChem Express #AN-2728, lot #16616; Tofacitinib, ApexBio #477600-75-2, Batch No 01). The next morning, fresh media containing inhibitors and cytokine cocktail stimulation were added basolaterally for 24 h stimulation. Tissue was harvested and processed for RNA isolation and qRT-PCR.

### **2.4 Data Processing and Statistical Analysis**

All statistics were analysed by GraphPad Prism v7 software using non-parametric two-tailed t-test with Mann-Whitney (95% confidence).

## **3. RESULTS AND DISCUSSION**

### **3.1 Skin characterization in *ex vivo* culture**



HESC can be maintained for up to 9 days without stimulation with minimal impact to structural tissue integrity based on necrosis and epidermal/dermal separation and metabolic activity based on ribosomal 18s gene expression (Neil et al., 2020). Here, skin integrity was assessed following either Th1, Th2, or Th17 cytokine stimulation with haematoxylin and eosin staining of tissue to determine integrity in an inflammatory environment. The stimulation cocktail primes the naïve skin resident T cells with Cluster of Differentiation 3 and 28 (CD3/CD28) antibodies with additional cytokines to direct the inflammatory stimulation toward either a Th1, Th2, or Th17 signalling pathway. Skin was stimulated and then harvested at Day 1, Day 2, Day 3 and Day 4 in culture. The images show that up to Day 3 in culture the tissue maintains visual integrity with little or no evidence of spongiosis, necrosis, parakeratosis or epidermal/dermal separation (Figure 1). However, Day 4 post-stimulation spongiosis becomes apparent. These results suggest an abbreviated window available for tissue analysis after inflammation induction, but still provides sufficient time to allow multiple dosing.

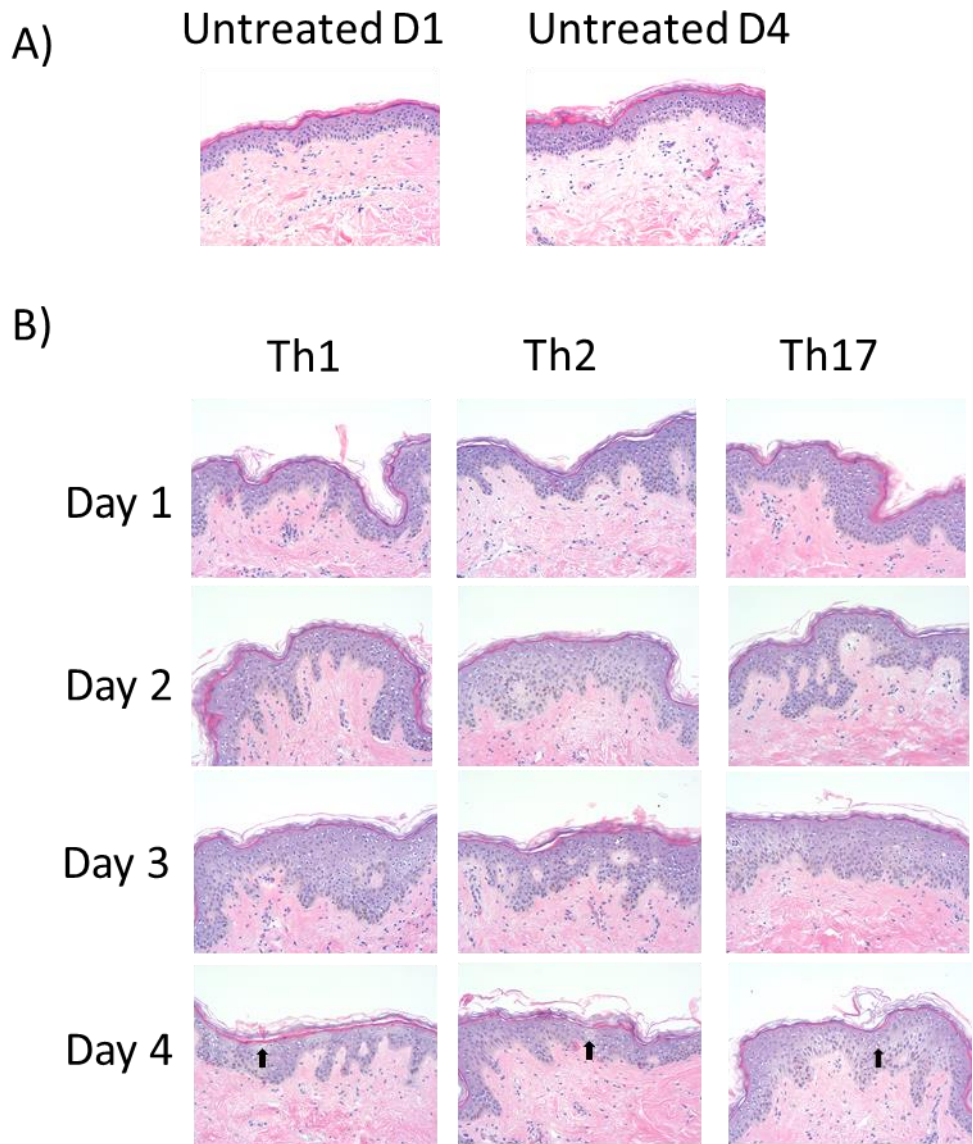


Figure 1. **Haematoxylin and eosin histology of tissue integrity over time following Th1, Th2, or Th17 stimulation.** A) Representative images from haematoxylin and eosin-stained untreated tissue at Day 1 (24 h post-stimulation) and Day 4 (96 h post-stimulation). B) Representative images from haematoxylin and eosin-stained tissue over time course with Th1, Th2, or Th17 stimulation. Images taken at 20X magnification. Solid arrow points to spongiosis (Day 4).

### **3.2 Assessment of gene induction with Th1, Th2 or Th17 cytokine cocktails**

In order to determine the level of stimulation attained with either the Th1, Th2 or Th17-mediated cytokine cocktails, an assay comparing low-, mid- and high- concentrations of each

cytokine stimulation was employed (Figure 2A, B, C) where each cytokine was either diluted or increased by 5-fold compared to the mid-level cocktail concentrations. To assess Th1-mediated stimulation, the cytokines IL12 and IL1 $\alpha$  and chemokines CXCL10 and CCL2 were quantified. To assess Th2-mediated stimulation, cytokines IL13 and IL31 and defensin DefB4 and chemokine CCL26 were quantified. To assess Th17-mediated stimulation, cytokines IL17 $\alpha$  and IL22 were quantified and DefB4 and chemokine CCL20. IFN $\gamma$  was evaluated for all three stimulation conditions to compare its expression and determine cross-stimulation (Figure 2D).

The Th1 stimulation cocktail did not indicate a plateau in any of the biomarkers assayed. The Th2 cytokines IL13 and IL31 expression peaked at the mid-level stimulation but downstream activation of DefB4 and CCL26 expression increased with higher levels of stimulation. The Th17 cytokine IL17 $\alpha$  showed maximal expression with the low stimulation cytokine cocktail, however the biomarkers IL22 and CCL20 showed higher expression with the mid-level stimulation cocktail. DefB4 continued to increase with the high-level Th17 stimulation.

IFN $\gamma$  gene expression was evaluated to determine the cross-pathway stimulation of the three cytokine cocktails. Whilst IFN $\gamma$  is highly Th1-associated and increased in a concentration-dependent manner, it is also expressed in the Th2 or Th17 systems, but was not attenuated by higher levels of stimulation.

All genes quantified achieved significance with the mid-level cytokine cocktail stimulation with the exception of Th2-induced DefB4. From these results, the mid-level cytokine cocktail was selected for all further evaluation, based on adequate levels for gene induction quantified across all the biomarkers assayed.

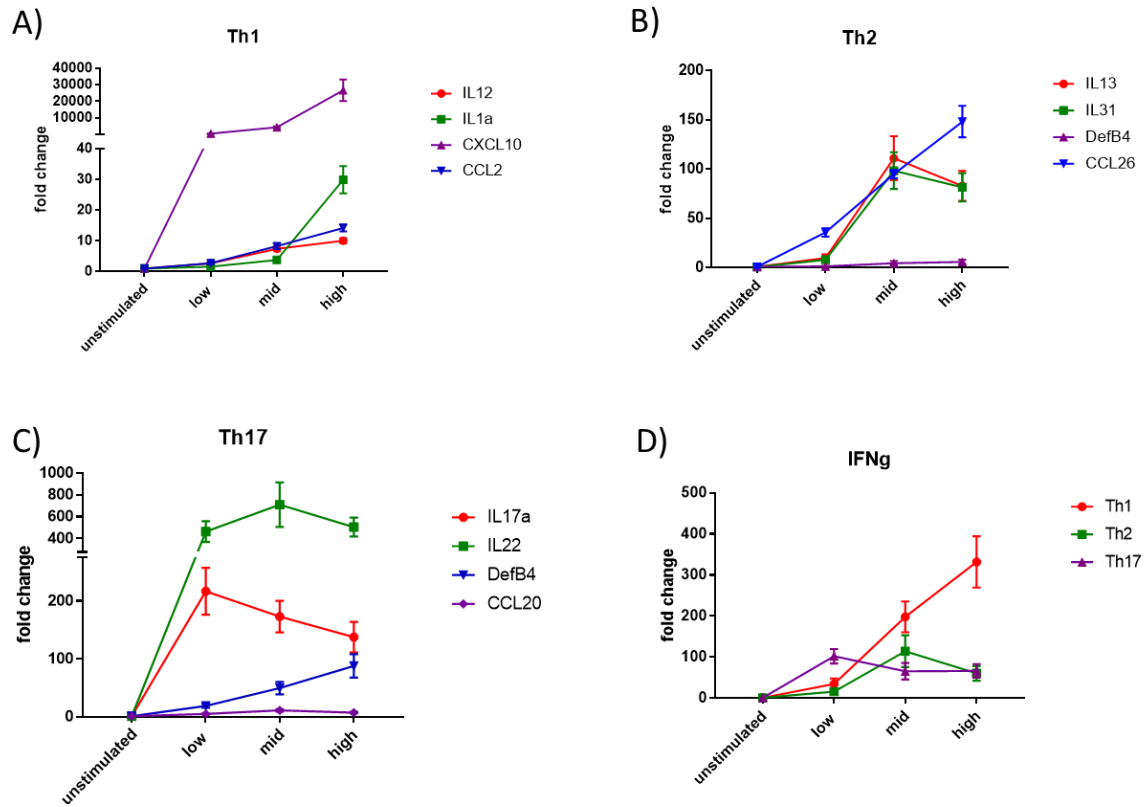


Figure 2. **Gene expression of the Th1, Th2, or Th17-associated inflammatory cytokines with low, mid, and high concentration cytokine cocktails.** Average fold change of three combined donors (4 replicates per donor) stimulated with low, mid and high dose Th1, Th2, or Th17 cytokine cocktails. Error bars represented as standard error of mean (SEM). A) Th1 comparing IL12, IL1a, CXCL10 and CCL2. B) Th2 comparing IL13, IL31, DefB4 and CCL26. C) Th17 comparing IL17a, IL22, S100A7 and DefB4. D) IFN $\gamma$  gene induction with Th1, Th2, or Th17 stimulation.

### **3.3 Biomarker characterisation of Th1, Th2 or Th17 stimulation**

To further characterize the biomarker response to Th1, Th2 or Th17 stimulation, 24 genes were chosen to illustrate Th1 and Th2-mediated inflammatory dermatoses, selected from multiple publications showing up- and down-regulated gene expression profiles in atopic dermatitis *in vivo* (B. Brandt, 2011, Neis et al., 2006, Suárez-Fariñas et al., 2015, Bianchi et al., 2012, Rozenblit et al., 2014, Gittler et al., 2012, Leung et al., 2004, Sonkoly et al., 2006).

While many of the assayed biomarkers are closely associated to either Th2 or Th1 inflammation, they are not mutually exclusive (Grewe et al., 1998). Similarly, a further 26 genes were chosen to illustrate the Th17-mediated disease state in psoriasis *in vivo* (Chiricozzi et al., 2011, Guilloteau et al., 2010, Keermann et al., 2015, Russell et al., 2014, Starodubtseva et al., 2011, Sobolev V, 2015, Suárez-Fariñas et al., 2010, Swindell et al., 2013, Villanova et al., 2013, Zaba et al., 2007, Zhou et al., 2003). Time-dependent (post-stimulation) changes in expression of these biomarkers are shown in Figure 3.

While hundreds of genes are reportedly associated to Th1 and Th2-mediated dermatoses, several are consistent biomarkers across publications and techniques. As such, genes coding for multiple inflammatory pathways were chosen as follows:

- T cell activation; IL2, IL4, IL5, IL6, IL10, IL13, IL16, IL17a, IL17f, IL22, IL31 and IFN $\gamma$
- Chemokine activation; CCL17, CCL22, CCL26, CXCL1, CXCL10
- Macrophage and dendritic cell activation; IL1b, IL12, IL23p19, CCL4, ARG2, MMP9, MMP12
- Epidermal cell activation; CCL20, CXCL1, CXCL6, IL8, IL36G, GM-CSF, IVL, LOR, S100A7, S100A9, S100A12, DefB4, Fil, LCN2, SerpinB4, TNF $\alpha$
- Monocyte and neutrophil activation: IL19, IL10, LCN2 and DefB4.

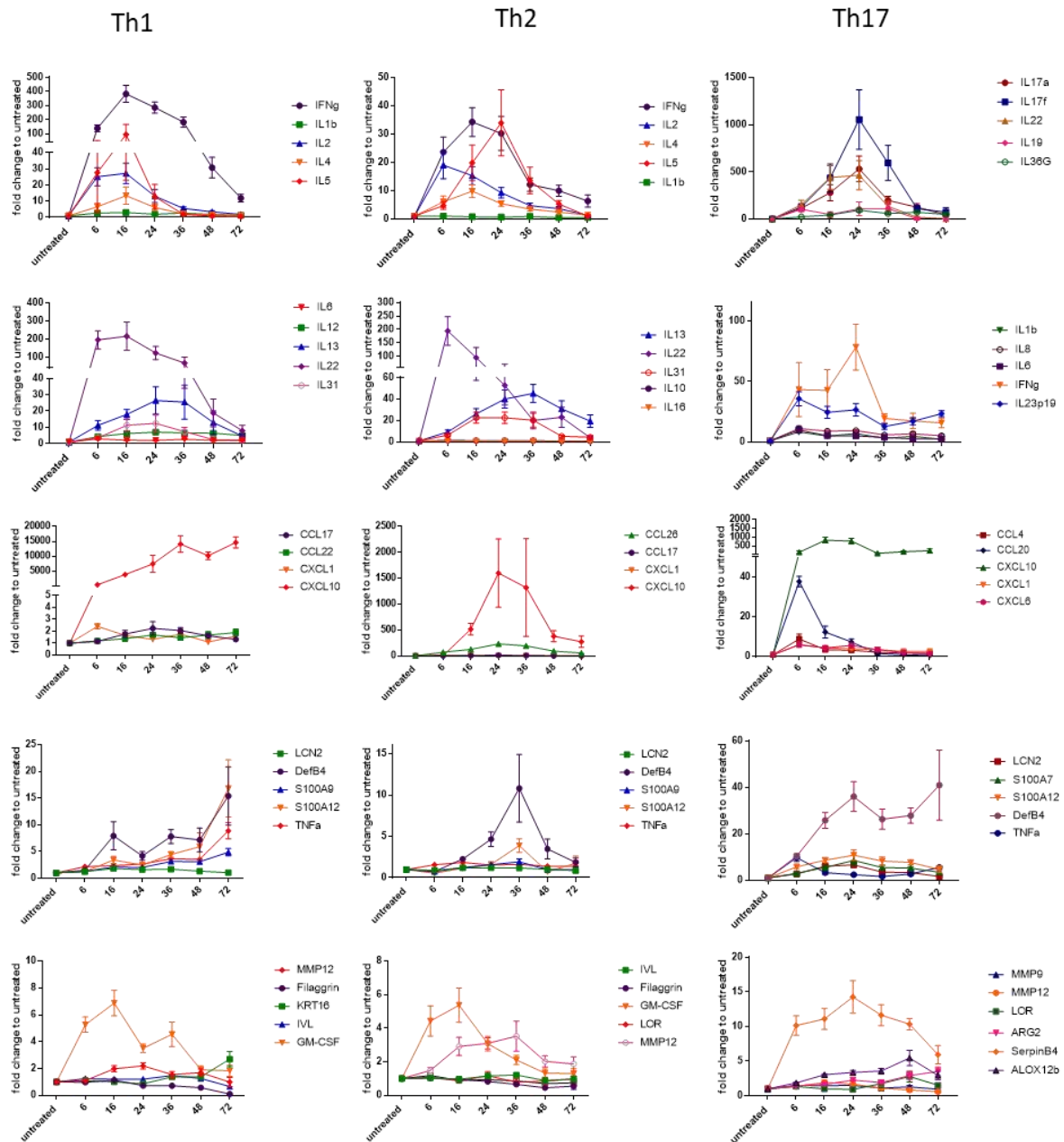
These biomarkers do not work in isolation but coordinate across signalling pathways to perpetuate the inflammatory response. This model retains the immune functions regularly described in inflammatory dermatoses such as T cell activation and expression of interleukins, monocyte and fibroblast activation and expression of chemokines, and keratinocyte activation and expression of antimicrobial peptides. Early responders in the Th1-mediated inflammatory pathway responsible for Th1 cell differentiation are interleukins IL2,

IL6, IL1 $\beta$ , IL12 and IFN $\gamma$ . IL12, produced primarily by macrophages and monocytes, is significantly higher in chronic dermatitis than acute stage disease (Hamid et al., 1996). The late-stage chemokine CXCL10 is consistent with the longer-term inflammatory condition. The antimicrobial genes S100A9, S100A12 and LCN show the tissues response to the prolonged inflammatory state as well as the ubiquitous TNF $\alpha$ . The barrier function genes that are typically down-regulated in chronic atopic dermatitis, IVL and FILAGGRIN, were downregulated 72 h post-stimulation. This observation further validates the similarity of this model to a clinical disease phenotypic state of barrier dysfunction characterized by disorganization of the protective keratin and structural proteins (Agrawal and Woodfolk, 2014). All genes quantified achieved significance by 16 h post-Th1 stimulation with the exceptions of IL31, INV, KRT16. FILAGGRIN gene expression did not reach significant down-regulation until 72 h post-stimulation.

Gene expression indicative of the Th2-mediated acute atopic dermatitis profile includes the expected IL4, IL5, IL13 and IL31 interleukins associated with early dermatosis disease-state (Gittler et al., 2012. Silverberg and Kantor, 2017, Bieber, 2020). Interleukin 31 is unique in its association to pruritus presented in dermatitis (Sonkoly et al., 2006, Furue et al., 2018). Also observed was an increase in CCL17 and CCL26 consistent with the early recruitment of T cells and eosinophils, respectively, to the site of initial inflammation. The fact that the Th2-dominant biomarkers IL-13 and IL-31 were also induced by the Th1 cytokine cocktail shows the lack of exclusivity with this cocktail, probably due to the large resident Th1 memory T cell population known to reside in the skin being reactivated in the inflammatory state (Clark et al., 2006). All genes quantified achieved significance by 16 h post-Th2 stimulation with the exceptions of S100A12, INV, LOR, and FILAGGRIN gene expression.

Some of the earliest responding biomarkers after Th17-mediated stimulation were the interleukins IL22 and IL23p19; the chemokines CCL20 and CXCL10; and the pro-

inflammatory serine protease inhibitor SerpinB4. The cytokine TNF $\alpha$  has long been associated with autoinflammatory diseases leading to the development of anti-TNF antibodies as injectable biological therapeutics. Upregulation of TNF $\alpha$  in psoriasis is known to induce keratinocyte derived CXCL1 as well as IL19 as seen in our model. IL23p19 is implicated in the survival and expansion of Th17 cells and remains upregulated throughout the study period. Another early induced cytokine, IL22, enhances production of DefB4 and S100A7, but also inhibits keratinocyte differentiation resulting in thickening of the epidermis (Grine et al., 2015). The Th17 model emulates this pathology, evidenced by the lack of LOR gene expression, a biomarker of keratinocyte terminal differentiation. Lastly, IL36G peaks at 24 h but maintains a high profile instead of downregulating like IL17a and IL17f. This cytokine is implicated in maintenance of the psoriatic phenotypic state by perpetuating an IL17, TNF $\alpha$  and IL36G feedback loop (Towne and Sims, 2012). As a trend, the chemokine responses peaked at 6 h post-induction, suggesting efficient recruitment of immune cells similar to an *in vivo* inflammatory state. The antimicrobial-associated biomarkers DefB4, LCN2, S100A7 and S100A12 tended to peak between 16 and 24 h and remained steady thereafter. The ECM-associated biomarkers such as MMP9, MMP12, ARG2 and ALOX12B showed very low-level induction with Th17 stimulation, however SerpinB4, known to act as a triggering autoantigen in psoriasis, activated as early as 6 h post-stimulation and increased up to 48 h. All genes quantified achieved significance by 16 h post-Th2 stimulation with the exceptions of LOR, and IFN $\gamma$  which achieved significance at 24 h.



**Figure 3. Gene expression of inflammatory dermatoses biomarkers induced by Th1, Th2, or Th17 stimulation cocktails.** Average fold change of three combined donors; 4 replicates per donor per time point, stimulated by Th1, Th2, or Th17 cytokine cocktail over a 72 h time course. Error bars represented as standard error of mean (SEM).

### **3.4 Comparison of the Th1, Th2, or Th17-mediated inflammatory dermatoses to lesional biopsies *in vivo* and demographic differences**



Gene expression of Th1 and Th2-mediated inflammatory dermatoses biomarkers in lesional biopsies with moderate to severe dermatitis has been reported (Suárez-Fariñas et al., 2015, Bianchi et al., 2012, Rozenblit et al., 2014, Neis et al., 2006). From Figure 3, IFN $\gamma$  and IL12 were selected for the Th1-mediated inflammatory dermatoses state and IL13 and IL31 as representative Th2-specific biomarkers for comparison with published *in vivo* biopsy data. To correlate the Th17-mediated model to clinical findings, the cytokines IL17a and IL17f were selected as being preferentially produced by Th17 T cells (Ouyang et al., 2008).

The HESC Th1 and Th2-induced inflammation resulted in greater inflammatory gene expression compared to that reported in lesional biopsies (Table 1) and provides a robust disease-like inflammatory state for evaluating topical therapeutics that allows for clear and quantifiable down-regulation of the resulting gene expression. Three literature reports provide the fold-change of IL17a and/or IL17f in Th17-mediated psoriatic lesional biopsies compared to non-involved tissue (Russell et al., 2014, Sobolev V, 2015, Suárez-Fariñas et al., 2010). While the induction of gene expression is robust in these models, the resulting gene expression creates an exaggerated inflammatory state compared to the lesional biopsy tissue reported here. For example, Th17 stimulation induced a 2-3 times greater change in IL17a compared to data from *in vivo* biopsies whereas IFN $\gamma$  increased between 13 and 230 times that reported in the literature after Th1 stimulation.

<b>Th1</b>		Neis	Rozenblit	Suárez-Fariñas	HESC
<i># of patients</i>		33-56	17	18	35-44

IL12			2.04	2.71	13.4±21.4
IFN $\gamma$		22.01	1.26	1.39	289±253
<b>Th2</b>	Bianchi	Neis	Rozenblit	Suárez-Fariñas	HESC
<i># of patients</i>	23	33-56	17	18	53-60
IL13		14.3	5.03	7.57	59.2±85.5
IL31	29.51±39.38	3.18			53.1±67.8
<b>Th17</b>		Sobolev	Russell	Suárez-Fariñas	HESC
<i># of patients</i>		10	24	15	45-49
IL17a		54.4	54*	72	149±140
IL17f			64*		197±194

±standard deviation where reported

\*approximate value derived from graphical data

**Table 1. Published results of the fold-change in gene expression for inflammatory markers from atopic dermatitis and psoriasis biopsies compared to non-involved skin and the average change in expression of the same markers using our Human Ex Vivo Skin Culture model**

To further characterize the reproducibility of the human *ex-vivo* skin culture with exogenous stimulation, inter-(Supplemental Data Figure 1) and intra-(Supplemental Data Figure 2) donor response variability was evaluated in 36-60 individual skin donors; as well as gene expression by race (Supplemental Data Figure 3) and age (Supplemental Data Figure 4). The only statistical difference observed between race occurred with Th17-induction by Student's t-test. Caucasian vs African American gene expression of IL17f resulted in a p value of 0.0308 and African American vs Hispanic gene expression of IL17f resulted in a p value of 0.0408. While trends in the decrease in gene expression with stimulation were observed in the age groups over 40 yrs, the only statistical significance by Student's t-test was observed with IFN $\gamma$  gene expression after Th1 induction, with a significant decrease in gene expression (p=0.0489) between the 40-59 yrs of age and those >60 yrs of age.

### **3.5 Target specific inhibition with Th1, Th2, or Th17 stimulation**

Inflammatory dermatoses are managed by both broad and target specific inhibitors. To correlate the results from our HESC inflammatory dermatoses model with *in vivo* data from known therapeutics, four established anti-inflammatory molecules were selected alongside a new Janus kinase therapeutic currently in clinical trials. Each inhibitor was applied basolaterally to maintain molar equivalence (1 $\mu$ M) when assessing anti-inflammatory activity of the compounds; the drugs have different permeability coefficients when applied topically which would result in dissimilar fluxes and hence concentrations in the culture media.

Clobetasol propionate, a highly potent corticosteroid targeting the glucocorticoid receptor is effective through its immunosuppressive, anti-inflammatory and antiproliferative actions, but side effects include skin atrophy, striae, and thinning of all dermal layers (Uva et al., 2012).

Calcitriol, a vitamin D3 analogue, also exhibits immunomodulatory effects and binds to the vitamin D receptor to inhibit dendritic cell maturation, proinflammatory cytokine production and keratinocyte and lymphocyte proliferation (Datta-Mitra et al., 2014).

Pimecrolimus was developed to inhibit calcineurin activity and down regulate T cell proinflammatory cytokine release (Malecic and Young, 2016).

Crisaborole is a phosphodiesterase type 4 (PDE4) inhibitor; inhibiting PDE4 activity of intracellular cyclic adenosine monophosphate degradation downregulates nuclear factor associated T cell signalling pathways and cytokine release (Paller et al., 2016).

Janus kinases (JAKs) elevate proinflammatory cytokines via STAT transcription factor activation and nuclear factor  $\beta$  expression. Tofacitinib, currently only available as an oral therapy but has been proposed for topical application, inhibits JAK signalling thus decreasing inflammatory cytokine expression in psoriatic lesions (Gladman et al., 2017).

Biomarkers were selected based on gene induction and disease-relevance to evaluate individual inflammatory effects with Th1, Th2 or Th17 cytokine stimulation. IFN $\gamma$ , IL12,

FILAGGRIN, CXCL10, S100A12 and GM-CSF were chosen to represent the Th1-mediated disease state. IL13, IL31, FILAGGRIN, CCL26, MMP12 and GM-CSF were used for Th2 cell-specific activation. IL17a, IL1b, DefB4, CCL20, SerpinB4, and IL8 were chosen to reflect the Th17-mediated disease state. These biomarkers represent multiple scenarios in inflammatory dermatoses including T cell activation, keratinocyte activation, antimicrobial expression, chemoattraction of lymphocytes, and barrier function (Figure 4).

The steroid clobetasol propionate inhibited multiple signalling pathways including IFN $\gamma$ , GM-CSF, IL13, IL31, CCL26, MMP12, IL17a and IL8 ( $p < 0.0001$ ) corresponding to published clinical trial data of clobetasol effect on AD lesions with treatment (Guttman-Yassky et al., 2017). The steroid significantly increased the chemokines CCL26 and CCL20. A similar result has been reported previously with an increase of chemokine expression following dexamethasone treatment and appears to be chemokine specific (Kato et al., 2009). It should also be noted that, dependent on the dose administered, steroids can be either pro- or anti-inflammatory. The increase in FILAGGRIN expression ( $p = 0.0014$  to  $0.0029$ ) relates to an increase in initial barrier integrity proteins consistent with improvements in barrier function, the disorganization of which is known to preclude and exacerbate the dermatitis outcome (Sandilands et al., 2009). A recent study highlighted the ability of glucocorticoids to mediate keratinocyte differentiation in wound closure via the phospholipase/protein kinase C signalling pathway (Jozic et al., 2017).

The vitamin D3 analogue calcitriol inhibited Th2-cell specific cytokines IL13 and IL31 ( $p < 0.0007$  and  $p < 0.0317$  respectively) and significantly increased CCL26 ( $p < 0.0001$ ) and S100A12 ( $p < 0.0068$ ) gene expression, illustrating its Th2-dominant activity. The vitamin D3 analogue significantly inhibited IL17a, CCL20, and the chemokine IL8 ( $p = 0.0242$ ,  $0.0242$  and  $0.0023$  respectively) but proved ineffective in altering the response of any other biomarker with the Th17-mediated inflammation.

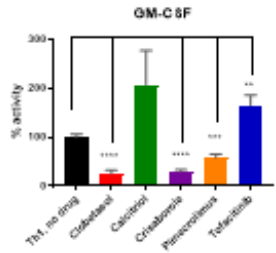
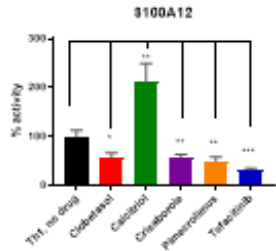
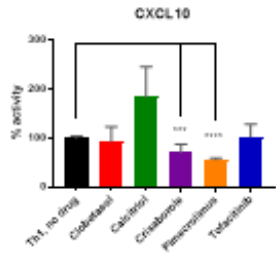
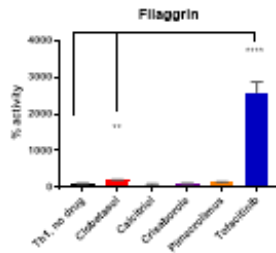
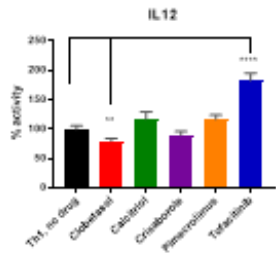
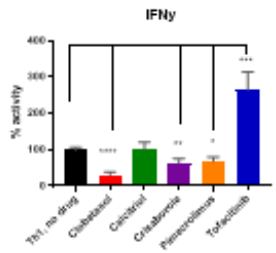
Crisaborole, the PDE4 inhibitor, significantly inhibited all Th2 associated biomarkers IL13, IL31, MMP12, and GM-CSF ( $p < 0.0001$ - $0.0014$ ) and increased CCL26 gene expression ( $p < 0.0001$ ), analogous to the gene expression profiles reported by Bissonnette, et al clinical application. Compared to the inhibition of IFN $\gamma$  ( $p = 0.0029$ ), S100A12 ( $p = 0.0068$ ), and GM-CSF ( $p < 0.0001$ ) with Th1-stimulation, and IL17a ( $p = 0.0018$ ), DefB4 ( $p = 0.0332$ ), and IL8 ( $p = 0.0011$ ) with Th17-stimulation, the PDE4 inhibitor was more effective in Th2 inflammatory down regulation, suggesting greater efficacy for Th2-mediated dermatitis. The decreases in IL13, IL31, IL17a, and MMP12 are similar to biopsy results following crisaborole ointment application on lesions (Bissonnette et al., 2019).

Calcineurin inhibition by pimecrolimus following Th1-mediated stimulation reduced IFN $\gamma$ , CXCL10, S100A12 and GM-CSF ( $p < 0.0001$ - $0.0387$ ). This reduction in inflammatory activity correlates to the clinical reduction of AD flares in children and adults and a mean Eczema Area Severity Index (EASI) reduction between 38-71 % in adults and 47-82 % in children in multiple clinical studies (Breuer et al., 2005). Calcineurin reduced Th2 T-cell specific activation of IL13, IL31, MMP12 and GM-CSF ( $p < 0.0001$ - $0.0036$ ) as well as significantly increased FILAGGRIN expression ( $p = 0.0387$ ) but had no effect on CCL26 eosinophil chemoattraction. With Th17-mediated inflammation the calcineurin inhibitor acted much like the vitamin D3 analogue in only inhibiting IL17a and IL8. The decreases in IFN $\gamma$ , MMP12, CXCL10, IL13, IL31, and IL17a are similar to those in from AD biopsies after topical pimecrolimus application (Guttman-Yassky et al., 2017).

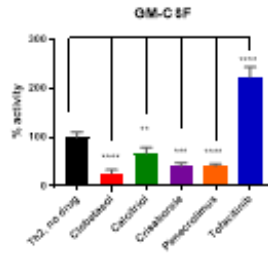
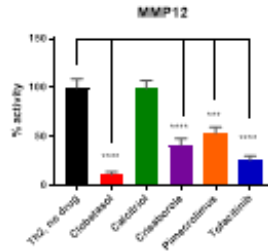
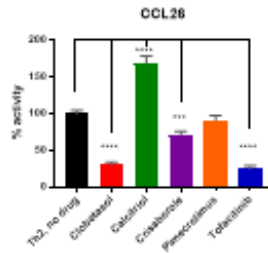
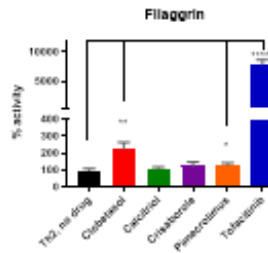
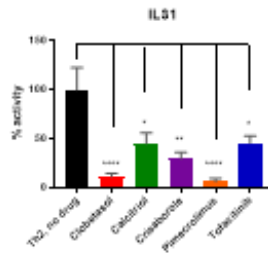
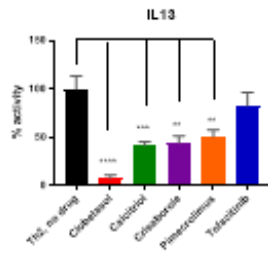
The increase in GM-CSF observed with JAK inhibition after tofacitinib application with both Th1 or Th2 stimulation, as well as the increase in IL12 and IFN $\gamma$  with Th1 stimulation can be explained by the positive feedback exhibited with IL12 induction. This activation induces the production of additional IFN $\gamma$ , IL12 and GM-CSF, suggesting a positive feedback loop indifferent to the downstream JAK inhibition (Beckeski and Grusby, 2007). Tofacitinib

significantly inhibited IL31, CCL26 and MMP12 gene expression ( $p < 0.0001-0.0332$ ) with Th2 stimulation, confirming that JAK inhibitors show promise in Th2-driven AD patients (He and Guttman-Yassky, 2019). The increase in FILAGGRIN expression ( $p < 0.0001$ ) suggests this compound may also be effective in restoring the stratum corneum barrier. Tofacitinib also gave marked inhibition of DefB4 and SerpinB4 ( $p < 0.0001$ ), both keratinocyte-associated, in the Th17 stimulated model, while upregulating IL1b, CCL20 and IL8. This differential effect on inflammatory biomarkers was reported in a clinical trial where reduction of T cell-associated cytokines such as IL17 and IFN $\gamma$  occurred relatively late in treatment while an earlier enhanced inhibitory effect was shown on keratinocyte-associated gene expression such as DefB4 and S100, suggesting a direct effect of JAK inhibitors on keratinocytes (Krueger et al., 2016).

# Th1



# Th2



# Th17

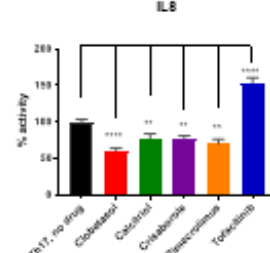
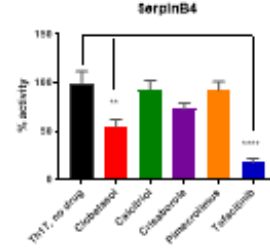
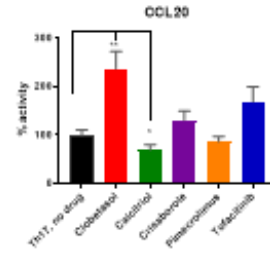
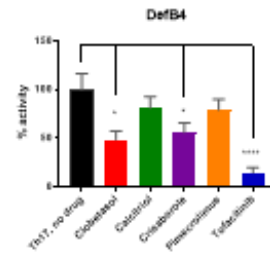
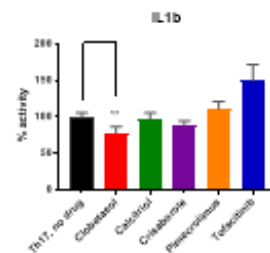
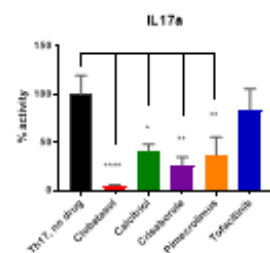


Figure 4. **Target specific inhibition of inflammatory gene expression with Th1, Th2 or Th17 stimulation.** Average percent activity of three donors; n=4 replicates per treatment per donor; stimulated with Th1, Th2, or Th17 cytokine cocktail and target specific inhibitors. Error bars represented as standard error of mean (SEM). Statistical significance determined by non-parametric t-test to treated. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001.

#### 4. CONCLUSION

The HESC inflammatory dermatoses models described herein remain viable for up to 9 days in culture without stimulation as shown in Neil et al (2020) and, separately, for up to 4 days in culture after inflammatory induction. This combined data suggests that multiple dosing paradigms could be employed pre- or post-stimulation to explore multiple interventions such as drug load, wash-out, and skin retention. The cytokine cocktails generate dose-dependent gene induction which was reproducible. While no *ex vivo* model can directly recapitulate the human inflammatory dermatoses state given the lack of vascularization and infiltrating immune cells, the ability to induce chemoattractants that are known to traffic circulating immune cells into the tissue mimics the inflammatory state found in human inflammatory dermatoses. Gene expression profiles elicited in the HESC reflected an enhanced inflammatory state compared to those published from lesional biopsies and were consistent with the known pathophysiology of the diseases. Common inflammatory dermatoses such as psoriasis and atopic dermatitis often involve the dysfunction of more than just immune cells, highlighted by the ability of non-immune biomarkers to be regulated in this model such as DefB4, S100A12, and MMP12. The utility of the model was demonstrated following drug treatment; target engagement and decreased inflammatory gene production and was again consistent with clinical biopsy data. With the addition of *in vitro* permeation testing, this model can be extended to evaluate pharmacokinetic/pharmacodynamic relationships for formulation optimization by exploring drug delivery and target engagement. Further characterization of this model is underway to determine translational significance to clinical data to guide future bioequivalence research. In summary, the model is a robust pre-clinical



tool for evaluating and selecting new molecules or formulations for a range of inflammatory dermatoses and thus has the potential of de-risking costly and time-consuming clinical trials.

**Acknowledgements:** This work was funded by MedPharm Ltd. We thank Alison Caserta and Lynde Ring for their scientific contributions.

We dedicate this paper to our long-time friend and colleague, Dr. Ken Walters, who passed away in 2021. Ken understood how to use fundamental science to bring topical therapeutics to patients and we shall miss his insights and support that he so generously gave.

**Supplementary Material:** Supplementary Information is available for this paper.

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## Supplementary Data

Supplemental Data Figure 1 **Intra-donor gene expression fold change of individual samples among multiple donors with Th1, Th2, or Th17 stimulation.** Each point represents a single sample. Error bars represent mean with STDEV. The average standard deviation (STDEV) of single samples across multiple donors calculated as the average standard deviation of replicates within each donor, including the range of lowest and highest observed fold change in the total data set. The percent coefficient of variation (%CV) calculated as the average % CV of replicates within each donor.

Supplemental Data Figure 2. **Inter-donor gene expression fold change of multiple individual donors with Th1, Th2, or Th17 stimulation.** Each point represents a mean of a single donor. Error bars represent mean with STDEV. The average fold change and standard deviation (STDEV) of combined donors calculated as the average of the fold change of each donor and average of the standard.

Supplemental Data Figure 3. **Average gene expression fold change of donors within specified ethnic groups with Th1, Th2, or Th17 stimulation.** Average gene expression fold change of donors with Th1 (a), Th2 (b), or Th17 (c) stimulation by RT-qPCR by race. n=number of donors.

Supplemental Data Figure 4. **Average gene expression fold change of donors within specified age groups with Th1 (a), Th2 (b), or Th17 (c) stimulation.** The average fold change and number of donors within specified age groups. Error bars represent standard deviation of the mean (stdev).

Supplemental Data Figure 5. **Commercially available primers used in RT-qPCR from Invitrogen life Technologies.**

