Novel ex vivo human skin inflammatory pathway models for developing topical medicines to treat autoimmune dermatological disease

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Declaration of original authorship

‘Declaration: I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.’

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At the time this PhD was being pursued, I was an employee and all work was conducted at MedPharm Ltd; in Durham, NC, USA.

Dedication

This thesis is dedicated to my parents whom without none of this would have been possible. They have provided all a loved one needs throughout their life; compassion and unwavering support, no matter the hairbrained idea.

Acknowledgments

I am extremely grateful to my mentors; Professor Marc Brown and Professor Adrian Williams, whom not only guided with experience and knowledge, but also with boundless patience. I am also indebted to MedPharm for the opportunity to pursue this undertaking to further my understanding of not just science but deductive thinking and problem solving that will surely benefit me lifelong. Last but not least, to Dr. Jon Lenn; mentor, colleague, confidant, but most importantly a great friend.

Abstract

Background: The development of in vitro and ex vivo models to mimic human illness is important, not only for scientific understanding and investigating therapeutic approaches, but also to mitigate animal testing, bridge the inter-species translational gap, and reduce costly development and clinical trial time and expense. The life span of a new molecular entity from inception to commercial introduction can span over 15 years, with high failure rates at key milestones including safety, stability, thermodynamics, and clinical efficacy. Dermatological development accounts for $50 million dollars in clinical trial expense alone. This expense is heavily dependent upon the later stage clinical trial aspect depending on number of patients needed to establish treatment effects and number of pivotal trials required to support marketing approval, each variable contributing to higher accrued costs of running the trial (Moore et al., 2020). While in vitro models can facilitate high-throughput and cost-efficient evaluation of novel therapeutics, more complex ex vivo systems can better predict both desirable and adverse in vivo effects. The lack of translation from simpler in vitro models to complex in vivo disease as well as the discrepancies between animal physiology and human due to immune system, skin pathology, and drug interaction lend to the need for more complex ex vivo models to better characterise the human disease state.

Aim: The objective of the work described in this thesis was to develop a human ex vivo skin culture (HESC) model to explore the pathophysiology of inflammatory dermatoses and for preclinical testing of potential therapeutic treatments.

Results: Here a human ex vivo skin culture model is described in which pathological tissue integrity, barrier function, and metabolic stability over time has been characterised and shown success up to 9 days in culture. Following exogenous stimulation, tissue integrity and ability to induce inflammatory gene expression including interferons, interleukins, chemokines, and antimicrobial pepetides was retained, and stimulant concentrations and duration were optimised to correlate with published data from clinical biopsies of inflammatory dermatitis and psoriasis patients. The validity and utility of the model was demonstrated when challenged with 5 drugs; clobetasol, calcitriol, pimecrolimus, crisaborole, and tofacitinib; where inflammatory biomarkers were regulated in a manner consistent with the drugs' reported in vivo mechanisms of action. The steroid clobetasol propionate inhibited multiple signaling pathways including IFNg, GM-CSF, IL13, IL31, CCL26, MMP12, IL17a and IL8 corresponding to published clinical trial data of clobetasol effect on AD lesions with treatment

(Guttman-Yassky et al., 2017). The vitamin D3 analogue calcitriol inhibited Th2-cell specific cytokines IL13 and IL31 and significantly increased CCL26 and S100A12 gene expression, illustrating its Th2-dominant activity. Crisaborole, the PDE4 inhibitor, significantly inhibited all Th2 associated biomarkers IL13, IL31, MMP12, and GM-CSF and increased CCL26 gene expression, analogous to the gene expression profiles reported by Bissonnette, et al clinical application. Calcineurin inhibition by pimecrolimus following Th1-mediated stimulation reduced IFNg, CXCL10, S100A12 and GM-CSF. 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). Tofacitinib significantly inhibited IL31, CCL26 and MMP12 gene expression with Th2 stimulation, confirming that JAK inhibitors show promise in Th2-driven AD patients (He and Guttman-Yassky, 2019). 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. Similarities included reduction of IFNg 95% in vivo compare to 97% in the HESC model by clobetasol and reduction of IL13 68% in vivo compare to 70% in the HESC model by pimecrolimus.

Conclusion: Through characterisation of the HESC model including tissue integrity, viability, cellular activity, inflammatory stimulation, demographic variability and topical therapeutic action, a comprehensive understanding of the capabilities of the model as a preclinical ex vivo drug development tool have been established. 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 i.e., New Chemical Entity and formulation screening and optimisation and the characterisation and comparison of such drugs and formulations with those already marketed has the potential of de-risking costly and time-consuming clinical trials. In addition, upon further validation such inflammatory HESC models may provide an ex vivo approach to demonstrate therapeutic bioequivalence for commonly prescribed drugs allowing for faster and cost-efficient regulatory approval.

List of publications and conferences

Peer-reviewed publications

Neil, J. E., Brown, M. B., & Williams, A. C. (2020). Human skin explant model for the investigation of topical therapeutics. Scientific Reports, 10(1). https://doi.org/10.1038/s41598-020-78292-4

Neil, J.E., Lenn, J. D., Brown, M. B., & Williams, A. C. (2022). A new ex vivo skin model for mechanistic understanding of putative anti-inflammatory topical therapeutics. International Journal of Pharmaceutics, 617, 121610. https://doi.org/10.1016/j.ijpharm.2022.121610

Neil, J. E., Brown, M. B., Lenn, J. D., & Williams, A. C. (2022). Accelerating topical formulation development for inflammatory dermatoses; an ex vivo human skin culture model consistent with clinical therapeutics. International Journal of Pharmaceutics, 618, 121648. https://doi.org/10.1016/j.ijpharm.2022.121648

Conference presentations

Neil, J., Powell, K., Volmer, J., Brink, A., Ring, L., Lenn, J., Brown, M.B. Translation Of Ex-Vivo Human Skin Model To Lesional Psoriatic Skin As A Preclinical Model Of Psoriasis. AAPS, 2017. San Diego, CA. Unpublished conference poster.

Neil, J., Powell, K., Volmer, J., Brink, A., Ring, L., Lenn, J., Brown, M.B. Comparison Of Ex-Vivo Human Skin Model Of Th2 Driven Inflammation As A Preclinical Model Of Acute Atopic Dermatitis. AAPS, 2018. Washington, DC. Unpublished conference poster.

Neil, J. E. Preclinical Model of Inflammatory Infection in Ex Vivo Human Skin. AAPS, 2018. Washington, DC. Rapid fire presentation.

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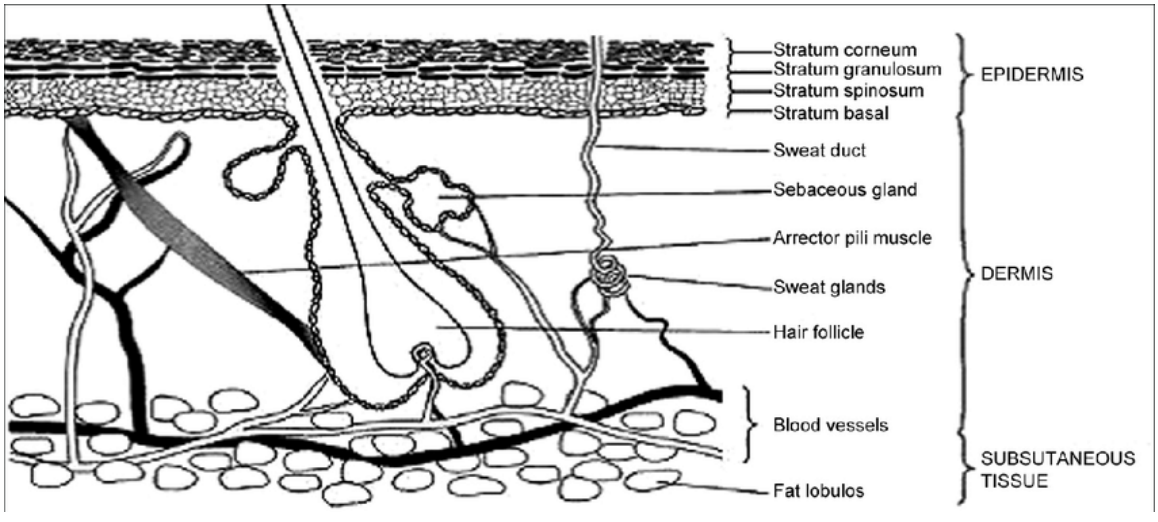
CHAPTER 1 : Introduction

1.1. Structure and Function of Human Skin

The skin is a large complex organ responsible for protection against physical, chemical and biological insults as well as moisture retention, metabolic and immunologic functions, and sensation perception. At the basic level, skin is comprised of three layers, the stratum corneum, epidermis and dermis (depicted in Figure 1), each contributing to the sensory, homeostatic and highly effective barrier functions of this organ (Benson & Watkinson, 2012). The homeostatic function of the skin supports thermoregulation of the internal environment whilst allowing for adaptation to varying climatic conditions. Protection from the external environment is also offered through the physical protective features of the skin which guards against chemical and bacterial toxins (Madison, 2003). It has been reported that a change in the these features can promote the ingress of foreign bodies, such as allergens, and may contribute to the development of inflammatory skin conditions (Cork et al., 2009). An understanding of the structure and function of healthy skin must first be established to appreciate the nature of the skin barrier to the absorption of drugs, the altered defenses of diseased skin and the action of topical treatments when applied to skin.

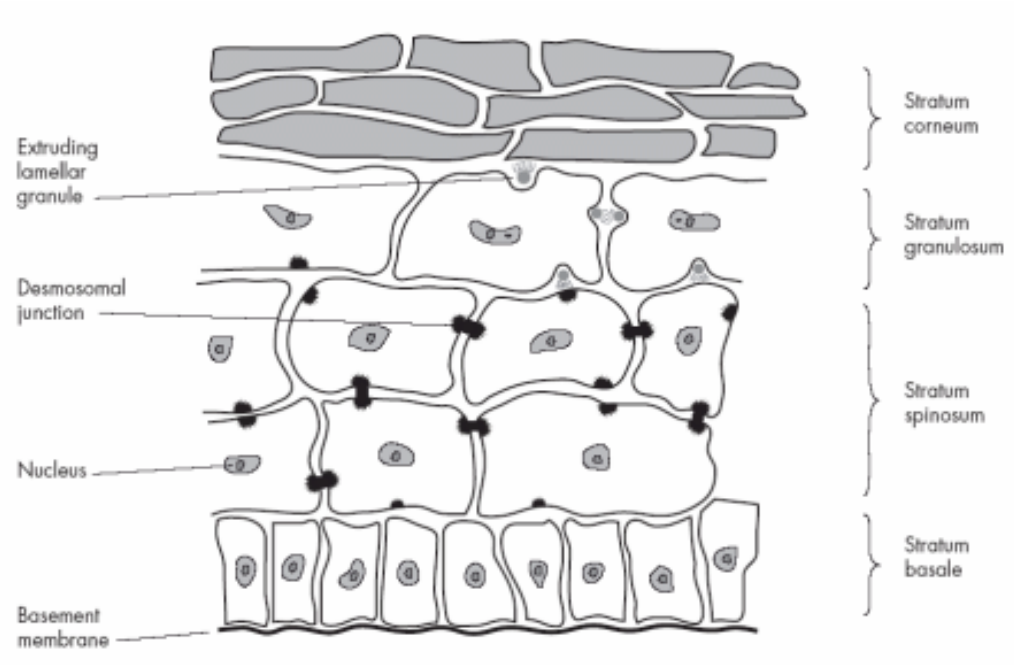
Figure 1: Illustration of the three main layers of human skin (the epidermis, dermis and subcutaneous tissue) and skin appendages (hair follicles, sweat ducts, etc) (Brown & Williams, 2019).



1.2. The Epidermal Barrier

The epidermal layer provides the foremost barrier properties of the skin, initially hypothesized by Homolle (1853) and Duriau (1856) who found that the skin was not entirely impermeable. The epidermal layer can be divided into several histologically distinct layers as depicted in Figure 2: the stratum basale, stratum spinosum, stratum granulosum and the outermost stratum corneum (Wickett & Visscher, 2006).

Figure 1: Illustration of the distinct layers of the epidermis: stratum corneum, stratum granulosum, stratum spinosum, and stratum basale (Brown & Williams, 2019).



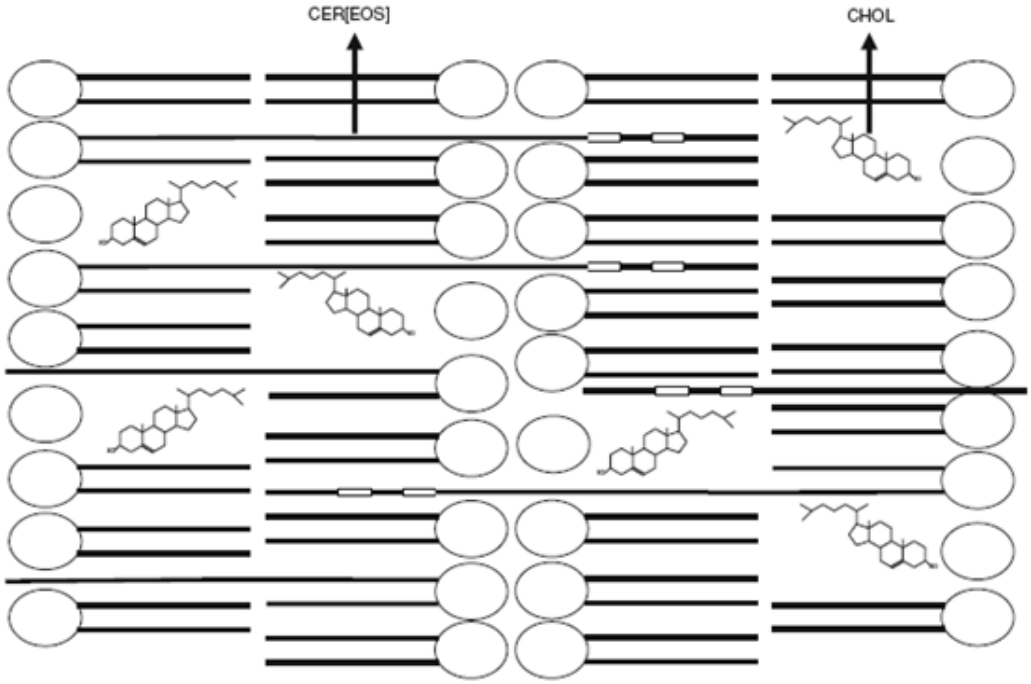
The primary barrier function of the epidermis was determined to be in the stratum corneum, following extensive research involving layer removal by sandpaper and measuring transepidermal water loss (TEWL) as a determining factor of barrier integrity (Windsor & Burch, 1944). The structure of the stratum corneum, often referred to as “bricks and mortar” (Michaels et al., 1975), is 10-15 µm thick (Bouwstra & Gooris, 2010) and constructed via keratinocyte terminal differentiation into annucleated corneocytes with a lipid enriched extracellular domain. These keratin-rich corneocytes are further bonded by desmosomes to promote cellular adhesion (Serre et al., 1991). Corneocytes undergo differentiation from

keratinocytes at the stratum granulosum and help control water loss and maintain adequate hydration of the stratum corneum (Rawlings & Harding, 2004). An integral constituent of corneocyte function, filaggrin, encourages the aggregation of keratin intermediate filaments, promoting the collapse of corneocytes into flat cells for organised packing (Wickett & Visscher, 2006). This densely packed construction of corneocytes creates a formidable barrier against penetrating microbes and molecules (Rawlings & Harding, 2004). The loss of filaggrin function has shown to correlate with dermatoses with barrier disruption attributes such as atopic dermatitis, as well as reduction of natural moisturizing factors and TEWL (Cork et al., 2009, Harding & Rawlings, 2005; Kezic et al., 2008; Seguchi et al., 1996). In concert with penetration-blocking properties of the stratum corneum, hydration retention is also maintained by natural moisturizing factors (NMF) including degraded filaggrin, amino acids, salts and lipids, to maintain elasticity and flexibility (Jokura et al., 1995).

The lipid constituents that make up the stratum corneum include cholesterol, ceramides, and free fatty acids (Jungersted et al., 2010) Figure 3). Cholesterol provides stabilizing support through synthesis by the enzyme 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase), which has been reported to be upregulated following an acute barrier disruption (Harris et al., 1997). Ceramides can present over a thousand different chemical structural entities in the stratum corneum lending to multiple functions (Bleton et al., 2001). The classification of these ceramides has resulted in 12 subclasses being identified, all sharing a sphingoid base coupled to a fatty acid chain (Masukawa et al., 2008; van Smeden et al., 2011). Of particular interest is the coupled ω-hydroxy (EO) fatty acid chain, which can link to linoleic acid to increase the chain length, and has been strongly associated with atopic dermatitis pathology (Macheleidt et al., 2002). In addition to providing protection from water loss, ceramides also assist in anchoring corneocytes to the extracellular matrix to provide structural support (Raith et al., 2004). The final lipid constituent of the stratum corneum, free fatty acids, are known to assist in barrier repair after disruption (Mao-Qiang et al., 1993), and contain ionizable head groups lending to the structure and pH of the layer (Jungersted et al., 2008; Wickett & Visscher, 2006).

As the primary barrier defense against environment insult, the stratum corneum is a powerful wall preventing penetration from pathogens and molecules, that relies on a delicate balance of differentiated corneocytes and lipid constituents. The complexity of this “brick and mortar” construction allows for the remarkable barrier properties afforded by the uppermost dermal structure to protect the underlying epidermis and dermis.

Figure 2: Schematic representation of ceramide packing within SC lipid matrix (Kessner et al., 2008)**. CER(EOS) extends into adjacent layers providing increased mechanical strength. The variation in chain length of ceramides promotes intercellular lipid space allowing for the incorporation of cholesterol into the matrix.**



The underlying epidermis is comprised of the stratum granulosum, stratum spinosum, and stratum basale. Keratinocyte differentiation initiates in the lower stratum basale where they undergo cell division and begin synthesizing keratin pairs as they migrate upward to the stratum granulosum. In addition to keratinocytes, the stratum basale houses melanocytes, Langerhans and Merkel cells anchored to the basement membrane by hemidesmosomes. As the keratinocytes migrate into the stratum spinosum they begin forming keratin filaments known as tonofilaments, and ultimately desmosomes for self-anchoring. Once in the stratum granulosum, the presence of keratin containing granules (keratohyalin) and differentiation membrane coating granules are noted, containing profilaggrin and intercellular lipid lamellar precursors for transport to the stratum corneum (Brown & Williams, 2019; Eckert & Rorke, 1989).

1.3. The Dermal Layer

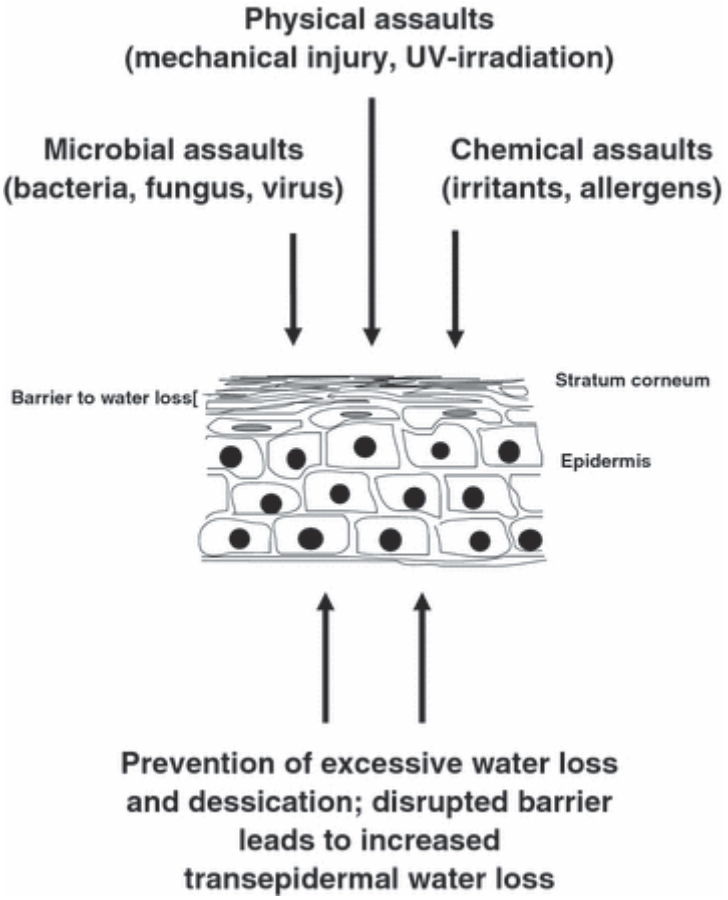
The dermal layer of the skin is 3-5 mm thick and contains an increased heterogeneous mix of cells and structures. Within the dermis are vascular blood vessels, lymphatic vessels, nerve endings, hair follicles, sebaceous glands, and sweat glands. The vasculature in the dermal

layer contributes to thermoregulation, delivery of oxygen and nutrients to the skin and clearance of drugs, toxins and waste from the skin. The extracellular matrix offers mechanical protection by cushioning with tightly packed collagen and elastic fibers and is produced by abundant fibroblasts in the dermis, constituting the connective tissue and lending to immune cell recruitment and inflammation at the site of injury (Brown & Williams, 2019).

1.4. Function of the Immune System in Skin

The skin in itself is a physical barrier efficient at preventing microorganism entry due to the tightly packed corneocytes of the stratum corneum, as well as acidic pH and immune cell recruiting chemokines. Within the corneocytes of the stratum corneum reside lipids whose conversion to sphingosine and dihydrosphingosine exert antimicrobial activities against bacterial strains such as *Staphylococcus aureus, Streptococcus pyogenes, Micrococcus leutus,* and *Propionibacterium acnes* (Bibel et al., 1992). Due to the stratum corneum dual function of both water retention and pathogenic defense, Proksch et al. (2008), coined the dichotomy "inside-outside" and "outside-inside" to categorize its functions. In the event of barrier disruption due to either mechanical damage or disease state, the ability of the stratum corneum to both prevent water loss and prohibit external insult is decreased (Figure 4). Serine protease within the stratum corneum contribute to the degradation of lipid-processing enzymes and corneodesmosome-constituent proteins which can contribute to an abnormal formation of lipids and desquamation, associated with broken barrier disease states such as atopic dermatitis (Elias et al., 2008).

Figure 3: Functions of the epidermal "inside-outside" and "outside-inside" barrier (Proksch et al. 2008**).**



Below the stratum corneum reside receptors programmed to recognize pathogenic associated molecular patterns (PAMP's) which bind pattern recognition receptors (PRRs) and lectins to recognize surface sugars common to microorganisms. The prominent keratinocytes that make up the human skin strata can produce antimicrobial peptides (AMPs) such as psoriasin, cathelicidin, lactoferrin, lipocalin and defensins to further resist pathogen invasion (Baroni et al., 2012). The most studied cathelicidin, LL-37, modulates the immune response via multiple functions including inducing differentiation of dendritic cells for cytokine production, inducing keratinocyte proliferation and migration as well as synergizing with inflammatory mediators such as Interleukin-1ß (IL-1ß) (Nguyen & Soulika, 2019). Keratinocytes are capable of synthesizing both Interleukin-1α (IL-1α) and (IL-1ß) within the epidermis. Interleukin-1 can be released by cytokine activation or cell damage and serves as a potent immunomodulator via activation of monocytes, dendritic cells, B and T cells and fibroblasts (Salmon et al., 1994). In addition to keratinocytes, fibroblasts are capable of expressing toll-like receptors that can recognize pathogens and activate inflammatory signaling pathways, AMPs, and cytokines

Interferon (IFNγ), Interleukin-6 (IL-6), and Interleukin-8 (IL-8) (Bautista-Hernández et al., 2017).

Immune cells are major players in the pathophysiology of skin biology. Langerhans cells are derived from the bone marrow and migrate to the skin to make up 2-4% of epidermal cells. These cells express membrane adhesion molecules such as intercellular adhesion molecule-7 (ICAM-7) and lymphocyte function-associated antigen-3 (LFA-3) as well as class II MHC molecules capable of stimulating T cell-dependent immune responses and are responsible for delayed-type hypersensitivity responses. Once Langerhans cells detect antigen presence, they induce resident T cell activation as well as migrate out of the skin to regional lymph nodes to induce T lymphocytes proliferation and differentiation (Salmon et al., 1994). In addition to Langerhans cells there are macrophages, mast cells, eosinophils, and lymphocytes. Macrophages are both resident in the skin dermis and supplied by circulating monocytes. Their primary function is removal of cellular debris, however they also express Interleukin-10 (IL-10) suggesting immunoregulatory function both anti and pro-inflammatory. Macrophages are capable of secreting inducible nitric oxide synthase (iNOS), tumor necrosis factor alpha (TNFα), IL-1ß, and IL-6 (Mills, 2015). Mast cells and eosinophils, found mainly in the dermis, are highly associated with allergic reactions due to the ability to release histamine, prostaglandins (PGDs), and leukotrienes. B cells comprise a sparse population of the skin lymphocyte population and are unlikely skin residents, however are present in skin dermatoses by expression of immunoglobins IgM, IgE, and IgG (Nguyen & Soulika, 2019).

Of these different immune cells, the resident memory T cells (T_{RM}) are known to be potent mediators against infection, but also autoimmune disease. There are around 20 billion memory T cells in skin of an adult human, with an estimated 1 million in each cm^2 (Clark et al., 2006; der Veen et al., 2011; Lowes et al., 2014). The majority of these T cells reside in the dermis (90%), with less than 2% of the T cell population residing in the epidermis (Salmon et al., 1994). Of these, CD69 and CD103 are among the markers that are robustly expressed. When analyzing the cellular populations of normal human skin explants, the isolated T cells were also shown to primarily express the memory T cell marker CD45RO, as well as the cutaneous lymphocyte antigen (CLA) and the chemokine receptor CCR4 (Clark et al., 2006). To further elucidate the function of these resident T cells, mitogen stimulation was used to determine the polarization of the cells via secreted cytokines and surface markers. It was found that the predominant skin resident T cell population secreted Th1 cytokines IFN-γ and IL-2, with a small sample also producing the Th2 cytokine IL-4 (der Veen et al., 2011). T cells are highly studied within the skin, primarily αß T lymphocytes. Aß T cells reside in the dermis and epidermis and are comprised of both CD8+ and CD4+ cell populations. CD8+ T cells are effective antiviral

agents by producing an IFNγ-mediated antiviral state, while CD4+ cells are immunoregulatory in hypersensitivity reactions (Nguyen & Soulika, 2019).

1.5. Autoimmune Dysregulation and Inflammatory Dermatoses

The human immune system is a complex and highly regulated defense mechanism against invading pathogens. While the immune system has developed over the course of human evolution to recognize exogenous entities and protect the body from harmful microorganisms, this mechanism has the propensity to become self-harming when it begins to recognize intrinsic epitopes as the enemy. When the negative selection of lymphocytes in the thymus designed to prevent self-recognition is dysregulated, it results in an autoimmune environment where lymphocytes begin attacking endogenous tissues. While autoimmune diseases vary drastically, approximately 3-5% of the general population suffer from some form of immune dysregulation which result in an array of physiological disorders involving the entire body. These include type I diabetes of the pancreas, rheumatoid arthritis of the joints, multiple sclerosis of the neurological system, Crohn's disease of the colon, coeliac disease of the intestine, Grave's disease of the thyroid, Addison's disease of the adrenal gland, Sjogren syndrome of the tear and salivary glands, and lupus involving immune attack of multiple organs simultaneously. Both genetic and environmental factors are often identified in the pathogenesis of autoimmune disease. Environmental culprits often include infectious agents such as bacteria, viruses, parasites and fungi, but also include ingested foods such as gluten and the human microbiome. Once activated by an environmental insult, immune cells may become self-responsive, attacking endogenous tissues and exacerbating to a chronic autoimmune disease state (L. Wang et al., 2015).

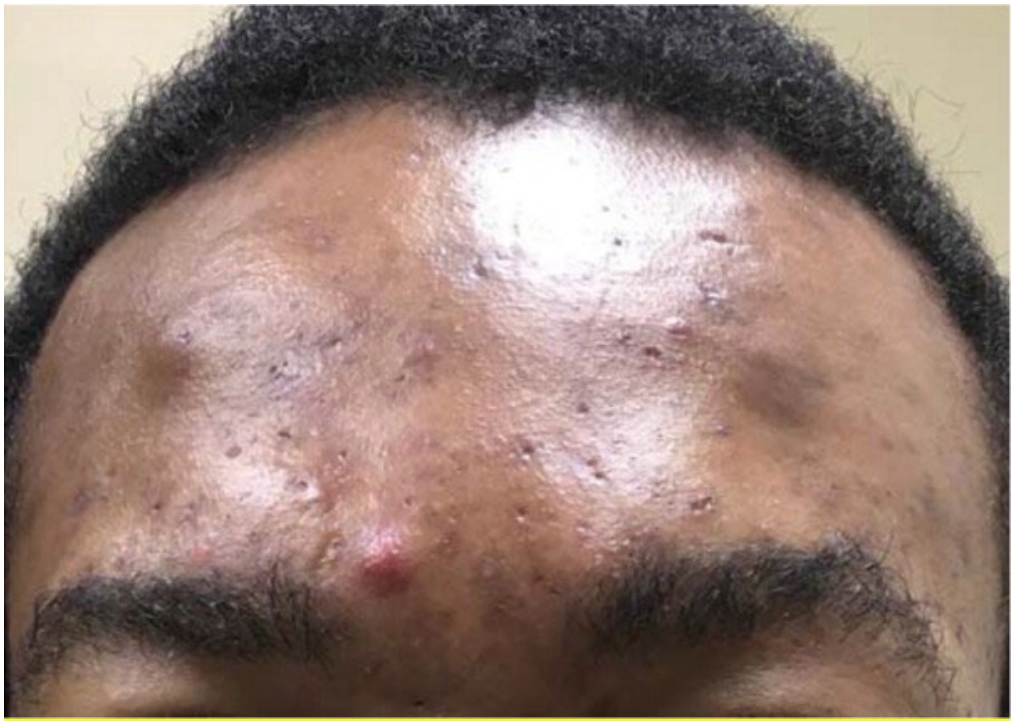
When autoimmune dysregulation results in inflammation within the skin strata, it is broadly known as inflammatory dermatoses. This can lead to an array of skin disease such as acne, bullous pemphigoid, granuloma annulare, rosacea, vitiligo, hidradenitis suppurativa, alopecia areata, lichen planus, and the more common dermatitis and psoriasis. The common denominator among these skin diseases is the presence of elevated immune infiltrates, cytokines, and chemokines lending to pruritis, pustules and tissue damage (Zeidler et al., 2019). While the predominant immune function and environmental insult varies among these dermatoses, three main T cell mediated states stand out; Th1, Th2, and Th17 pathways. Th1-mediated inflammation is associated with secretion of IL-2, TNF and IFNγ, activating surrounding immune cells such as macrophages to produce reactive oxygen intermediates and nitric oxide. This cell mediated immunity is commonly associated with delayed hypersensitivity responses (Skapenko et al., 2005). Th1 cell differentiation is achieved via T-box transcription factor (T-bet)

activation, while activation of transcription factor GATA-3 leads to Th2 cell differentiation. Th2 cells are known to secrete IL-4, IL-5, and IL13 and provide a stronger antibody response (Fang et al., 2018). Th17 activated lymphocytes are associated with IL-17 and IL-23 cytokine secretion and proinflammatory pathways leading to psoriasis, hidradenitis suppurativa, and alopecia areata. The Th17 pathway is often initiated by IL-23 activation of T cells via retinoid-related orphan receptor gamma (RORγ), and signal transducer and activator of transcription 3 (STAT3). Once activated, Th17 cells induce expression of NF-κB and mitogen-activated protein kinase (MAPK). Subsequent secretion of IL-17, IL-22, and TNFα lead to chemokines for lymphatic immune cell recruitment and additional molecules including antimicrobial peptides S100A7, defensins, and interferons (Liu et al., 2020).

1.5.1. Acne vulgaris

Acne vulgaris is the most common skin disease, affecting nearly 80 percent of adolescents and young adults. The presence of *Propionibacterium acnes* (P. acnes, recently renamed *Cutibacterium acnes*) is very common in the development of acne and often considered the main contributing factor. The underlying cause of acne vulgaris is multifactorial, often involving sebum overproduction, follicular epithelium shedding, inflammation, bacterial colonization, and hyperproliferation of keratinocytes in sebaceous follicles leading to the formation of comedomes. These lesions can result in scarring and hyperpigmentation of the tissue (Gollnick et al., 2003;Oge et al., 2019); see Figure 5

Figure 5. Severe inflammatory acne with open comedomes, cysts, and nodules (Oge et al., 2019)**.**

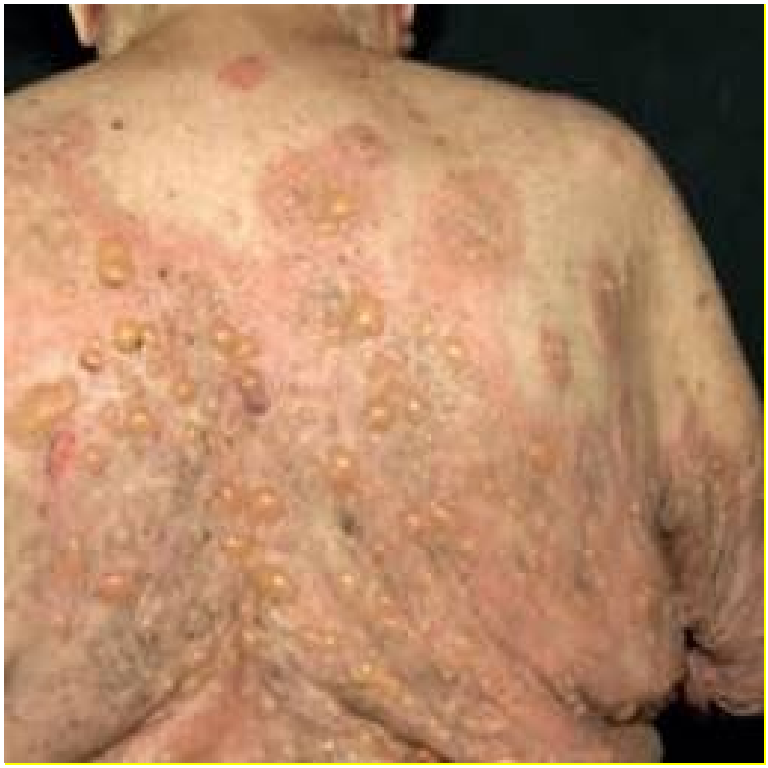


Increased numbers of CD3+ and CD4+ T cells as well as macrophages in uninvolved skin from acne patients suggest an underlying inflammatory state preclusive of comedone formation. The compounding presence of *P. acnes* triggers a proinflammatory state through the activation of toll-like receptors (TLRs) which detect exogenous microorganisms. The resulting signaling cascade includes Interleukin-12 (IL-12) and Interleukin-18 (IL-18) as well as antimicrobial defensins. In addition to TLRs, *P. acnes* has been shown to elicit IL-1α, IL-8, and matrix metalloproteinase as a result of protease-activated receptor-2 (PAR-2) activation. The sebaceous gland of the skin is also a culprit in acne inflammation, producing IL-1α and IL-1ß (Tanghetti, 2013). Treatment of acne is often based on severity and location, and has a multipronged approach including topical anti-inflammatories such as steroids and retinoids, antibacterials including Clindamycin and Erythromycin, as well as benzoyl peroxide and salicylic acid to target sebum production. Severe cases of acne may require systemic antibiotics Doxycycline and Minocycline (Oge et al., 2019).

1.5.2. Bullous pemphigoid

The primary blistering disorder of the skin, especially in adults over 70 years of age, is bullous pemphigoid (BP). This disease presents with pruritic fluid filled blisters (Schmidt et al., 2012); (Figure 6); and affects over 300 people per million over the age of 80 (Bernard & Antonicelli, 2017). Circulating autoantibodies to the proteins BP230 and BP180 are the most common cause of BP resulting in an inflammatory cascade compromising the integrity of the dermal-epidermal junction. The resulting inflammation cascade includes multiple cytokines such as CCL-2, CCL-17, IL-5, IL-6, IL-8, IL17, IL1-ß, and TNFα (Khalid et al., 2021). Underlying autoimmune diseases are often associated with later in life BP, such as psoriasis and lichen planus, lending to the endogenous inflammatory association (Miyamoto et al., 2019). Bullous Pemphigoid is a chronic disease often resulting in relapse after cessation of treatment. Treatment options include corticosteroids for their immunosuppressive and anti-inflammatory capabilities (Bernard & Antonicelli, 2017).

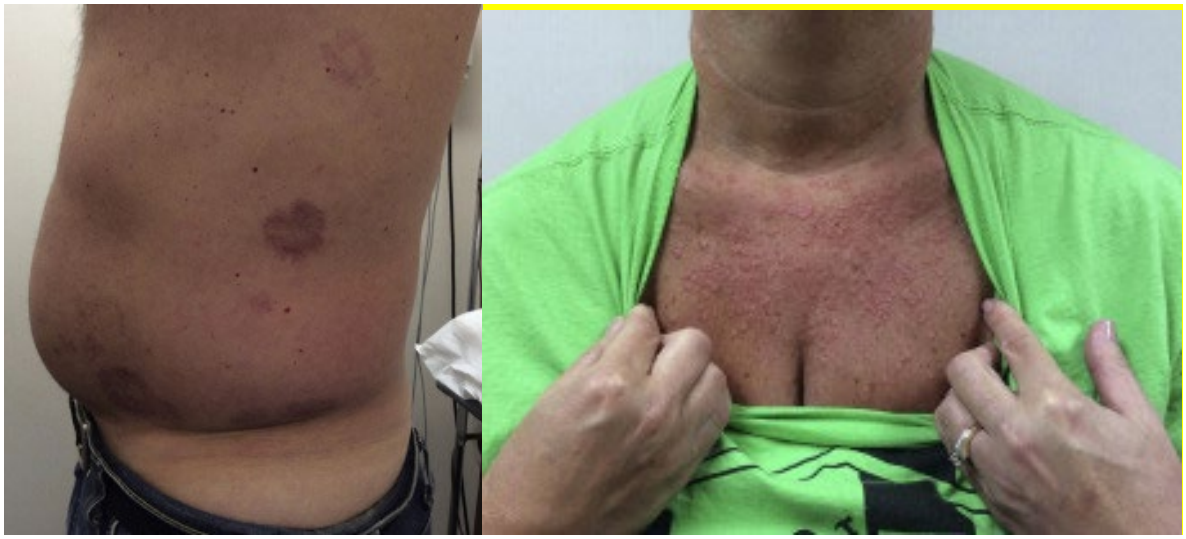
Figure 6. Bullous pemphigoid blisters on the trunk and limbs with erythematous and edema (Miyamoto et al., 2019)**.**



1.5.3. Granuloma annulare

Granuloma annulare (GA) is characterised by discolored plaques of granulation tissue. It is a relatively rare disease in the United States with an incidence rate of 0.04%, and can occur in both children and adults, however has a 2:1 prevalence in women over men. The most defining histological feature of GA is the presence of mucin deposits within the lesion (Joshi & Duvic, 2021); commonly seen as ring-like erythematous papules (J. Wang & Khachemoune, 2018); see Figure 7.

Figure 7. Granuloma annulare involving torso and chest (Blum & Altman, 2019).



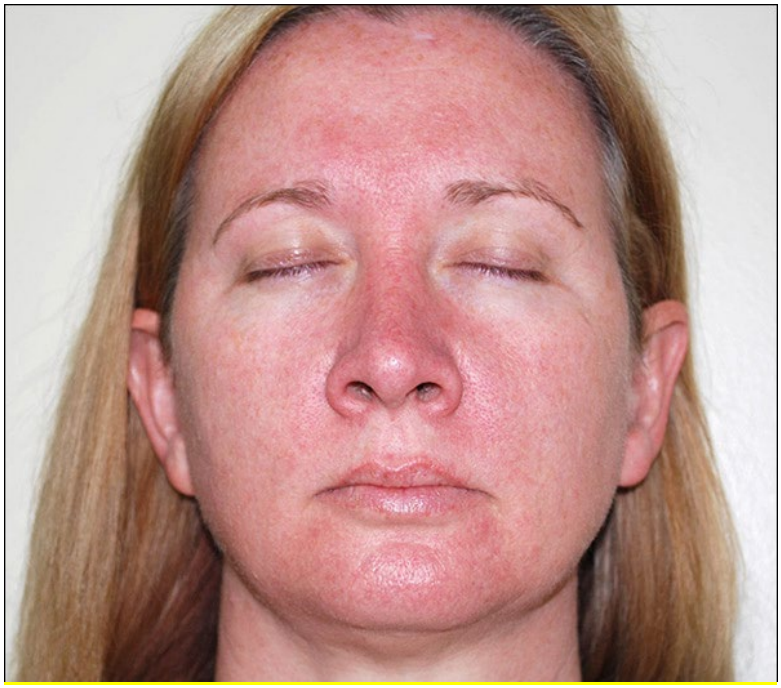
Immune dysregulation includes activation of the Th1, Th2, and Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathways, including TNFα, IL-1ß, IFNγ, IL-4, and IL-31 (Min et al., 2020). As an immune regulated inflammatory disease, GA is often treated with topical and injectable corticosteroids. However cryosurgery and phototherapy have proven effective alternatives (J. Wang & Khachemoune, 2018).

1.5.4. Rosacea

Rosacea is a common chronic inflammatory disease associated with redness, dilated blood vessels, pustules, and sometimes skin thickening of primarily the face. Usually affecting women with lighter skin tone, it is categorized into four subtypes based on presenting symptoms; erythematotelangiectatic, papulopustular, phymatous and ocular. Erythemato-telangiectatic rosacea (ETR) often presents with persistent redness of the central face as well as sensitive skin with burning sensation. Papulopustular rosacea (PPR) shows the same redness as ETR, however is accompanied with papules and scaly red plaques. Phymatous rosacea varies by location and can affect the nose, chin, forehead, ears, and eyelids with thickened plaques and fibrosis. Lastly, ocular rosacea presents in and around the eye and causes dryness, irritation, blurry vision, eyelid thickening and can develop conjunctivitis. The redness associated with rosacea has both vascular and inflammatory mediators. Increased vascular permeability in the upper dermis with patients with rosacea causes the erythema. T-cells and macrophages in the skin are capable of releasing factors that contribute to vasodilation and recruitment of neutrophils and mast-cells found in the pustules (Mikkelsen et al., 2016). Common to people of European descent in the US and Europe, rosacea begins to manifest in the mid-20s and progresses thereafter, with approximately 10%

occurrence in the population presenting prevalently in women 3:1 compared to men (Culp & Scheinfeld, 2009).

Figure 8. Rosacea facial erythema (Oge et al., 2015).

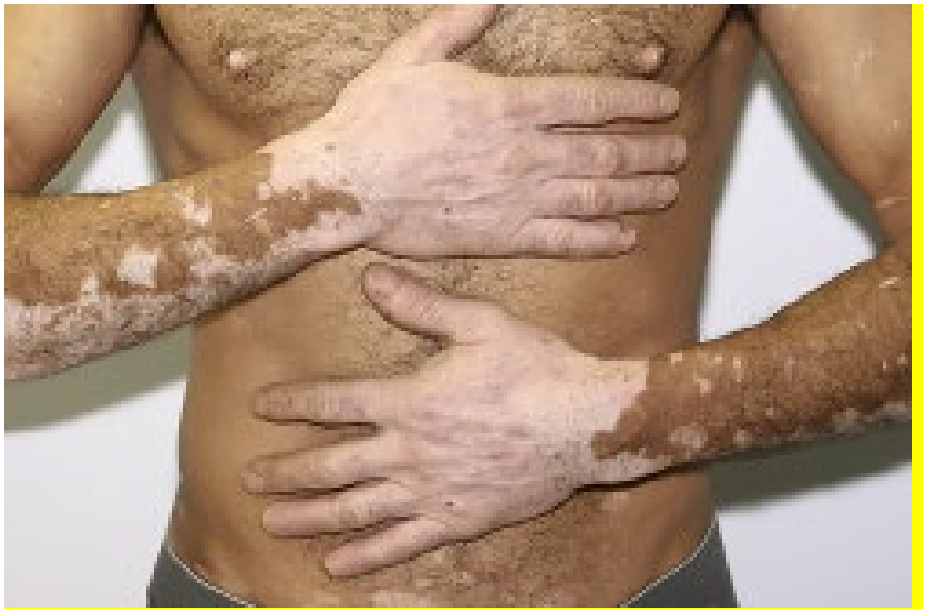


Rosacea is highly associated with a Th1/Th17 immune cell paradigm, with elevated gene expression of IFNγ, TNFα, IL-17a, IL-22, IL-6, and CCL20 (Kelhälä et al., 2014). The existence of Demodex mites in the microbiome is a known trigger for rosacea via toll-like receptors, notably, TLR-2 and PAR2 resulting in IL-1ß and TNFα increased expression (Casas et al., 2012). The current widely accepted treatment for rosacea is oral tetracycline and similar antibiotics, however the overall range of therapeutics is meager for this dermatosis (Culp & Scheinfeld, 2009). For relatively mild cases, some topical preparations are available, including metronidazole, ivermectin, and azelaic acid, however efficacies are variable (Rainer et al., 2017).

1.5.5. Vitiligo

Vitiligo can affect any part of the body marked by depigmentation in the epidermal layer due to loss of melanocytes. The lesion is typically a non-scaly chalky-white region with distinct margins. Vitiligo presents in all ethnic groups with a 0.5-2% prevalence in children and adults across the globe, equally affecting men and women (Bergqvist & Ezzedine, 2020).

Figure 9. Vitiligo depigmented macules of extremities (Bergqvist & Ezzedine, 2020).

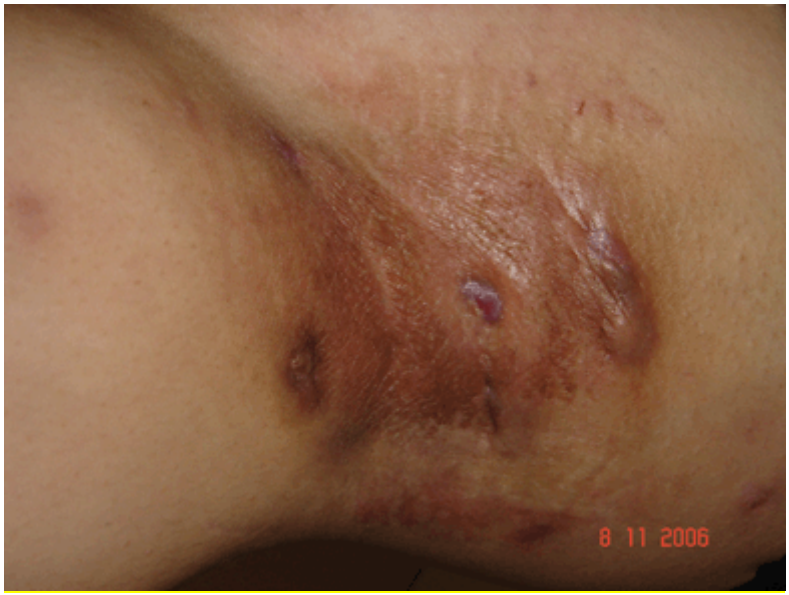


The loss of melanocytes is highly associated with CD8 T cells expressing elevated levels of IFNγ and TNFα. These autoreactive T cells are capable of inducing apoptosis of melanocytes and detachment from the basal epidermal layer (Boukhedouni et al., 2020). Increased activation of Th17 cells correlate with severity of depigmentation in concert with enhancing the effects of TNFα, IL-1ß, and IL-6 on melanocyte destruction (Campione et al., 2020). Treatment of vitiligo focus on reducing oxidative stress and autoimmune inflammation to allow for repopulation of healthy melanocytes. These include corticosteroids, calcineurin inhibitors, vitamin D analogs and UVA or UVB phototherapy. Emerging treatments include targeted anti-inflammatory molecules such as Janus kinase inhibitors often used for rheumatologic autoimmune disorders and procedural laser dermabrasion (Manga et al., 2016).

1.5.6. Hidradenitis suppurativa

Hidradenitis suppurativa (HS) is found in the apocrine-bearing areas of the body including the armpit, areole of the breast, and anogenital region. First described by French surgeon Arisride Verneuil in 1954, HS is caused by follicular occlusion and keratinous plugging of the apocrine glands causing inflamed lesions. This inflammatory disease results in comedones, relapsing nodules and painful fistulous. While considered an orphan disease, there is a global prevalence of 1% in the general population (Revuz, 2009).

Figure 10. Hidradenitis suppurativa sinus tract and pyogenic granuloma (Revuz, 2009).

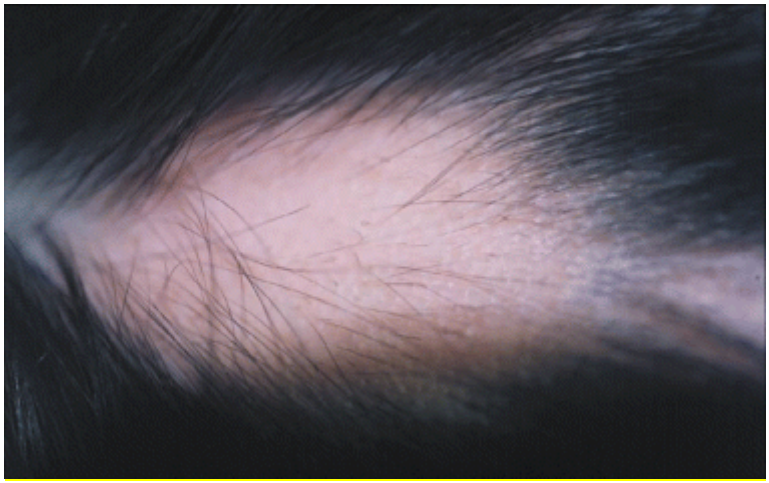


TNFα levels have been correlated to disease severity, as well as elevated IL-1ß, IL-17, and IL-23 (Campione et al., 2020). Tumor necrosis factor alpha inhibitors such as adalimumab have shown promise in alleviating the disease, supporting the strong TNFα association. Due to the severity of HS, both therapeutic and surgical interventions are often used in the treatment. Antibiotics and anti-inflammatories are used to reduce inflammation and swelling, however excision of the tissue may be needed to remove sinus tracts or recurring cysts at fixed locations (Vossen et al., 2018).

1.5.7. Alopecia areata

Alopecia areata (AA) is an autoimmune disorder leading to localized or diffuse hair loss. Affecting nearly 2% of the population at some point in the lifespan, it typically occurs in patchy hair loss due to inflammatory cell infiltrate concentrated around the hair follicle (Pratt et al., 2017); see figure 11. The scalp is the most common afflicted area for both men and women and appears in well demarcated localized regions. Afflictions of the nails are sometimes observed including pitting, trachyonychia, and thinning or thickening (Wasserman et al., 2007).

Figure 11. Localized patch of alopecia areata on scalp (Wasserman et al., 2007).

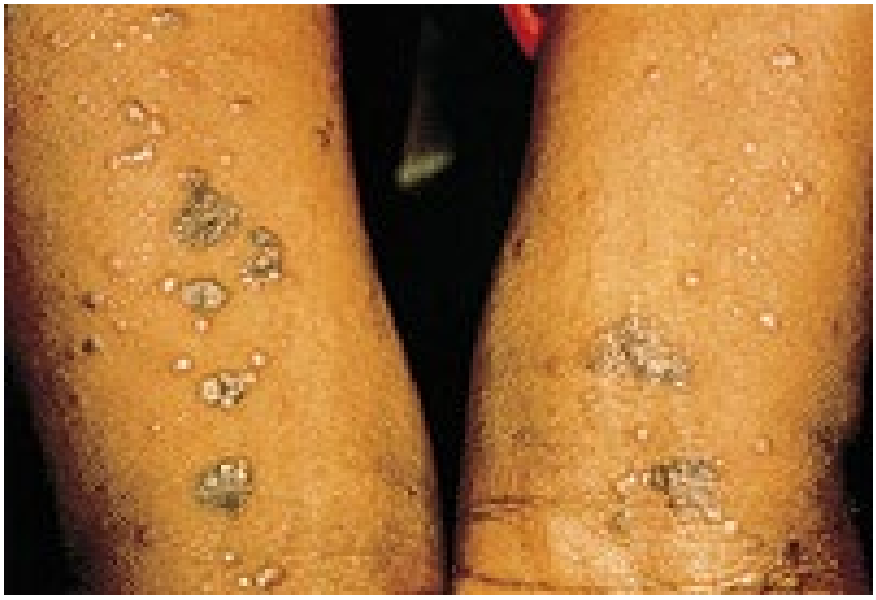


Real-time quantitative polymerase chain reaction (RT-qPCR) revealed multiple upregulated inflammatory pathways in AA patients including chemokines CCL13, CCL18, CXCL10 and CXCL12 related to B and T cell-lymphocyte recruitment. In addition, overexpression of human leukocyte antigens and immunoglobulins contribute to a leukocyte rich inflammatory environment leading to follicular damage and hair loss (Michel et al., 2017). Autoimmune antibodies upregulated in dermatoses such as vitiligo, lichen planus, and celiac disease point to autoimmune dysregulation (Wasserman et al., 2007). While spontaneous remission can occur in cases of mild AA, often topical or systemic corticosteroids are administered to dampen inflammation leading to follicular damage to slow hair loss and optimally allow regrowth. Recent trials to explore the use of immune suppressants such as methotrexate and tacrolimus, as well as antibody therapeutics adalimumab, infliximab, and etanercept have unfortunately been ineffective at treating alopecia areata (Pratt et al., 2017).

1.5.8. Lichen planus

Lichen planus is a T-cell mediated autoimmune disease presenting in over 20 different clinical manifestations. Often manifesting in the stratified squamous epithelium, it presents with pruritic papules or plagues and erosion of mucous membranes; see Figure 12. A very rare disease, it is estimated to occur in less than 1 percent of the population, most commonly in middle-aged adults (Katta, 2000).

Figure 12. Cutaneous lesions of lichen planus (Katta, 2000).



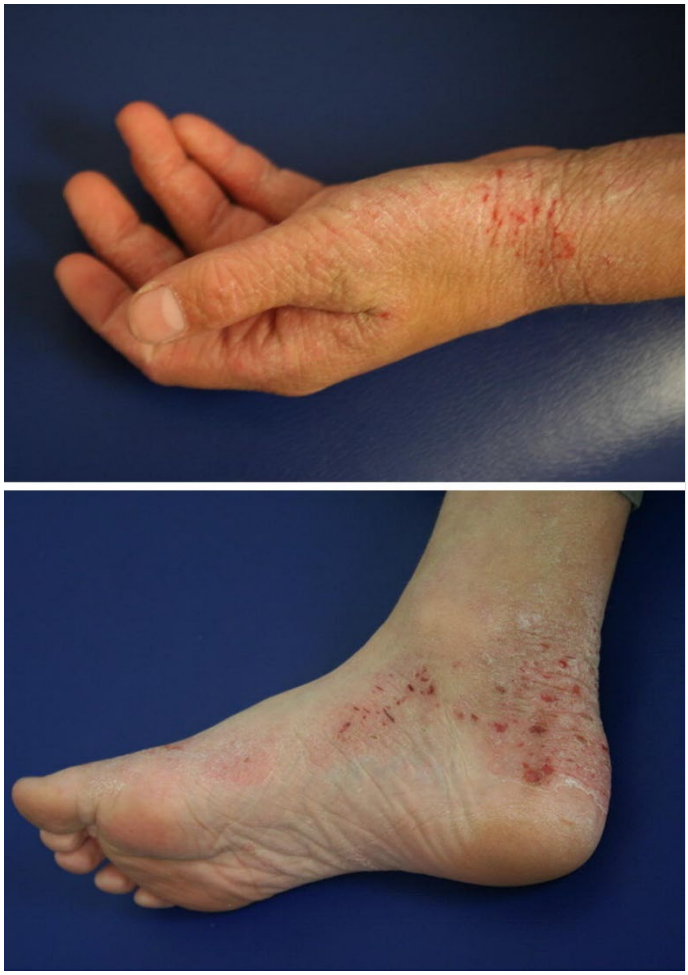
Increases in protease expression of matrix metalloproteinases MMP-2, MMP-7 and MMP-9, as well as chymase and tryptase lead to the disruption of the basement membrane resulting in lichen planus. Additional autoimmune dermatoses such as alopecia areata and vitiligo are often represented in patients with lichen planus. Alterations in cytokine expression have included IL-5, IL-6, IL-8, IL-10, IL-12, IL-17 and IL-22, as well as TNFα, TGFβ, and IFNγ. Chemokines CXCR3, CXCL10, CXCL12, CCR4, and CCL17 are also unregulated (Boch et al., 2021). Evidence suggests a strong Th1-mediated inflammatory response of $CD8^+$ T lymphocytes including elevated type I interferons leading to the recruitment of cytotoxic T cells and keratinocyte damage (Wenzel et al., 2006). Potent topical corticosteroids are the primary medication prescribed for lichen planus, while oral steroids may be prescribed in severe cases. Calcineurin inhibitors tacrolimus and pimecrolimus have been found efficacious for mucosal erosion, while retinoids show promise but tend to be too irritating for mucosal application. Phototherapy has been suggested for individuals lacking steroid tolerance, however in a small subset, UVA exposure has caused lichen planus reaction. Additional drugs being tested as therapeutics include hydroxychloroquine, dapsone, and anti-TNFα antibody treatment (Husein-El Ahmed et al., 2019).

1.5.9. Atopic dermatitis

Atopic dermatitis (AD), an immune regulated disease resulting in eczematous skin lesions accompanied by pruritis, is the most common skin disease worldwide affecting an estimated 3% of the adult population and as high as 30% of children depending on demographic (Bin & Leung, 2016). In addition to the well-established “atopic march” through allergic

comorbidities (e.g. food allergies, asthma, seasonal allergies), there is increasing evidence that adults with long-standing atopic dermatitis (AD) have systemic inflammatory proteins which may be a risk factor in rheumatoid arthritis, inflammatory bowel disease and cardiovascular disease (Leung et al., 2004).

Figure 13. Atopic dermatitis of the hands and feet (Kapur et al., 2018).



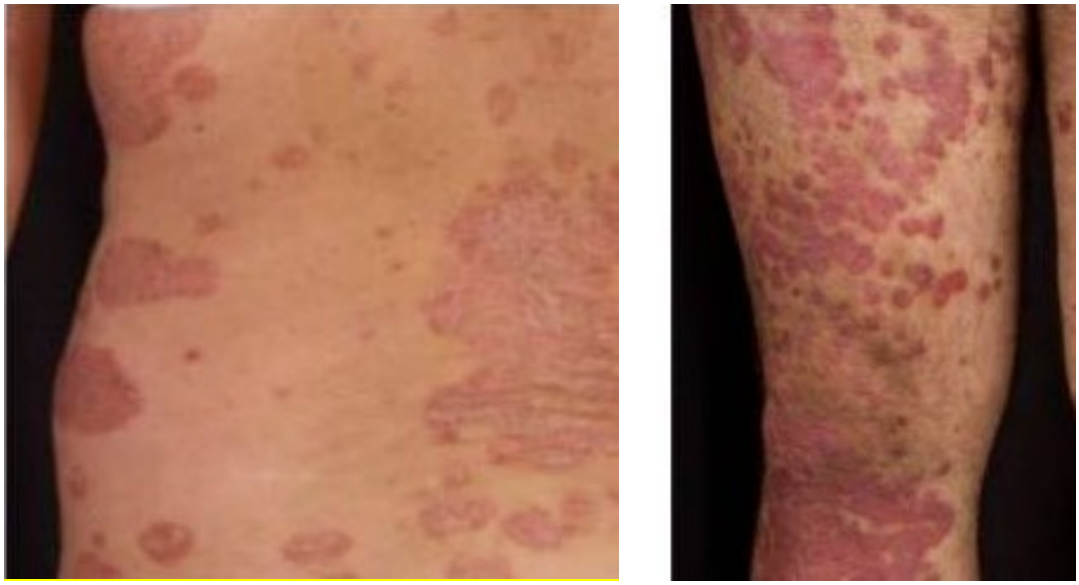
The initial acute stage of AD is characterised by a predominantly Th2-mediated inflammatory state that progresses to a Th1-dominated inflammation as the disease becomes chronic (Leung et al., 2004). The onset of AD can be spontaneous but is often linked to environmental stimuli. The epidermal barrier is impaired, with genetic mutations such as loss of filaggrin gene expression often underlying this impairment. The barrier dysfunction, along with an inherent hyper-reactive epidermal state, leads to activation of both the innate and the adaptive immune systems (Eyerich & Novak, 2013). In conjunction with elevated IgE antibodies, the keratinocytes of AD lesional skin produce increased levels of thymic stromal lymphopoietin (TSLP) cytokine which helps to drive the pro-inflammatory disease state (Leyva-Castillo et al., 2013). Concomitant with the increase in TSLP production and the compromised barrier function,

(Klonowska et al., 2018) elevated Th2 cytokines cause a lack of loricrin and involucrin expression and a down-regulation of antimicrobial peptides resulting in increased susceptibility to bacterial infection, especially by *Staphylococcus aureus* (Howell et al., 2008; Kim et al., 2008). The cytokines upregulated in the Th2 pathway contributing to contact or acute AD include IL-4, IL-5, IL-13 and IL31, while later stage Th1 cytokines such as IFNγ, IL12 and TGFß are often upregulated in chronic AD. The therapeutics being explored for the treatment of AD are as varied as the pathology of the disease, targeting multiple pathways to alleviate inflammation. While corticosteroids are often the first line of defense as ambiguous inflammation modulators, multiple target-specific therapies are being explored. Thymic stromal lymphopoietin has been shown to be an early sensitizing marker to AD and has been explored with the antibody therapy, tezepelumab, to decrease circulating TSLP (Klonowska et al., 2018). Other keratinocyte signaling mechanisms are also being targeted by antibody-specific therapeutics, such as bermekimab for IL-1α and fezakinumab for IL-22. An early-stage response in AD is the aryl-hydrocarbon receptor (AhR) responsible for upregulation of several inflammatory signaling pathways, especially JAK-STAT. Tapinorof is an AhR-modulating agent specifically to prevent the receptor activation. Microbiome modulators such as antimicrobial defense peptides to curb overgrowth of *S. aureus* are currently in Phase I trials. Given the broad role janus kinase (JAK) play in cell signaling, multiple drugs have been developed to target the JAK pathway to inhibit activation. These include topical non-selective drugs such as delgocitinib and cerdulatinib, as well as dual inhibitors ruxolitinib, and systemic abrocitinib selective for JAK1 (Bieber, 2022).

1.5.10. Psoriasis

Psoriasis was first classified as a separate disease distinguished from leprosy by Thomas Bateman in 1836, based on the observations of British dermatologist Robert Willan (Nestle et al., 2009). It is a chronic skin disease affecting approximately 2-4% of the population, with a greater prevalence in adults compared to children with a dual peak of incidence at 30-39 years and ~60 years of age (di Meglio et al., 2014). Comorbidities include depression, obesity and arthritis. This condition presents with well-demarcated plaques and silvery scales caused by hyperproliferation of the epidermis. Often presented is a redness associated with erythematosus indicating an underlying inflammatory state. These hyperproliferative keratinocytes take on an innate immune role by producing antimicrobial peptides, chemokines, and proinflammatory cytokines (Nestle et al., 2009).

Figure 14. Psoriasis of the trunk and limbs (Rendon & Schäkel, 2019).



A closer evaluation of the underlying mechanism for psoriasis reveals that the disease has both an autoimmune and autoinflammatory component. The adaptive immune system controlling the activation of T and B cells contributes to an autoimmune dysfunction, however the reactivity to endogenous signals such as LL-37/cathelicidin and metabolic mediators such as phospholipase A2 group IVD portrays an autoinflammatory response, relegating psoriasis as a complex inflammatory disease (Liang et al., 2017). The most abundant T cell identified has been Th17, known to produce Interleukin-17 (IL-17), closely followed by IFNγ-producing Th1 cells. Keratinocytes respond to these activated T cells and cytokines by increasing proliferation and producing additional proinflammatory cytokines and chemokines. These keratinocyte-derived effectors act upon dendritic cells and T cells perpetuating a chronic inflammatory state (Hawkes et al., 2017)(Lowes et al., 2014). Treatment options range depending on severity, with topical emollients, corticosteroids and vitamin D analogues such as calcipotriol for mild cases and phototherapy to systemic immunosuppressants for moderate to severe. More recent biologic therapies target key inflammatory mediators such as alefacept for CD2 inhibition, etanercept for TNFα, and secukinumab for IL-17. Phosphodiesterase 4 (PDE4) and JAK inhibitors have also gained popularity, apremilast and tofacitinib respectively (di Meglio et al., 2014).

1.6. Animal Models of Human Inflammatory Dermatoses

Safety and efficacy must be demonstrated prior to human testing, requiring in vivo, in vitro and ex vivo models for quantitation of toxicity and target engagement (Maeda-Chubachi et

al., 2020). All regulatory authorities base their decision on marketing authorization of a medicine on its quality, safety and efficacy. For topical products this is extensively reviewed by Brown and Williams (Brown & Williams, 2019). The testing of topical cosmetic and pharmaceutical excipients, active pharmaceutical ingredients (APIs) and final products for possible irritation effects and potency (for medicines) has seen a slow evolution since their inception and requirements in the early 1900's. In 1944 the first U.S. Food and Drug Administration requested that animal testing was implemented. Known as the "Draize test" by toxicologist Dr. John Henry Draize, it utilized rabbits as test subjects for ocular and skin irritation of cosmetics and personal care items. While advances in animal testing allowed for the decrease from six test subjects to one to three rabbits per test, animal rights activists and government legislation continues to denounce the practice of animal testing for topicals. In 1959, Universities Federation for Animal Welfare scholars Russel and Burch proposed the concept referred to as the three Rs (Russel & Burch, 1959). These principles include Replacement; the substitution of live animals for insentient material; Reduction; reducing the number of animals necessary to acquire the data; and Refinement; the decrease in severity of inhumane procedures (Hubrecht & Carter 2019). The United States Food and Drug Administration (FDA) and European Medicines Agency (EMA) has adopted the 3Rs principles to minimize animal testing, while the 7th amendment to the European Union Cosmetics Directive now forbids animal testing of cosmetics in Europe (Lebonvallet et al., 2010).

Elsewhere in the pharmaceutical arena proponents for the discontinuation of animal testing argue that for local irritation and toxicity and pharmacokinetic studies the dissimilarities between animals and humans make such tests illegitimate. The differences include skin thickness, density, and immune system irregularities, all of which could provide false negative or positive results when testing topically applied active pharmaceutical ingredients and excipients. In 1988 the European Centre for the Validation of Alternative Methods (ECVAM) Skin Irritation Task Force published a report evaluating new in vitro testing methods for the classification of skin irritants (Botham et al., 1998). From this report it was suggested that monolayer keratinocyte cultures, while useful, lack the inherent barrier function making them less than ideal for routine testing of chemicals for skin irritation. However, to uphold the 3Rs principles, these cultures are routinely used to investigate skin irritation while accepting the known model limitations.

With regards to an early assessment of the potency or efficacy of a topical API and/or product, multiple animal models have been explored for chracterising dermatoses such as atopic dermatitis, utilizing mainly mice and dogs. The first and most widely published mouse model of AD is the NC/Nga mouse. This is an inbred strain that spontaneously develops AD-like lesions

and immune response when housed in uncontrolled conditions that allow for pathogen accessibility. This model, like most of the animal AD models available, is based on a Th2 immune response more closely associated to human acute or contact AD, instead of the later stage Th1-mediated chronic AD in humans. The NC/Nga mouse model presents with itching, erythema, scaling and alopecia at two months of age. These symptoms increase with age and are accompanied by increased serum IgE levels and mast cell population. This effect was found to be solely dependent on allergen induction, as mice housed in a pathogen-free environment did not develop lesions. The next most popular model for AD in mice is the hapten-induced disease state. This model closely recapitulates contact dermatitis and requires hypersensitization prior to any pathological response. One sensitization reagent is 2,4,6-trinitrochlorobenzen (TNCB), another is oxazolone. This treatment elicits epidermal hyperplasia, increased mast cell and T cell accumulation and elevated serum IgE. Gene expression analysis with TNCB treatment shows an initial Th1 immune response followed by a shift to a chronic Th2 inflammatory state, highlighting the differences in the typical human AD response which shift from an acute Th2 to a chronic Th1 state (Shiohara et al., 2004). Several versions of transgenic mice models have been developed to explore specific genetic modification and how it influences disease state. These include over-expressing IL-4, IL-31, IL-18 and TSLP mouse models, as well as RelB and cathepsin E knockout mice. While each of these models recapitulate some aspect of human AD, they lack certain intrinsic characteristics. The IL-31 transgenic mouse model showed thickening of ear skin, acanthosis and inflammatory cell infiltrate, but no increase in IgE serum levels. The TSLP transgenic mice show much of the pathologic signs of AD, but it was discovered that the inflammation in this model is T cell independent, unlike the human pathology. The RelB knockout mouse spontaneously develops dermatitis and immune cell infiltration, but no discernible pruritis (Jin et al., 2009).

While each of these models represent some features of human AD, they include disadvantages such as reproducibility, requirement for repeated treatments, innate differences in immune function compared to human, as well as differences in skin thickness and permeability for topical compound treatment (Shiohara et al., 2004). Physiologically, humans have melanocytes located in the basal layer of the epidermis to provide pigmentation and protection from ultraviolet radiation, but mice melanocytes are located mainly at the base of hair follicles embedded in the dermis (Avci et al., 2013). Of note in the skin are the expression of antimicrobial peptides and defensins in the skin. Neutrophils in human skin provide defensins for protection against exogenous pathogens, however mouse neutrophils do not express defensins. Also, the predominant T cell in mouse skin are γ/δ T cells while human are α/ß T cells. Along with the difference in T cell populations are the differences in mice and humans to cytokine

signaling. Mutations in the common gamma chain which interacts with IL-2, IL-4, IL-7, IL-9 and IL-15 can cause severe immunological effects in humans and a decrease in T cells and natural killer cells, however B cell development maintains normal. This mutation in mice causes a marked decrease in B cell development. As Janus Kinase 3 (JAK3) is the major signal transducer for common gamma chain, this can have a profound effect on the efficacy being evaluated for new JAK-targeting therapeutics for skin diseases such as psoriasis and atopic dermatitis. The skewing of T cell populations during inflammation is another factor delineating mice from humans. In response to viral infection, IFNα is secreted by several cell types as a protective measure to induce Th1 differentiation via STAT4 activation in humans, but in mice IFNα does not activate STAT4 nor induce Th1 differentiation. The anti-inflammatory cytokine IL-10 is considered a Th2-specific cytokine in mice, while in humans both Th1 and Th2 cells can produce IL10. In mice, endothelial P-selectin can be strongly up-regulated by TNF and LPS to induce leukocyte rolling, whereas in humans this is nonresponsive. Human T cells express MHC class II molecules on activated T cells as well as a calcium flux mediated by a potassium channel, however murine T cells lack MHC class II expression and potassium channel function. Chemokines, the regulators of immune cell movement and infiltration can vary considerably between mice and humans. While this system is full of redundancy, mice lack the human chemokines CXCR1, CXCL8, CXCL11, MCP4, CCL15 and CCL23, while humans lack the mouse-expressed CCL6, CCL9, CXCL15 and MCP5 (Mestas & Hughes, 2004).

An alternative to mouse models of atopic dermatitis are canine models, which present spontaneous AD in about 10% of the population and is the most commonly diagnosed atopic disease in dogs. While it is an allergen-based reaction similar to contact or acute dermatitis, it has been shown to be familial and house a genetic predisposition much like human AD. While most human acute AD is accompanied by elevated IgE serum levels, canine AD is less consistent in the IgE increase. When considering secondary infections in the disease state, both dogs and humans often present with Staphylococcus bacterial infection. Langerhans and T cell infiltrates and accumulations in lesional skin is also similar between dogs and humans, as well as pruritis. The most common approach for the canine AD model is the use of house dust mite sensitization to elicit an allergen-induced AD disease state (Marsella & Olivry, 2003).

In the pursuit of an adequate model to simulate human psoriasis, animal models have been an invaluable tool in understanding the pharmacodynamics of the disease. Unfortunately, the only animals known to recapitulate psoriasis are rhesus and cynomolgus monkeys. Dogs and pigs have also shown signs of psoriasis; however these are sporadic and unreproducible. As such, the majority of animal models rely on mouse strains categorized as spontaneous, genetically modified, induced and xenotransplantation. Spontaneous and genetically modified

mice present the disease state without exogenous stimulation, whereas induced mice require induction of disease state usually by surgery, chemical or biological application. Xenotransplantation is considered the humanization of mice by surgical grafting of human lesional tissue to immunocompromised mouse strains. Since psoriasis is not naturally occurring in mice, each model reflects a slightly different mechanism of the disease. The differences in human and mouse skin also complicate the translatability of the model, including the thicker dermis of human skin and multiple epidermal layers compared to mouse. The majority of immune cells found in human epidermis are Langerhans cells and T lymphocytes, however mice show a majority population of dendritic cells and lack an influx of T cells to the skin evident in human psoriasis (Bocheńska et al., 2017).

Current spontaneous mouse models include the homozygous asebia mouse, flaky skin mouse and chronic proliferative dermatitis mouse. The homozygous asebia mouse presents hyperkeratosis and a loss of sebaceous glands. This results in epidermal acanthosis, increased vascularization and influx of mast cells. This model is limited by the influx of T cells and neutrophils to the skin. Flaky skin mice successfully induce inflammation with hyperkeratosis and an influx of neutrophils to the skin, however the limited lifespan of these mice limit their usefulness. The closest representation of human psoriasis in a spontaneous mouse model is the chronic proliferative dermatitis mutation. This mouse presents inflamed skin with expanded blood vessels with an increase in eosinophils, macrophages and mast cells, however does not respond to immunosuppressant cyclosporine A treatment as humans do. Dozens of genetically modified mouse models are available to look at specific functions in the psoriasis disease state. Many of these models target epithelial cell markers, however important inflammatory factors such as cytokines and interferon are absent from the disease state. Other models target immune cell function such as Th17 and chemoattractants. While these models can often recapitulate both the inflammatory state and indirect keratinocyte involvement, they often lack other key features such as vascularization (Bocheńska et al., 2017).

Induction of a psoriatic phenotype by imiquimod is the most common chemical induction model. Daily application of 5% imiquimod to mice results in skin lesions, erythema, epidermal proliferation, neoangiogenesis and an influx of immune cells to the skin. The downside to this model is the lack of underlying autoimmune dysfunction that comprises the psoriatic disease in humans. To date, the best mouse models for psoriasis research have been xenotransplantation. This model overcomes the lack of morphological similarities between human and mouse skin. A transplantation of human lesional tissue to an immune deficient mouse strain such as the athymic nude mouse or severe combined immunodeficiency (SCID) mice can be maintained for up to 2 months while retaining signature histological features of psoriasis. However the graft loses

retention of the stratum corneum and parakeratosis. Even considering these limitations, the xenotransplantation model mimics the most complete spectrum of phenotypes to model psoriatic disease (Bocheńska et al., 2017).

The limitations of these mouse models include skin morphology and genetic manipulation. Rete ridges present in human epidermis are also absent in mouse skin, as well as differences in density and length of hair follicles, antigen expression, epidermal turnover rate and immune cell population. In addition, mice present a panniculus carnosus or layer of striated muscle that is absent in human skin. Genetic modification is introduced on animals of a homogeneous genetic background under controlled environmental conditions. This varies wildly from the diverse genetic background in which psoriasis manifests in humans as well as the environmental impact that pathogens and exogenous insult can have on disease severity. Phenotypic penetrance also plays a part in the mouse model variation with differing levels of inflammatory reaction dependent on the varying levels of transgene expressed. This could be affected if the genetic background is altered through breeding or if environmental factors are changed. This has been observed when the psoriasiform phenotype differed between animals of different facilities with the same transgenic modification (Schon, 2008).

The mouse models developed for the evaluation of drug activity are not suited to permeability studies due to the decreased thickness of mouse skin. Human skin is over 100µm thick compared to only 25µm in mice (Zomer & Trentin, 2018). When evaluating the permeation characteristics of different topical therapeutics prior to clinical application, stark differences have been observed between species that must be considered. One study compared four compounds of different polarities to highlight the differences between human skin, reconstructed human skin (RHE), rat skin and pig skin. A typical penetration experiment was conducted using Franz diffusion cells over a 48 hour period with receptor solution sampling every eight hours. All compounds were applied in a similar vehicle at 1% concentration. The antifungal terbinafine permeated 55-fold higher through RHE, 50-fold higher through rat skin, and 2-fold higher through pig skin over human. Clotrimazole, also an antifungal compound, permeated 200-fold higher through RHE, 3-fold higher through rat skin, and gave the same flux through pig skin as human. The common corticosteroid hydrocortisone permeated 200-fold higher through RHE, 50-fold higher through rat skin, and had a 0.5-fold lower flux through pig skin than human skin. Finally, fluxes were higher for salicylic acid, however still permeated 7-fold higher through RHE, marginally higher through rat skin, and had a 0.5-fold lower flux through pig skin than human skin. These trends observed between all four compounds highlight the need to focus on ex vivo human skin for pre-clinical evaluation of topical drug delivery (Schmook et al., 2001). In

a separate study, the flux of fluoxetine formulations with various penetration enhancers showed a 2-3.5 fold increase in flux using rat skin over human skin (Jung et al., 2013).

1.7. In Vitro Skin Models

Beginning in the 1980's the development of reconstituted human epithelium (RHE) skin models promised conscientious and unequivocal human irritancy studies. These cultures allowed for the differentiation of an intact stratum corneum at an air-liquid interface that more closely resembled that of the human skin barrier via differentiation of human keratinocytes into a stratified epidermis. These models quickly gained interest for topical testing in both pharmacotoxicology studies and dermal irritation evaluation. Commercial availability has now expanded to include EPISKIN (L'Oreal) and EpiDerm (MatTek Corporation). Recently they have evolved to include collagen, fibroblasts, and melanocytes (EPISKIN.com) (MatTek.com). The disadvantage of these models is the lack of any immune-associated cell types, either resident or carried by vascularisation, that are present with in vivo human skin clinical studies. While RHE have proved valuable in replacing animals in skin irritation models and characterizing keratinocyte development, they lack the intrinsic immune cell population necessary for a disease-relevant inflammatory response and the barrier impermeability of human skin.

Several in vitro models have been designed to mimic the human inflammatory state for the purpose of both characterizing the pathology and testing therapeutics. The simplest of these models are the two-dimensional engineered cell models. These consist of monolayer keratinocyte cultures that serve as easy and reproducible early development tools. In an effort to reproduce the psoriatic state, investigators attempted to isolate and culture keratinocytes from psoriatic patients, however these cultures proved difficult to culture and often lost the gene expression profiles during expansion. Reconstructed human epidermal models were soon developed to include keratinocytes and fibroblasts to better recapitulate the skin strata and intercellular cross-talk. These cultures can be induced to differentiate into basal, granular and cornified epidermal layers by the addition of growth factors, however they still lack any immune cell presence. Coculture of the reconstructed human epidermal models with polarized Th1 and Th17 T cells showed promising similarities to the psoriatic gene profile, however are hindered by the uniform application of the immune cells and migration into the extracellular matrix and the media requirements for coculture (Desmet et al., 2017).

Hydrogel systems create a more complex scaffold for coculturing fibroblasts and keratinocytes, using collagen and other extracellular matrix proteins, to create a 3D assembly of cell culture and promote keratinocyte differentiation. Because of the scaffold structure, hydrogel

systems can evaluate epidermal behaviour, chemical cytotoxicity, and cellular interaction; however, dependant on the complexity of the biological components, they can create high variability (Klicks et al., 2017). One such study expanded this model to observe the interaction between keratinocytes and immune cells in an inflammatory state, exhibiting cell migration and inflammatory cytokine expression, though pathological hyperproliferation of the keratinocytes indicative of a psoriatic disease state was not observed (van den Bogaard et al., 2014). Additional complexities are starting to be incorporated such as vasculature, appendages, pigmentation, innervation, immune cells and hypodermis to better recapitulate the human skin strata (Souci & Denesvre, 2021).

1.8. Ex Vivo Skin Models

One of the first papers to characterise the ex vivo culture of human skin explants was published in 1965 by Reaven et al. This paper summarized the previous work undertaken on human ex vivo skin culture (HESC) as well as exploring the morphological changes inherent to the skin culture using histological methods (Reaven & Cox, 1965). In 1968 a follow up publication looked at the effects of tape stripping, temperature, oxygen tension, pH and serum to further describe the inherent means of culturing and manipulating the HESC for research purposes (Reaven & Cox, 1968). Since then, multiple improvements have been made to the system to optimize for research of therapeutics and formulations without the need for animal testing. A study in 2001 compared the ability to culture the HESC in submerged versus air-liquid interface cultures and the effect of cytokine stimulation (Companjen et al., 2001) and is one of the first papers to exhibit the viability of the HESC in culture as well as the addition of cytokine stimulation for disease state representation. A study published in 2009 examined the longevity of HESC in air-liquid interface up to 4 weeks in culture (Steinstraesser et al., 2009), while a paper in 2010 touted the preference of HESC cultures over reconstructed human epithelial models (RHE) to investigate the effects of environmental stress, aging and skin diseases. (Lebonvallet et al., 2010). In 2012 a separate laboratory used HESC to explore the potential for wound healing models (Xu et al., 2012). A research team in France published one of the earliest in-depth papers suggesting the ability to generate a psoriatic model using the HESC and a complex combination of stimulatory cytokines resulting in the involvement of not only immune cells but also the release of chemokines, antimicrobial peptides and keratinocyte differentiation markers associated with clinical psoriasis (Rabeony et al., 2014). Since these early studies, interest in using HESC to investigate pharmaceutical formulations for the treatment of a range of skin

ailments from irritation screening to wound closure and infection has been explored to generate relevant preclinical models.

While keratinocytes can proliferate and differentiate over a wide range of extracellular calcium concentrations, fibroblast growth is strictly regulated and requires above 1mM Ca^{2+} to maintain proliferation and begin losing viability below 0.15mM. As fibroblasts and endothelial cells are a source of several keratinocyte growth factors, it is necessary to supplement the culture media of human explant skin cultures (HESC) with extracellular calcium to maintain overall health (Varani, 1998). Culture media pH also proved crucial for tissue integrity, with a pH at 7.0 sufficient for survival, however above 7.6 showed increases in keratohyalin granuoles and at a pH above 7.8 survival decreased sharply (Reaven & Cox, 1968).

The closest morphological representation of human skin are ex vivo explant models. This tissue is typically removed during elective abdominoplasty surgery and contains not only differentiated keratinocytes, but also fibroblasts, adipose tissue, Langerhans cells, dendritic cells and resident T cells (Bocheńska et al., 2017). Several versions of the human ex vivo skin culture (HESC) model have been proposed (Lebonvallet et al., 2010; Park et al., 2015; Vostálová et al., 2018). Many have reported increased epidermal thickness over the course of the first week in culture with a decrease in tissue integrity and keratinocyte differentiation occurring after day 14. The loss of keratinocyte proliferation is accompanied by an increase in keratinocyte apoptosis evident after the first week (Xu et al., 2012). Intact epithelial barrier function has been observed out to 28 days in culture using topically applied transgene expression (Steinstraesser et al., 2009). For the basis of the HESC presented in this thesis, components from Vostálová et al., 2018 and Smith et al., 2016 were incorporated including the basis for the modified Cornification media and Th17 stimulation cockatil respectively, as well as tissue preparation described in Smith et al., 2016.

1.9. Methodology

All 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. Study Title: Healthy volunteer skin donation for in vitro experimentation). Written informed consent was obtained from all subjects or, if subjects are under 18, from a parent and/or legal guardian. All donors were healthy and not currently taking any form of systemic corticosteroid treatment. Only abdominal tissue from elective abdominoplasty was used. No identifying information beyond ethnicity and age were provided, when available. Donors above the age of 60 were excluded for all but age-related analysis in Chapter 3. Due to privacy

regulations ethnicity and age were often not provided, however of the information provided, the majority of donors were female. Each data set represents a separate subset of donor tissue with the exception of Figures 1 and 2 in Chapter 2 and Figures 2 and 4 in Chapter 4 which represent data from the same donors. Tissue was maintained in humidified incubators at 37°C and 5% CO2 in either Franz cells (~0.63 cm2 tissue surface area) or Costar Transwell 0.33 cm^2 Permeable Support (Thermo Fisher Scientific; #3470). Tissue was kept chilled until processing for culture (between 16 and 24 hours post-removal) and processed for use within 24 hours of surgery. Tissue was defatted and dermatomed to a thickness of 750±100 µm (Integra Padgett Slimline SB). Tissue exhibiting abnormalities such as edema, abrasion, or heavy striation were discarded. Tissue was further cut into 1cm^2 sections for mounting onto Franz cells or a 7mm punch biopsy was used for transwell studies. The well was then filled with modified Cornification media resulting in an air-liquid interface ex vivo culture. Media was changed no less then every 48 hours. Human tissue explants were cultured in a modified Cornification media consisting of DMEM/Hams F12, 2% fetal bovine serum, antifungal/antimycotic, insulin, adenine, and additional nutrient supplements. The composition of the cytokine stimulation cocktails were comprised of CD3/CD28 antibodies for initial T cell activation and additional cytokines chosen to act upon the primed T cells to encourage differentiation into specific subsets. These cytokines were chosen as upstream mediators of T cell differentiation toward Th1, Th2, or Th17 maturation. Inhibiting antibodies were included in the cytokine stimulation cocktails to inhibit differentiation into an inflammatory pathway not specified by the cocktail. The exact composition of the stimulation cocktails cannot be disclosed due to proprietary intellectual property restrictions. Application of the drugs was undertaken either basolaterally as in Chapter 3 by first dissolving the drug in DMSO at 1000x concentration and then adding to the Cornification media, or topically, as in Chapter 4, by applying the formulation to the tip of a syringe plunger by displacement pipet and gently applying to the top of the tissue to ensure consistent coverage.

At time of harvest tissue was placed into RNALater preservative to allow permeation overnight at 4°C. Following tissue mincing and homogenization 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 & Schmittgen, 2001). Data was normalized internally using the housekeeping gene GAPDH. 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. Methodologies for individual data sets including number of donors, treatment paradigms and quantitative analysis is included with each chapter to reflect data set specifics.

1.10. Aims

The overarching aim of this work was to prove the translatability of a stimulated HESC model as a preclinical tool for the evaluation of novel therapeutics as a cost saving and risk mitigation tool in drug development. Initially, the work performed was designed to determine whether this HESC model could not only be stimulated to express immune-specific biomarkers, but also to create an inflammatory tissue environment to elicit dermatoses-like responses from keratinocytes, fibroblasts, macrophages and dendritic cells. The ability of this exogenous stimulation of HESC to translate to the human disease state, better than in vitro models or animal models, was then evaluated by thorough characterisation to provide a better understanding of both the pros and cons of using HESC for preclinical therapeutic testing.
To fully characterise the HESC model, a bottom-up approach was utilized.

- First, prove the ability to culture human ex vivo skin while maintaining tissue integrity, viability, and metabolic activity since these basic attributes are necessary to ensure the tissue maintains in vivo characteristics lending to translatability of findings between the ex vivo skin culture and human condition as useable data sets.
- Second, explore the ability to induce an inflammatory state and compare that state to naturally occurring dermatoses in the human population. Multiple stimulatory cocktails were employed to mimic the early phase inflammation seen in Th1, Th2, and Th17-mediated diseases and quantitate the gene expression output compared to known human transcription levels in dermatoses. Within this data set, intra and inter-donor variability was calculated, as well as age and ethnicity profiles to create a comprehensive picture of the HESC model.
- Lastly, the model was benchmarked against a published clinical trial of two commercially available topical formulations for the treatment of atopic dermatitis; Elidel and Dermovate, to show the correlation of the gene expression modulation using these formulations in the inflamed HESC compared to human application.

CHAPTER 2 : Human skin explant model for the investigation of topical therapeutics

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Human skin explant model for the investigation of topical therapeutics

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Jessica Neil was responsible for 90% of conception and design, 90% of data collection, 95% of data analysis and conclusions and 70% of manuscript preparation

ABSTRACT

The development of in vitro and ex vivo models to mimic human illness is important not only for scientific understanding and investigating therapeutic approaches but also to mitigate animal testing and bridge the inter-species translational gap. While in vitro models can facilitate high-throughput and cost-efficient evaluation of novel therapeutics, more complex ex vivo systems can better predict both desirable and adverse in vivo effects. Here we describe an ex vivo cultured human skin explant model in which we have characterised pathological tissue integrity, barrier function and metabolic stability over time. Our findings suggest that human skin can be successfully cultured for pharmacodynamic use up to and beyond 9 days without any adverse physiological consequence.

INTRODUCTION

The testing of topical cosmetic and pharmaceutical excipients and active ingredients for possible irritation effects and potency has slowly evolved since its inception in the early 1900's. In 1944, the first U.S. Food and Drug Administration recognized animal testing was implemented; known as the "Draize test" after toxicologist Dr. John Henry Draize, it utilized rabbits as test subjects for ocular and skin irritation of cosmetics and personal care items. While advances in animal testing allowed a decrease from 6 to 1-3 rabbits per test, researchers, consumers and pressure groups refuted the need and value of such tests, and ultimately government legislation is moving

to ban animal testing for topical products. The 7th amendment to the European Union Cosmetics Directive now forbids animal testing of cosmetics in Europe [1]. Notably, the dissimilarities between animal and human tissue raises questions regarding the legitimacy of data that is generated; for skin, these differences include the thickness of the tissue itself and its constituent layers, density of the hair follicles and other appendages, and immune system irregularities.

In 1988 the European Centre for the Validation of Alternative Methods (ECVAM) Skin Irritation Task Force published a report evaluating new in vitro testing methods classifying skin irritants [2]. From this report it was suggested that monolayer keratinocyte cultures, while useful, lack the inherent barrier function and properties of skin and in particular its outermost stratum corneum, making them unsuitable for routine testing of chemicals for skin irritation. From the 1980's, reconstituted human epithelium (RHE) skin models were developed, in part for human irritancy studies. These cultures allowed differentiation of an intact stratum corneum in an air-liquid interface that more closely resembled the in vivo human skin barrier. These models quickly gained interest for pharmacotoxicology studies and dermal irritation evaluation with commercially produced systems such as EpiSkin (L'Oreal) and EpiDerm (MatTek Corporation) available. However, these models still lack immune-associated cell types, either resident or carried by vascularisation, that are present with in vivo human skin clinical studies.

In addition to the prominent keratinocytes which can produce antimicrobial peptides such as psoriasin, lipocalin and defensins, immune cells play a major role in the pathophysiology of skin disorders. In the absence of cell migration from the circulatory and lymph system into the dermis, resident immune cells remain; lymphocytes, dendritic cells and Langerhans cells. Of these, the resident memory T cells are known to be potent mediators against infection, but also function in autoimmune disease. There are approximately 20 billion memory T cells in the skin of an adult human, with an estimated 1 million in each cm^2 [3-5]. To further elucidate the function of these resident T cells, mitogen stimulation was used to determine the polarization of the cells via secreted cytokines and surface markers. It was found that the predominant skin resident T cell population secreted Th1 cytokines IFN-γ and IL-2, with a small sample also producing the Th2 cytokine IL-4 [5].

Organ culture of human skin dates back over 50 years [6] and has undergone significant improvements to optimize the tissue for research studies developing therapeutics and formulations without the need for animal testing. The ability to maintain a Human Explant Skin Culture (HESC) in submerged versus air-liquid interface cultures and the effects of cytokine stimulation have been studied [7] and more recently the viability of HESC at an air-liquid interface was shown for up to 4 weeks in culture [8]. The benefits of using HESC cultures over reconstructed human epithelial models (RHE) were evident from varied studies investigating the

effects of environmental stress, aging and skin diseases [1] and as a model for wound healing [9]. Cytokine stimulation of HESC provoked immune cell responses with release of chemokines, antimicrobial peptides and keratinocyte differentiation markers associated with clinical psoriasis [10]. However, in the HESC model literature [1,6,11-13], a general increase in epidermal thickness is noted over the first week in culture with a decrease in tissue integrity and keratinocyte differentiation occurring after ~14 days. In addition, loss of keratinocyte proliferation is accompanied by an increase in keratinocyte apoptosis evident after the first week [9]. In contrast, the epithelial barrier function was reportedly intact up to 28 days in culture, when assessed using topically applied transgene expression [8]. Given the growing interests and need for HESC models for toxicity, irritation, and therapeutic efficacy testing across diverse disciplines including the pharmaceutical, cosmetic and agrochemical sectors, here we assess tissue viability as the analysis of tissue integrity by histology and sustainment of housekeeping gene expression, mechanical barrier properties by TEER and metabolic activity by gene expression activation of HESC with time in culture.

RESULTS AND DISCUSSION

Assessment of skin viability over time

To determine tissue integrity over the 20-day time course in culture, haematoxylin and eosin staining of the tissue cross-section was performed (Figure 1). Images were scored for spongiosis, necrosis, parakeratosis and epidermal/dermal separation (Figure 2). Potential thickening of the epidermal layer (hyperplasia) was measured but no increase in epidermal thickness was observed over this period (data not shown). The histology presented is representative of one patient, Donor 1, of the three donors used for quantitative visual assessment.

The appearance of vacuolar fluid-filled cells in the epidermis indicates that spongiosis occurred on Day 9 in culture and steadily increased in prevalence up to day 15 ($P<0.0001$). Thereafter, necrosis of the keratinocytes diminished the appearance of spongiosis. (Figure 2A). Necrosis (determined by condensation of nuclei and bright red staining) was first apparent at Day 9 in culture and increased up to day 20 ($P<0.0001$). Parakeratosis, the thickening and sloughing of the stratum corneum was also a later event first observed on day 13 in culture. While an increase in parakeratosis was observed, it was not uniform across the whole sample with approximately 60% of the tissue area affected by day 20 ($P<0.0001$). Finally, separation of the epidermal and dermal layers is attributable to disintegration of the dermo-epidermal junction (basement membrane) and again was not uniform across the whole sample but extended to ca. 20% of the total tissue area by day 20 ($P=0.0211$). In combination, the histological analysis

demonstrates that tissue integrity is maintained unimpaired for up to 9 days in culture under the described conditions.

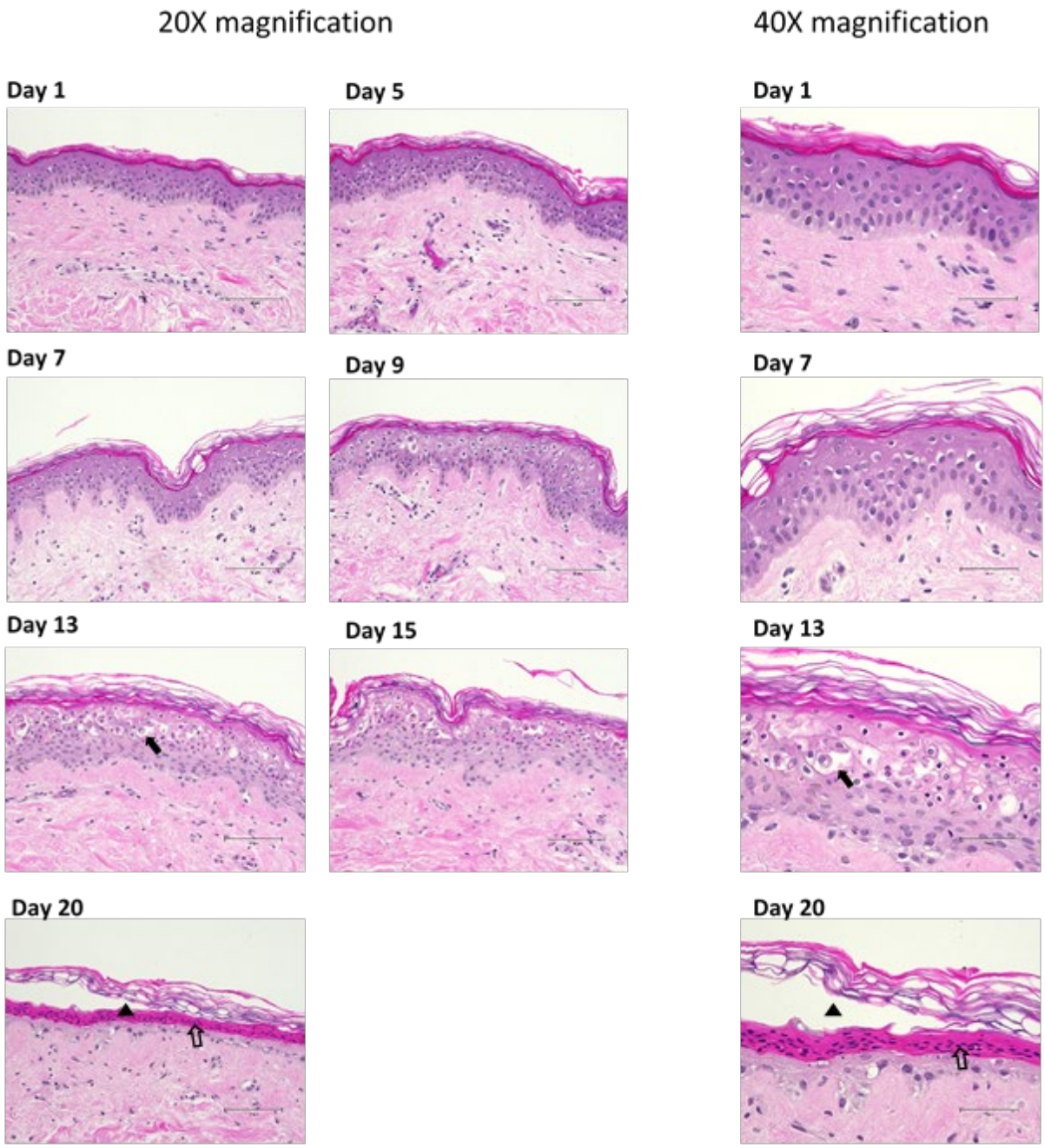

Figure 1. **Haematoxylin and eosin staining of tissue integrity over 20 days observing spongiosis, necrosis, parakeratosis and epidermal/dermal separation**. Representative images from Donor 1 haematoxylin and eosin stained tissue over time course. Days 1, 5, 7, 9, 13, 15 and 20 exhibited at 20X magnification (scale bar = 100µm). Days 1, 7, 13 and 20 exhibited at 40X magnification (scale bar = 50µm). Solid arrow points to spongiosis (Day 13). Empty arrow points to necrosis (Day 20). Solid triangle points to parakeratosis (Day 20).

To assess skin barrier integrity, Trans-Epithelial Electrical Resistance (TEER) is commonly measured [14]. Based on this approach electrical resistance can be quantified using an alternating current (AC) across the membrane to observe the integrity of tight junctions and involvement of any barrier dysfunction (Figure 2B). Combined donors resulted in a 53.5% ± 16.5 increase from

Day 0 (day of fresh tissue harvest) to Day 3 in culture. This change in TEER increased to 101% ± 68.5 by Day 7.

TEER readings are correlated with observed barrier disfunction of parakeratosis and epidermal/dermal separation in Figure 2C. The increase in parakeratosis and epidermal/dermal separation observed is not mirrored by the loss of TEER measured resistance beginning on Day 13. However, it is evident that the histological scoring captured morphological dysfunction earlier than Day 13, suggesting that TEER cannot detect early tissue integrity loss, though electrical resistance measurements remain useful for assessing gross barrier integrity of thinner tissue samples, such as those used for in vitro permeation studies.

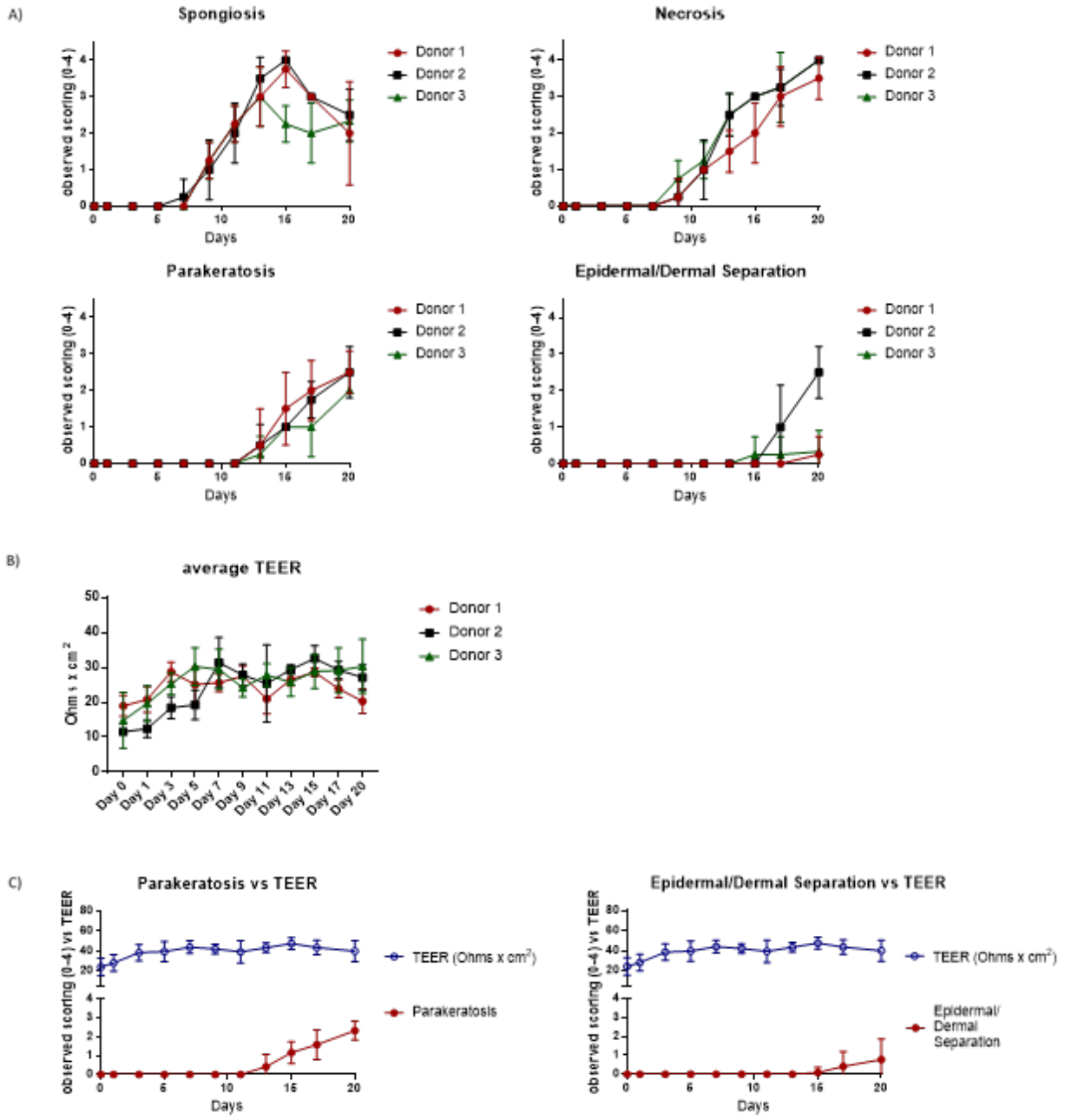

Figure 2a) **Histological analysis of tissue with haematoxylin/eosin staining over time course.** Average score measurement over time in culture. N=3 donors, 4 replicates per donor per time point. Error bars represent mean ± standard deviation. 2b) **The change in trans-epithelial**

electrical resistance (TEER) as a measure of barrier integrity. Average TEER measurement over time in culture. N=3 donors, 4 replicates per donor per time point. Reported TEER as Ohms*cm^2 tissue surface area. Error bars represent mean ± standard deviation.2c) **Ratio of parakeratosis and epidermal/dermal separation to TEER.** Comparison of average score measurement of parakeratosis and epidermal/dermal separation over time in culture versus reported TEER as Ohms*cm2 tissue surface area. N=3 donors, 4 replicates per donor per time point. Error bars represent mean ± SD. Scoring values were analysed using unpaired non-parametric two-tailed Mann Whitney t-test with GraphPad Prism software.

Tissue metabolic activity

To assess cell viability and metabolic capacity over time, RT-qPCR of common housekeeping, cell cycle and proliferation-associated genes was performed (Figure 3). The housekeeping genes ribosomal 18s (r18S), a ribosomal subunit integral in all protein translation within the eukaryotic cell, and cyclin-dependent kinase inhibitor 2A (CDKN2A), a biomarker of cellular senescence were compared [15]. Gene expression of r18s increases gradually from Day 1 to Day 9 (ca. 3.7 fold; $P<0.0001$), suggesting an increase of cellular metabolism. However, on Day 9 a spike in CDKN2A mRNA was observed (ca. 5.7 fold; $P=0.2145$), followed by an immediate arrest of r18s increase, suggesting a switch in the tissue metabolic paradigm.

Keratin 14 is a commonly used biomarker of dividing basal keratinocytes and tends to decrease as the cells differentiate and migrate to the stratified epithelium [16]. Keratin 16 is constitutively expressed at low levels in the epithelium but expression is induced upon injury [17]. Here, using healthy adult human skin, the expression of KRT14 remained at low levels and did not change throughout the tissue culture period suggesting no induction of keratinocyte proliferation. In contrast, the wound indicative expression of KRT16 rose steadily from Day 5 in culture reaching maximal levels at Day 15 (ca. 13.3 fold; $P=0.0043$).

Involucrin (IVL) gene expression occurs after the maturing keratinocytes have left the basal layer but before the onset of envelope cross-linking in the upper epidermal layers and so IVL is a good biomarker for early keratinocyte differentiation [18]. Loricrin (LOR) expression is a later stage marker of keratinocyte terminal differentiation as a major component in the cornified barrier structure [19]. The observed gradual increase in IVL expression from Day 1 to Day 9 (ca. 8.2 fold; $P<0.0001$) suggests a moderate return to keratinocyte maturation in the healthy tissue with a lack of terminal differentiation upregulation given the absence of late state LOR expression.

Matrix metalloproteinases function in tissue wound repair by facilitating extracellular matrix remodelling through the degradation of collagens, elastin, laminin and fibronectin[20]. Matrix metalloproteinase 9 (MMP9) is classified as a gelatinase prevalent in acute and chronic wounds

and secreted primarily by migrating keratinocytes [21]. Alternatively, matrix metalloproteinase 12 (MMP12) is classified as an elastase specifically expressed by macrophages [22]. MMP12 showed a fluctuating pattern of expression in the HESC with high variability, while MMP9 expression was not induced over the tissue culture time course (data not shown).

A major component of skin integrity is regulation of the inflammatory profile. Tumour necrosis factor alpha (TNFα) is a prevalent cytokine in tissue homeostasis. As a cellular signaling factor, TNFα can mediate cellular apoptosis, survival, proliferation and differentiation and regulation of TNFα levels is critical for proper tissue viability [23]. In contrast to its accepted anti-pathogenic properties, in vitro studies have shown the ability of TNFα to stimulate fibroblast proliferation [24]. Alternatively, TNFα can inhibit the proliferation of keratinocytes without inducing cell death [25]. While it is difficult to elucidate the exact function of the increase in TNFα in the HESC from day 5 to day 11 in culture (ca. 1.7 fold; P=0.9856), the lack of accompanying proliferation marker KRT14 and no observed necrosis by histology in this time frame suggest that survival may be one of the effects. Interferon-gamma (IFNγ) is a cytokine released by lymphocytes in response to antigen. No increase in IFNγ was observed suggesting a lack of unspecific T cell activation over the time course[26].

Interleukin 1α (IL1α) is produced by keratinocytes in the epidermis at higher levels than in any other tissue. It regulates keratinocyte differentiation and serves as an early signaling mediator of tissue damage [27]. Upon the release of IL1α due to stress or injury, a paracrine feedback loop consisting of the IL1α protein binding the IL1α receptors on the keratinocyte surface leads to the expression of additional IL1α mRNA [28]. Along with this function, IL1α is involved in wound healing, leukocyte recruitment and the induction of downstream cytokines such as TNFα, IL6 and IL8 [29]. In our study, the increase in IL1α gene expression up to Day 9 in culture (ca. 34 fold; P<0.0001) could be explained by the initial release of IL1α protein upon tissue damage during surgery, and the subsequent feedback loop described to induce additional mRNA expression. This also corresponds with the TNFα induction and suggests a correlation between the two cytokines. Unlike the constitutively expressed IL1α, interleukin 1ß is an induced inflammatory mediator secreted mainly by monocytes and macrophages in response to microbial infiltration and Toll-like Receptor activation and was not induced[30].

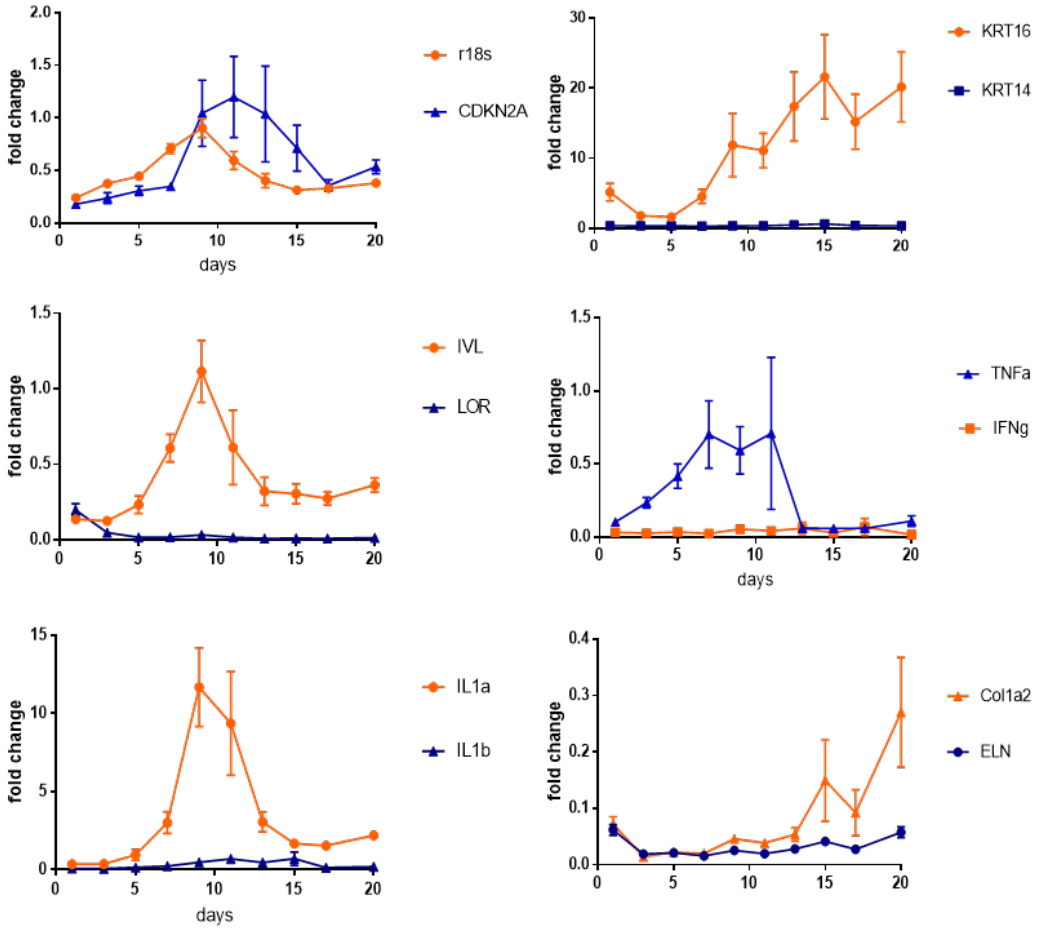

Figure 3. **Tissue metabolic activity measured by gene expression RT-qPCR**. Gene expression displayed as fold change compared to Day 0. N=3 combined donors; 4 samples per time point. Error bars represent mean ± standard error of the mean.

The most abundant extra-cellular matrix component is type I collagen (Col1a2) [31]. The gradual increase in Col1a2 expression beginning at day 13 up to Day 20 (ca. 5 fold; P=0.0050) could be in response to the loss of tissue integrity demonstrated by increased spongiosis and parakeratosis of the epidermal layers, causing an increase in collagen production as the wound healing mechanism. While elastin expression is typically low in the dermis of adult tissue, it can be upregulated by the overexpression of IL1ß [32]. The lack of ELN upregulation corresponds to the lack of IL1ß expression.

CONCLUSIONS

In conclusion, while some reports describe the cellular and morphological similarities and changes observed with human ex vivo skin culture, here we characterise the principle metabolic gene expression changes and correlate with the gross morphological changes to the tissue. Skin barrier integrity (as measured by TEER) could be maintained up to and possibly beyond 20 days

in culture but spongiosis, necrosis and parakeratosis showed morphological dysfunction prior to a loss in TEER barrier resistance.

Gene expression of critical biomarkers provides a comprehensive assessment of the metabolic challenges that the tissue experiences during culture. The housekeeping genes r18s and CDKN2A suggest a switch in metabolic activity on day 9. No induction of keratinocyte proliferation by KRT14 gene expression was quantified, however an increase in KRT16 gene expression was observed. In contrast, keratinocyte maturation quantified by IVL continued in the HESC, but terminal differentiation was not detected by LOR expression. While the expression of these biomarkers confirms cellular metabolic activity, there is evidence of a discrepancy between HESC and skin in vivo, requiring consideration when using this model as a test platform. A primary concern in the culture of human tissue is activation of the inflammatory cytokines which can be detrimental to cellular integrity over time. TNFα and IL1α gene expression was observed suggesting a response to the wounding and subsequent recovery, however the lack of additional expression of IFNγ and IL1ß shows minimal inflammatory reaction. Inflammatory markers may also be increased due to the surgical procedure as well as underlying obesity of the patient. Lastly the collagen extracellular matrix response also supports a wound healing mechanism in these cultures though additional characterisation of keratinocyte proliferation and maturation is merited.

This model augments on the previous work done by Vostálová et al whom described disinfecting fresh tissue and the basis for the culture medium, however differs in the use of porous membrane substrates to maintain an air-liquid interface. This approach was also adapted to allow for the mounting of tissue explants into Franz cells for the application of topical formulations without concern for lateral drug migration into the media. The data demonstrates that the HESC is structurally viable and metabolically active for up to 9 days in culture and can be employed for preclinical testing of delivery and efficacy of skin therapeutics under these conditions.

MATERIALS AND METHODS

Human Skin Explant

All 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. Study Title: Healthy volunteer skin donation for in vitro experimentation). Written informed consent was obtained from all subjects or, if subjects are under 18, from a parent and/or legal guardian. All donors were healthy and not currently taking any form of systemic

corticosteroid treatment. No identifying information beyond ethnicity and age were provided, when available. Tissue was maintained in humidified incubators at 37°C and 5% CO2 in either Franz cells or Costar Transwell Permeable Support (Thermo Fisher Scientific; #3470). Tissue was kept chilled until processing for culture (between 16 and 24 hours post-removal) and processed for use within 24 hours of surgery. Tissue was defatted and dermatomed to a thickness of 750±100 µm (Integra Padgett Slimline SB). Tissue exhibiting abnormalities such as edema, abrasion, or heavy striation were discarded. Tissue was further cut into 1cm^2 sections for mounting onto Franz cells or a 7mm punch biopsy was used for transwell studies. The well was then filled with modified Cornification media resulting in an air-liquid interface explant culture. Media was changed no less then every 48 hours. Human tissue explants were cultured in a modified Cornification media [12,33].

Tissue Integrity Time Course

Human tissue explants were plated onto static Franz cells and supplemented with modified Cornification media in a humidified incubator to be harvested at each respective time point. A total of three individual donors were assayed, four replicates per donor per time point. Eleven time points were harvested; Day 0, 1, 3, 5, 7, 9, 11, 13, 15, 17 and day 20. Day 0 explants were cultured 2 hours prior to harvest. At time of harvest, Cornification media was aspirated from the receptor chamber and replaced with pre-warmed 37°C phosphate buffered saline (PBS). The donor chamber was filled with pre-warmed 37°C PBS. Transepithelial Electrical Resistance (TEER) measurements were conducted using a Iso-Tech LCR821 Meter set at 0.0100 kHz Frequency and 0.200 Voltage. A single reading was recorded per tissue explant at each time point of harvest, as well as a Day 0 compromised explant for reference. TEER values are reported as Ohm (Ω) x cm2. Immediately after TEER measurement, PBS was aspirated from tissue and explant was removed from Franz cell and placed on a clean cutting board for dissection. Using a single edge blade, one half of the tissue was excised and placed in 10% Normal Buffered Formalin (NBF) for 48 hours at room temperature for fixation. After fixation in 10% NBF, the section was transferred to 70% ethanol for storage until histology processing. The remaining half of explant was immediately placed into a pre-chilled Eppendorf tube and stored at -80°C for future use.

Histology

Briefly, tissue was dehydrated in increasing concentrations of ethanol to xylene, then embedded in paraffin blocks and sectioned to a thickness of 5 µm and mounted on glass slides, 2

sections per slide. N=3 donors, 4 replicates per donor per time point. The sections were then stained using haematoxylin and eosin (H&E) per suppliers' protocol.

Tissue integrity over the time course was observed by brightfield microscopy using an Olympus CKX53 microscope and 4X, 10X, 20X and 40X objectives. A scoring parameter (0-4) was assigned for five integrity observations; vacuoles/spongiosis, dermal/epidermal separation, necrosis, parakeratosis, and epithelial hyperplasia. A score of 0 designates no observation, 1 designates observation less than <20%, 2 designates observation between 20% and 40%, 3 designates observation between 40% and 60%, 4 designates observation of greater than 60% of overall surface area. Objective scoring was conducted by assigning observed ranges in two serial sections of tissue per sample and taking the average of the score. Scoring values were analysed using unpaired non-parametric two-tailed Mann Whitney t-test with GraphPad Prism software. Representative images taken using Thermo EVOS M5000 microscope and 20X and 40X objectives.

RNA Isolation and RT-qPCR

Human skin explant samples were stored in RNALater (Invitrogen AM7021) to allow permeation overnight at 4°C before Isolate RNA was used per Qiagen RNeasy Mini Kit (Qiagen 74106) instructions. Reverse transcription used a High Capacity cDNA kit (Applied Biosystems 4368814). 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. Fold change was calculated as ratio of the power of the gene of interest divided by the average power of the samples at Day 0. Statistical testing used GraphPad Prism v7 software and Tukey's multiple comparisons test.

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Conflicts of interest: none declared

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CHAPTER 3 : A new ex vivo skin model for mechanistic understanding of putative anti-inflammatory topical therapeutics

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

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Statement of contributions:

Jessica Neil was responsible for 95% of conception and design, 90% of data collection, 95% of data analysis and conclusions and 70% of manuscript preparation

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 areata, 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 characterised 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). Jardet et al (Jardet 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. 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-medited 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_2 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 and 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^2 permeable supports, 0.4 µm pore size. The well was then filled with modified Cornification media resulting in an air-liquid interface ex vivo skin culture. Media was changed no less than every 48 h with ex vivo skin culture maintained in a modified DMEM/Hams F12 Cornification media (Vostálová et al., 2018). The stimulation cocktails; Th1, Th2 or Th17, were 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 µM 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

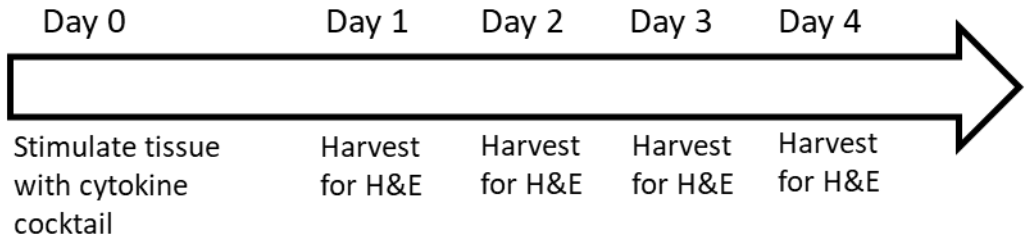
All statistics were analysed by GraphPad Prism v7 software using non-parametric two-tailed t-test with Mann-Whitney (95% confidence).

3. RESULTS AND DISCUSSION

3.1 Skin characterisation 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 signaling pathway. Skin was stimulated and then harvested at Day1, 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. However, Day 4 post-stimulation spongiosis becomes apparent (Figure 1). These results suggest an abbreviated window available for tissue analysis after inflammation induction, but still provides sufficient time to allow multiple dosing.

Treatment Schematic:



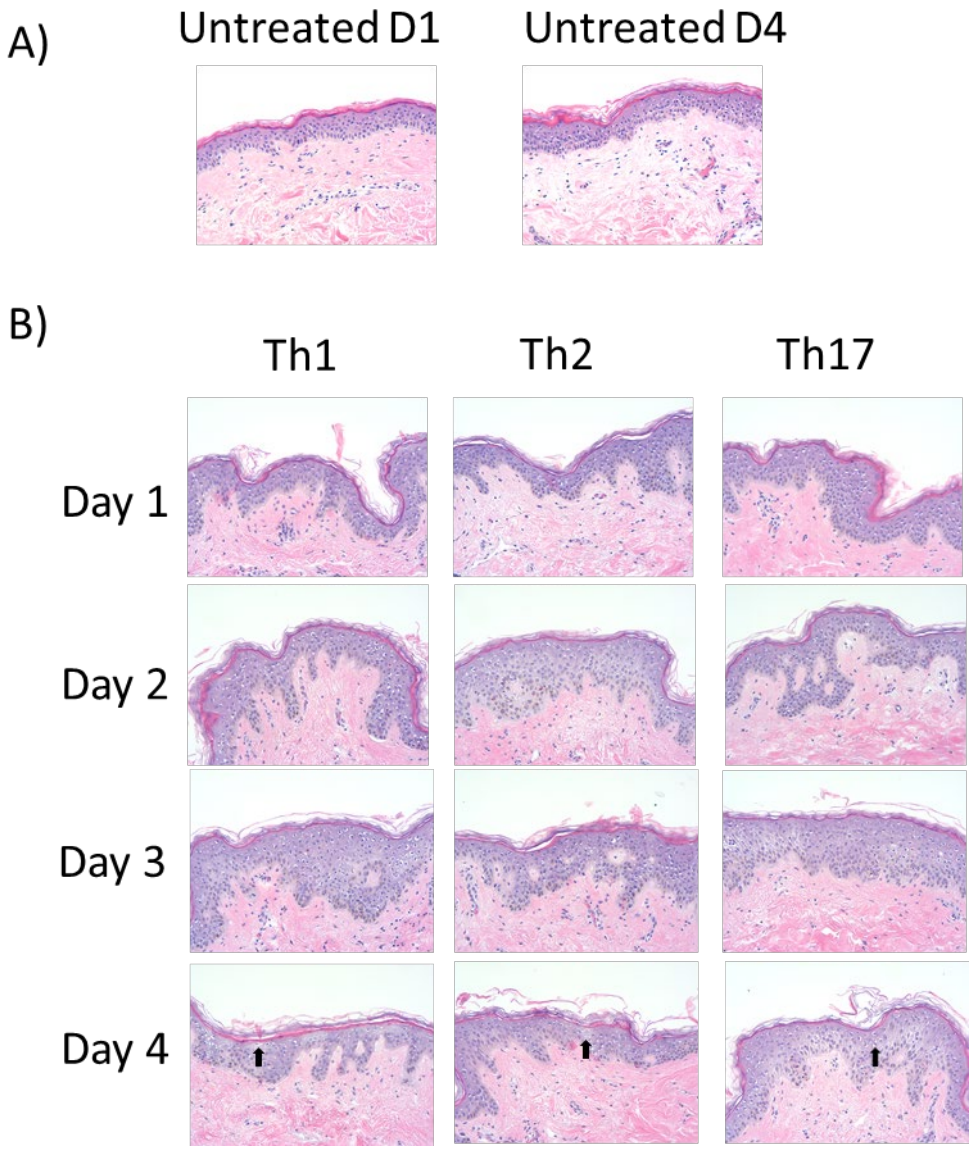


Figure 1. **Haematoxylin and eosin histology of tissue integrity over time following Th1, Th2, or Th17 stimulation.** *A)* Representative images from haematoxylin and eosin-stained untreated tissue at Day 1 (24 h post-stimulation) and Day 4 (96 h post-stimulation)**.** *B)* Representative images from 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 IL1a 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 IL17a and

IL22 were quantified and DefB4 and chemokine CCL20. IFNg 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 IL17a 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.

IFNg gene expression was evaluated to determine the cross-pathway stimulation of the three cytokine cocktails. Whilst IFNg 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.

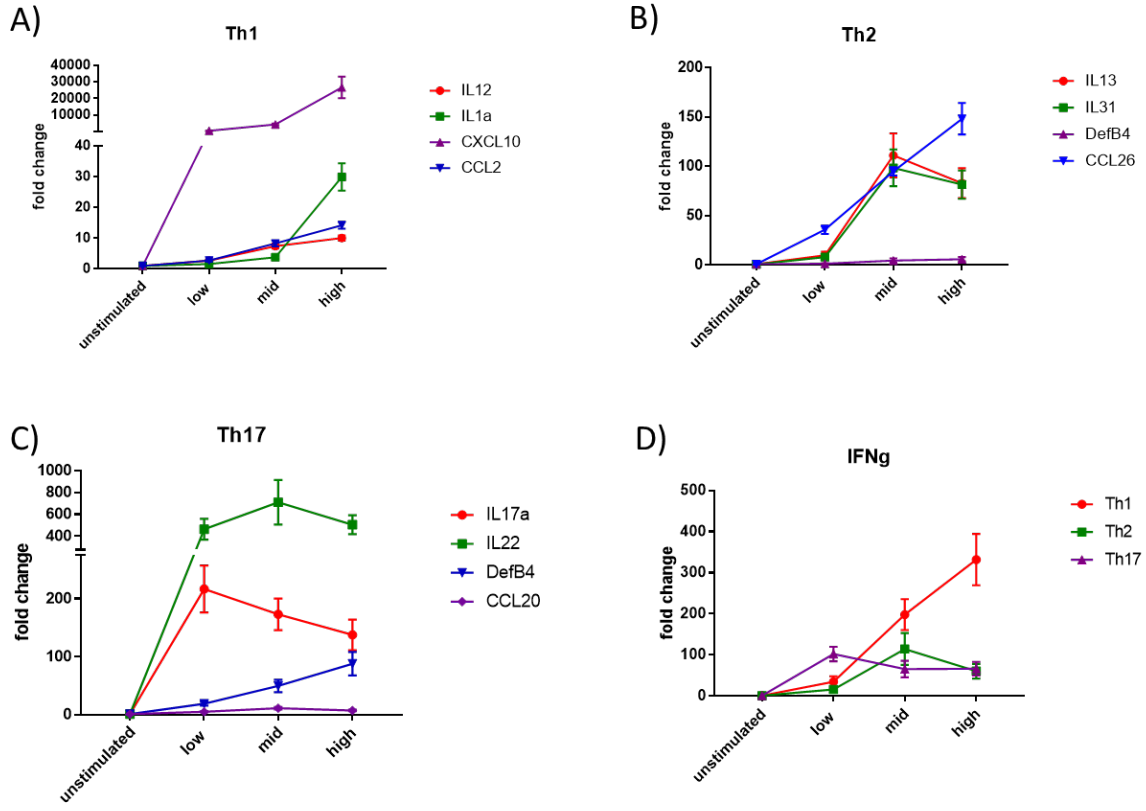


Figure 2. **Gene expression of the Th1, Th2, or Th17-associated inflammatory cytokines with low, mid, and high concentration cytokine cocktails.** Average fold change of three combined donors (4 replicates per donor) stimulated with low, mid and high dose Th1, Th2, or Th17 cytokine cocktails. Error bars 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) IFNg gene induction with Th1, Th2, or Th17 stimulation.

3.3 Biomarker characterisation of Th1, Th2 or Th17 stimulation

To further characterise the biomarker response to Th1, Th2 or Th17 stimulation, 24 genes were chosen to illustrate Th1and 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 IFNγ
- 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, TNFα
- Monocyte and neutrophil activation: IL19, IL10, LCN2 and DefB4.

These biomarkers do not work in isolation but coordinate across signaling 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, IL1ß, IL12 and IFNγ.

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 TNFα. 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 characterised 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 TNFa has long been associated with autoinflammatory diseases leading to the development of anti-TNF antibodies as injectable biological therapeutics. Upregulation of TNFa 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, TNFa 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 IFNg which achieved significance at 24 h.

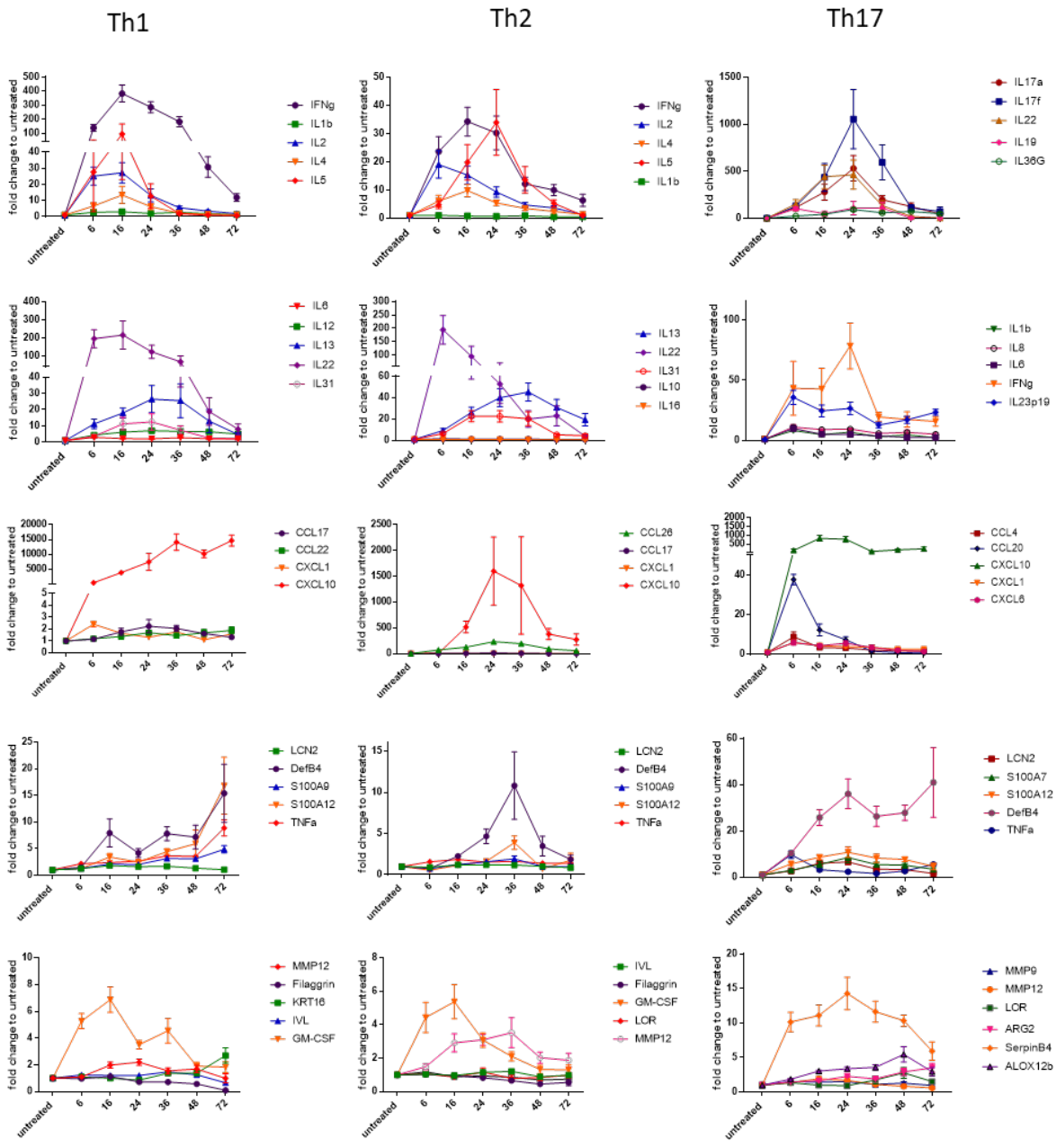


Figure 3**. Gene expression of inflammatory dermatoses biomarkers induced by Th1, Th2, or Th17 stimulation cocktails.** Average fold change of three combined donors; 4 replicates per donor per time point, stimulated by Th1, Th2, or Th17 cytokine cocktail over a 72 h time course. Error bars represented 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, IFNγ 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 IFNγ increased between 13 and 230 times that reported in the literature after Th1 stimulation.

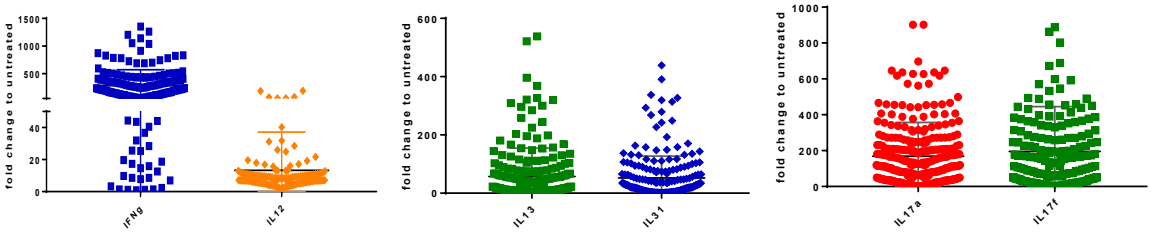
Th1		Neis	Rozenblit	Suárez-Fariñas	HESC
# of patients		33-56	17	18	35-44
IL12			2.04	2.71	13.4±21.4
IFNγ		22.01	1.26	1.39	289±253
Th2	Bianchi	Neis	Rozenblit	Suárez-Fariñas	HESC
# of patients	23	33-56	17	18	53-60
IL13		14.3	5.03	7.57	59.2±85.5
IL31	29.51±39.38	3.18			53.1±67.8

Th17		Sobolev	Russell	Suárez-Fariñas	HESC
# of patients		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

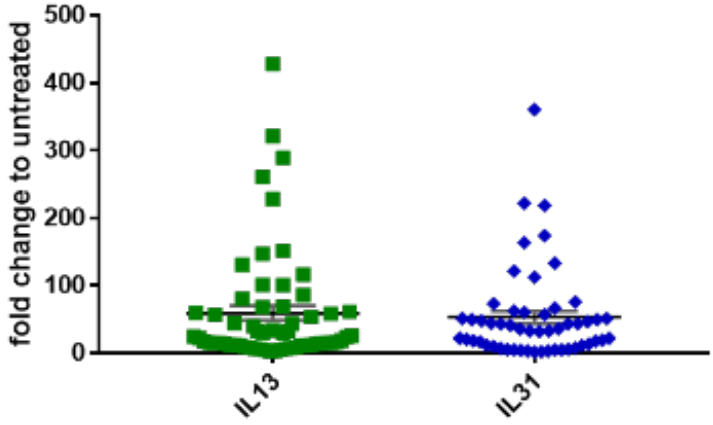
Table 1. Published results of the fold-change in gene expression for inflammatory markers from atopic dermatitis and psoriasis biopsies compared to non-involved skin and the average change in expression of the same markers using our Human Ex Vivo Skin Culture model

To further characterise the reproducibility of the human ex-vivo skin culture with exogenous stimulation, intra-and inter-donor response variability (Figures 4 and 5) was evaluated in 36-60 individual skin donors; as well as gene expression by age (Figure 6) and race (Figure 7). Post stimulation intra- and inter-donor variability was assessed by Th1, Th2 or Th17-induced skin samples. The Th1-associated cytokines IFNγ and IL12, Th2-associated IL13 and IL31, and Th-17-associated IL17a and IL17f were analysed by qRT-PCR for gene expression to determine the average fold change and standard deviation to be expected between donors. The intra-donor variability was characterised as the average standard deviation (STDEV) and average percent CV between individual replicates of a single donor. The percent coefficient of variation (%CV) was calculated to normalize the distribution of data points with differing means. All explants were supplemented with the modified Cornification media and stimulated with stimulation cocktail for 24 hours prior to harvest. Replicates range from four to six for each individual donor. Percent CV was calculated as (standard deviation/mean) * 100.

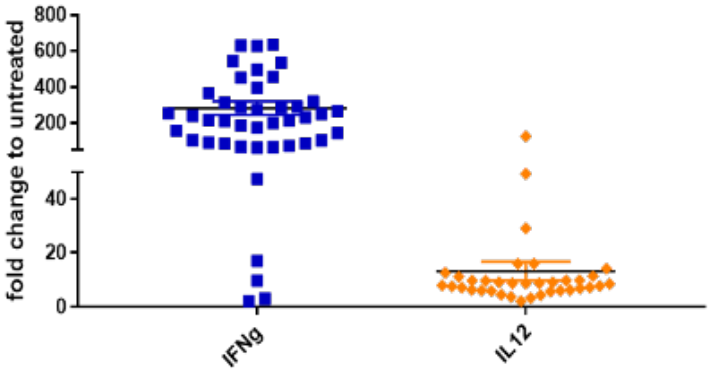


Biomarker	Number of donors	Avg STDEV of replicates (range)	%CV of replicates
IFNg	45	110.6 (1.08-460)	43.7
IL12	36	4.66 (0.28-73)	24.3
IL13	60	30.2 (0.99-302)	53.0
IL31	53	28.0 (1.4-116)	67.8
IL17a	49	80.8 (2.6-445)	62.0
IL17f	45	124.1 (3.2-701)	68.6

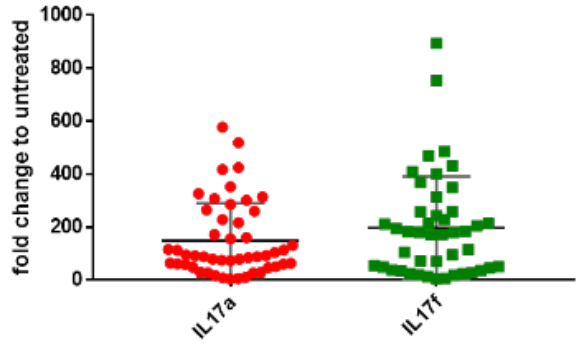
Figure 4. **Intra-donor gene expression fold change of individual samples among multiple donors with Th1, Th2, or Th17 stimulation**. Each point represents a single sample. Error bars represent mean with STDEV. The average standard deviation (STDEV) of single samples across multiple donors calculated 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.



Biomarker	Number of donors	Average FC of combined donors ± STDEV	% CV of combined donors
IL13	60	59.1 ± 85.5	144.6
IL31	53	53.1 ± 676.8	127.7



Biomarker	Number of donors	Average FC of combined donors ± STDEV	%CV of combined donors
IFNg	45	283 ± 254	89.7
IL12	36	13.3 ± 21.1	159



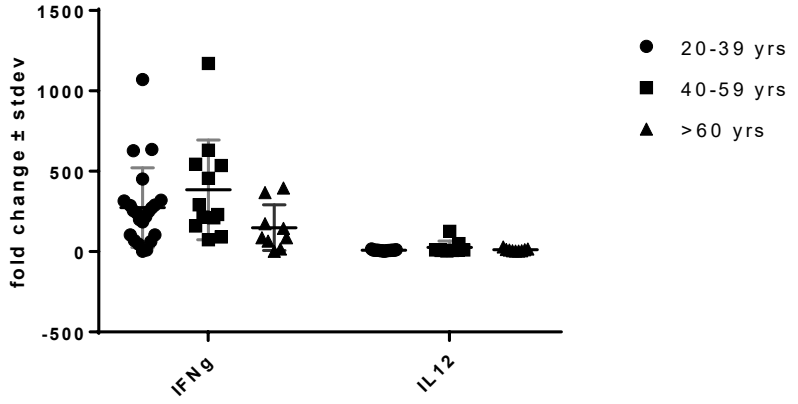
Biomarker	Number of donors	Average FC of combined donors ± STDEV	%CV of combined donors
IL17a	49	149 ± 140	94.3
IL17f	45	197 ± 194	98.4

Figure 5. **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 standard deviation of the data set.

Psoriasis can occur at any age, however it is most commonly diagnosed in a person's early twenties and persists throughout life. To evaluate the effect donor age has on immune response to the Th17 stimulation cocktail, samples were divided into age groups. A decrease in IL17a and IL17f gene expression with age is represented. While the incidence or severity of psoriasis does not increase over the age of 60, the literature does explain why a change in gene expression of these biomarkers may be expected. The most marked physical change observed in post-adolescent humans is thymic involution, described as the shrinking of the thymus with age.

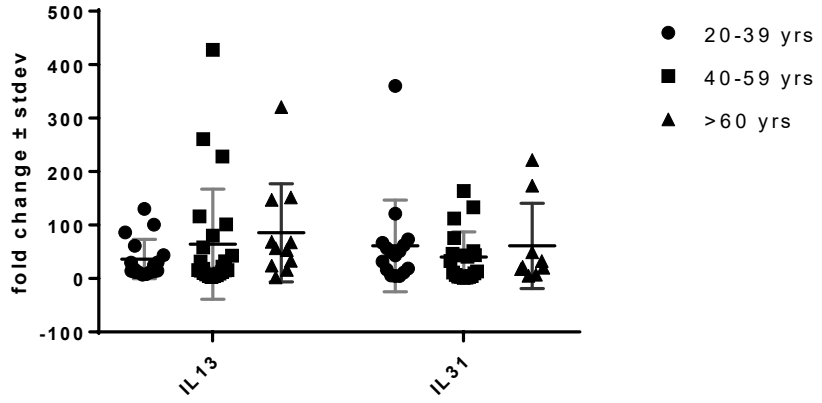
The thymic epithelial space can decrease from approximately 7 cm^3 in a young adult to about 1 cm^3 by 65 years of age. As such, the generation of naïve T cells is severely compromised by 50 to 60 years of age, especially evident in the CD8 T cell population (Goronzy et al, 2007). In contrast, a phenomenon termed 'inflamm-ageing' was coined to refer to the observed elevation of pro-inflammatory cytokines in adults over the age of 65 (Shaw et al, 2013). These contrasting immune changes with age result in a state of immunosenescence, affecting both innate immunity and, to a more profound level, adaptive immunity. The inherent loss of T cell stimulatory receptors such as CD28 and cytotoxic mediators like IL2 results in diminished capability for proliferation and antigen recognition (Arnold et al, 2011). There are some reports of an increase in IL10 cytokine production, leading to an anti-inflammatory environment further decreasing bacterial resistance (Fulop et al, 2005). Elevation of a similar anti-inflammatory co-receptor, CTLA4, can also explain the reduced ability of older immune cells to prompt a proper immune response (Pawelec et al, 2001). In addition, impairments in mitogen-activated protein kinases (MAPK) can result in decreased early inflammatory signal induction in the elderly (Whisler et al, 1996). It is suggested that this decrease in cellular signaling could be due in part to the loss of lipid raft fluidity with age, probably due to an increase in cholesterol in the plasma membrane. Loss of lipid raft fluidity can result in dysregulation of membrane signaling molecules, specifically TCR-specific signaling including CD3 and CD28 (Larbi et al, 2006). 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 Students t-test was observed with IFNg 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.

a)



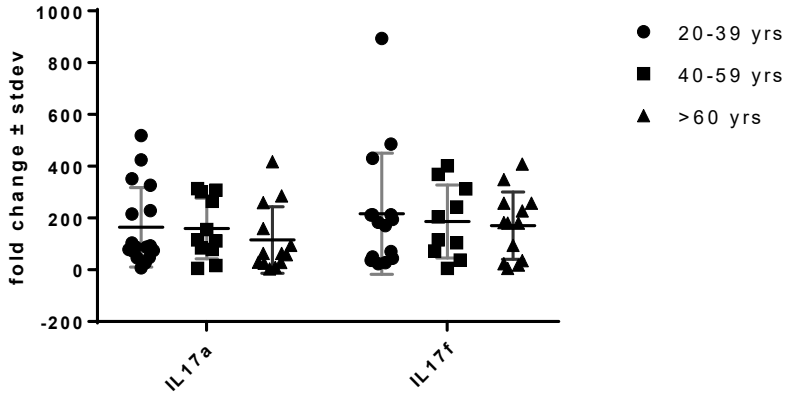
Biomarker	20-39 yrs	40-59 yrs	>60 yrs
IFNg	273.5 (n=22)	384.1 (n=12)	149.1 (n=9)
IL12	7.94 (n=17)	25.8 (n=9)	11.37 (n=9)

b)



Biomarker	20-39 yrs	40-59 yrs	>60 yrs
IL13	36.3 (n=17)	64.1 (n=24)	85.7 (n=11)
IL31	61.1 (n=16)	40.1 (n=20)	61.1 (n=9)

c)

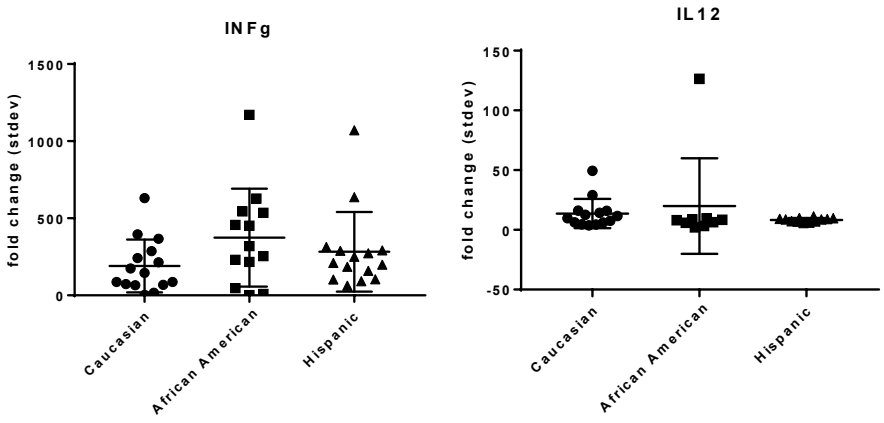


Biomarker	20-39 yrs	40-59 yrs	>60 yrs
IL17a	164.2 (n=17)	159.4 (n=11)	115.3 (n=13)
IL17f	216.4 (n=15)	186.5 (n=10)	170.8 (n=13)

Figure 6. **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).

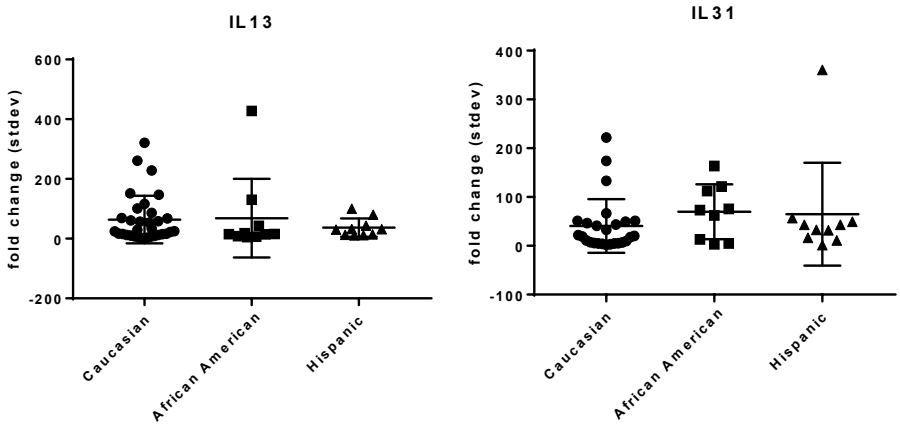
When evaluating ethnicity differences with Th17-mediated stimulation, an increase was seen in the immune response of African American tissue versus either Caucasian or Hispanic donors for both IL17aIL17a and IL17f gene expression. While not statistically significant in this data set with the exception of $p<0.0306$ by One-Way ANOVA for IL17f African American vs Hispanic, this is in contrast to published demographic literature that psoriasis is a Caucasian prevalent disease in the United States population. The 2009-2010 National Health and Nutrition Examination Survey (NHANES) estimated the prevalence of psoriasis in US adults to be 3.6% Caucasian, 1.9% African American and 1.6% Hispanic (Alexis and Blackcloud, 2014). This contrasting observation is most likely due to the limited sampling size (21 vs 9 vs 10 donors respectively for IL17a; 18 vs 9 vs 10 respectively for IL17f) available as well as the age disparity based on ethnicity. The mean age of the Caucasian sampling is 56.1 and 58.9 years for IL17a and IL17f respectively, while the mean age of the African American and Hispanic sample size is much lower; 41.7 and 37.5 years for both biomarkers respectively.

a)



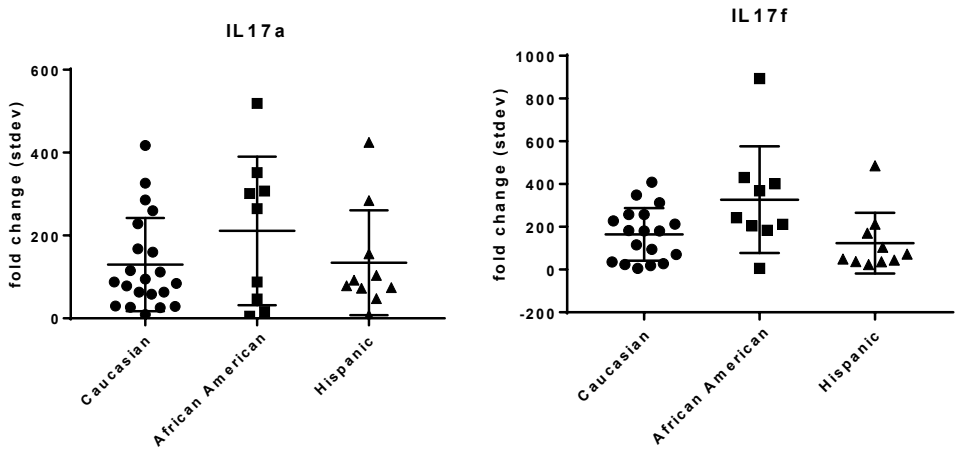
Biomarker	Caucasian ±stdev	African American ±stdev	Hispanic ±stdev
IFNg	190.5±172 (n=15)	374.5±317 (n=13)	282.7±258 (n=15)
IL12	13.6±12 (n=14)	19.9±40 (n=9)	8.30±2 (n=12)

b)



Biomarker	Caucasian ±stdev	African American ±stdev	Hispanic ±stdev
IL13	63.9±80 (n=32)	68.5±132 (n=10)	37.0±31 (n=10)
IL31	40.5±55 (n=26)	69.9±56 (n=9)	64.7±105 (n=10)

c)



Biomarker	Caucasian ±stdev	African American ±stdev	Hispanic ±stdev
IL17a	129.6±112 (n=21)	211.0±179 (n=9)	134.2±126 (n=10)
IL17f	164.4±123 (n=18)	362.9±250 (n=9)	123.6±142 (n=10)

Figure 6. **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.

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 (1μM) 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 signaling pathways and cytokine release (Paller et al., 2016). Janus kinases (JAKs) elevate proinflammatory cytokines via STAT transcription factor activation and nuclear factor ß expression. Tofacitinib, currently only available as an oral therapy but has been proposed for topical application, inhibits JAK signaling 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. IFNg, 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 7).

The steroid clobetasol propionate inhibited multiple signaling pathways including IFNg, 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 signaling 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 IFNg ($p=0.0029$), 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 IFNg, 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 IFNg, 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 IFNγ with Th1 stimulation can be explained by the positive feedback exhibited with IL12 induction. This activation induces the production of additional IFNg, IL12 and GM-CSF, suggesting a positive feedback loop indifferent to the downstream JAK inhibition (Becskei 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 IFNg 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).

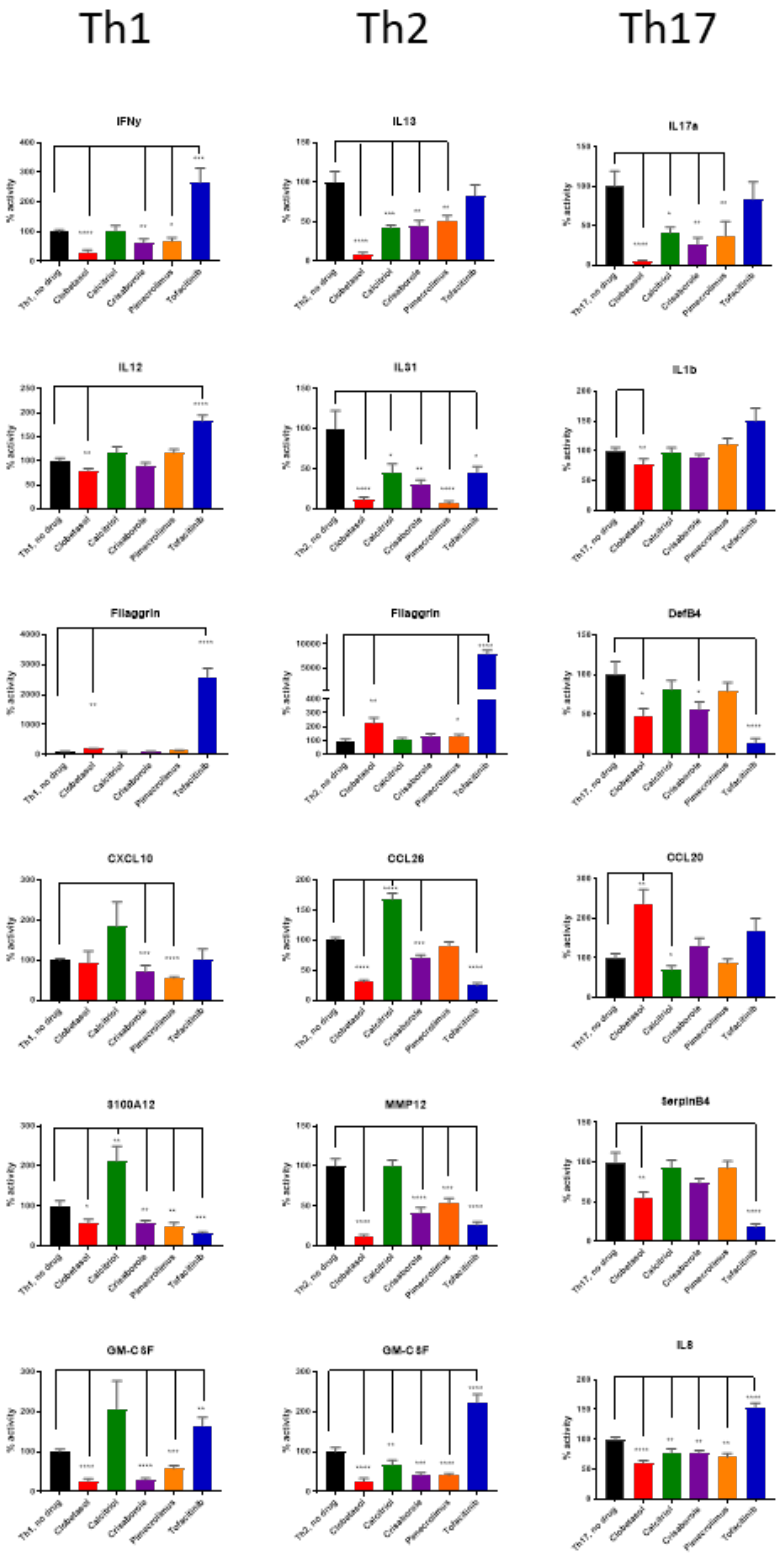


Figure 7. **Target specific inhibition of inflammatory gene expression with Th1, Th2 or Th17 stimulation.** Average percent activity of three donors; n=4 replicates per treatment per donor; stimulated with Th1, Th2, or Th17 cytokine cocktail and target specific inhibitors. Error bars represented as standard 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. 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.

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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.

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CHAPTER 4 : Accelerating topical formulation development for inflammatory dermatoses; a human skin ex vivo 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 areata 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 & 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 & 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 & Ley, 2002)(Zlotnik & 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 & 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 & 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 & 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 characterisation 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 & 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 & 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 (M. B. Brown & 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., 2020a). 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 & 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. Th1 stimulation cocktail 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.

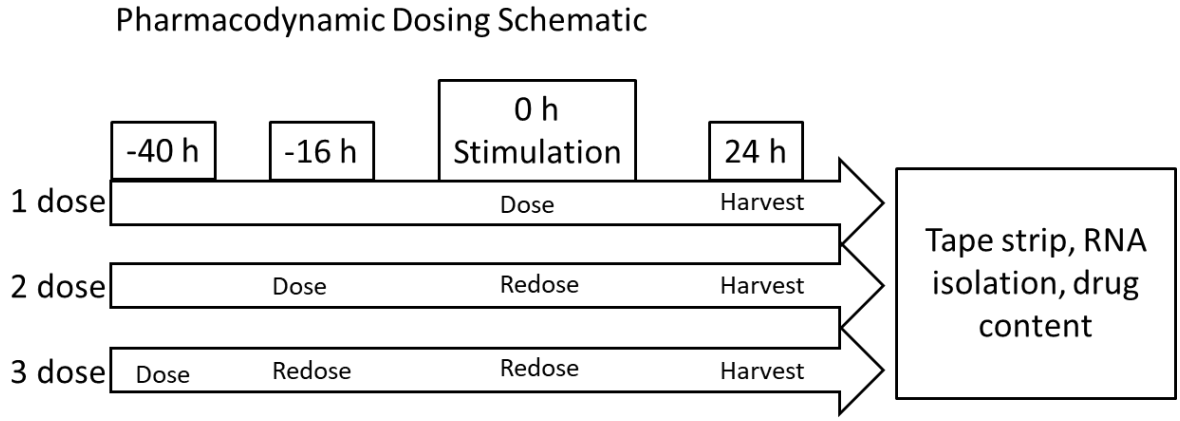

Figure 1. **Pharmacodynamic Dosing Schematic**. The dosing schedule for both active and vehicle formulations on human ex vivo skin culture. At time of stimulation; 1 dose; -16 h prior to

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 $\mu L/cm^2$ 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.

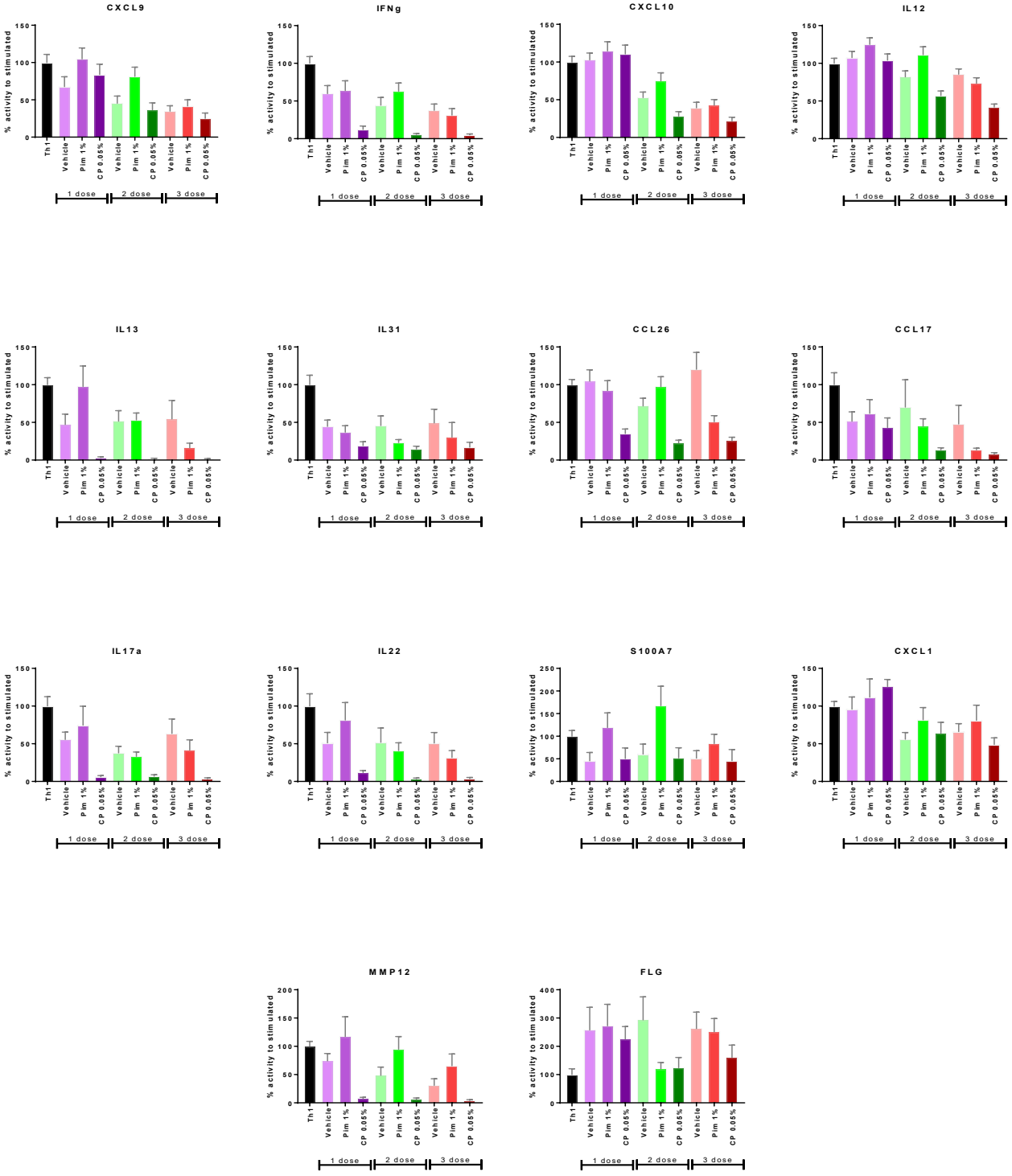

Figure 2. **Percent activity of gene expression with topical application of Elidel and Dermovate compared to Th1-mediated stimulation with one, two and three daily doses.** Average fold change of four combined donors stimulated by Th1 cytokine cocktail. N=4 samples per treatment per donor. Error bars 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 & 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 signaling (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.

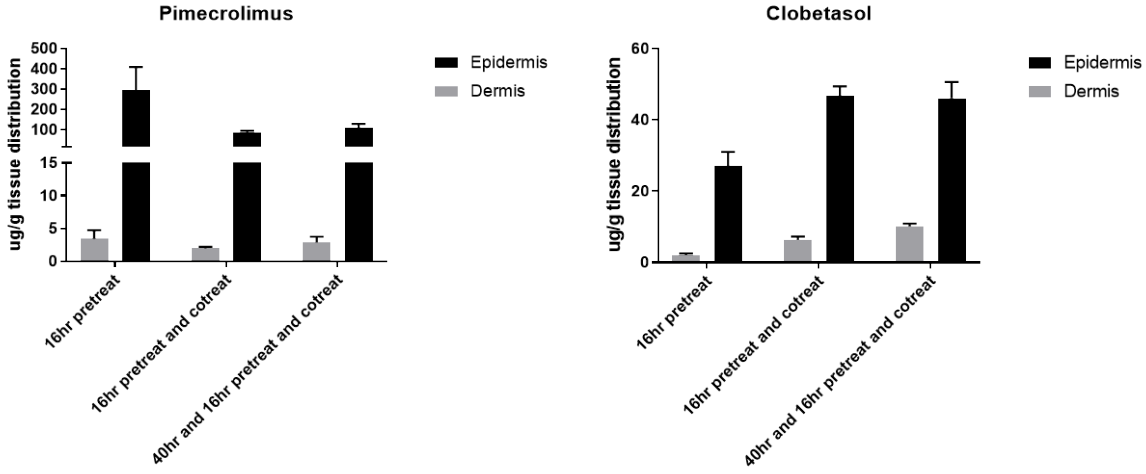

Figure 3. **Average tissue distribution of Elidel (Pimecrolimus) and Dermovate (Clobeatsol) (ug/g tissue) with topical application of one, two and three daily doses.** Tissue distribution

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.

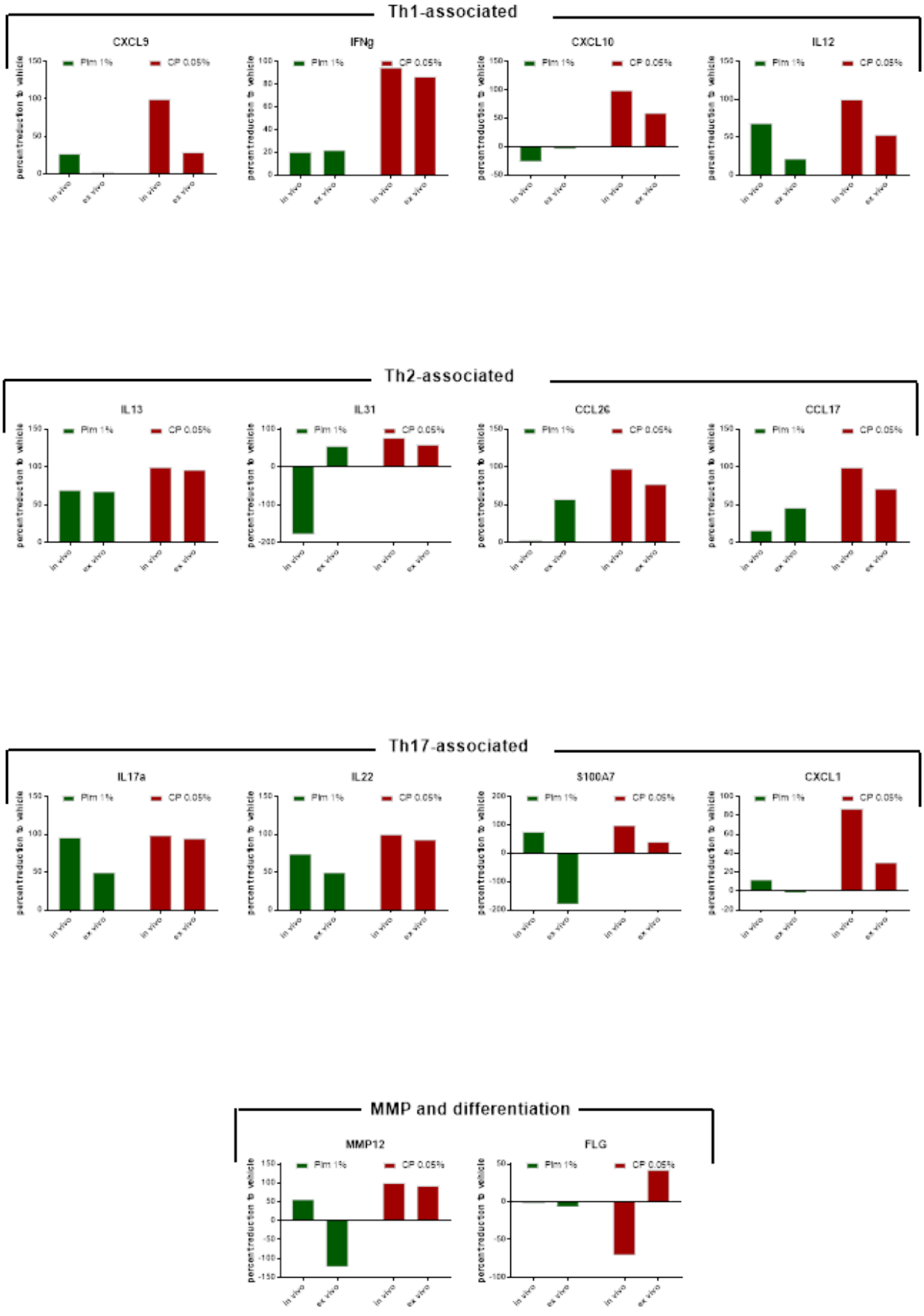

Figure 4. **Percent reduction in vivo and ex vivo in gene expression calculated from fold change with 3 daily doses of Elidel and Dermovate compared to Th1-mediated stimulation**. In Vivo (Guttman); n=30 donor clinical trial, single replicate; cream applied daily for 14 days.

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 characterisation 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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CHAPTER 5 : Discussion and future work

5.1 Review of prior model development

The evolution of tissue model development has spanned over five decades, with one of the first published human ex vivo skin models by Reaven et al in 1965 (Reaven & Cox, 1965), followed by Flaxman and Harper in 1975 (Flaxman & Harper, 1975). Since then, the evolution of science to better characterize and optimize these models has led to optimized nutrient supplements and conditions (Reaven & Cox, 1968), increased time in culture (Frade et al., 2015; Li et al., 1992), and the ability to induce inflammation to mimic disease states (Smith et al., 2016; Jardet et al., 2020). Considerations such as calcium concentration (Lee & Lee, 2018), air liquid interface (Zhou et al., 2018), and serum levels (Zeltinger & Holbrook, 1997) can determine tissue viability. All of these experimental paths have led to the human ex vivo skin (HESC) model that is gaining popularity. Current companies looking to optimize the HESC model for commercial use include Keratify and Ten-Bio Technologies in the United Kingdom.

Reconstructed in vitro cellular models have greater permeability coefficients in the absence of a fully developed stratum corneum and require exogenously introduced immune cells. Animal models often lack translation to human immunological responses or percutaneous absorption. In an attempt to capture the intricacy of the skin strata, the models have become incrementally more advanced. The earlier reconstructed human epithelium models (RHE) utilising differentiated keratinocytes proved beneficial for evaluating skin irritancy and corrosion to replace the rabbit Draize model of animal testing (Spielmann et al., 2007). The addition of cell types such as fibroblasts and melanocytes expanded the capabilities to test systemically applied drug products, however for novel topical therapeutics, this model lacks the barrier integrity to properly recapitulate drug penetration (Schmook et al., 2001). Often, in vitro models like those discussed herein, are too heavily relied upon to make translational decisions to human efficacy, leading to overconfidence in a drug products ability to be effective in a disease state. Human ex vivo skin includes not only the fully functional intact stratum corneum and epidermal layer, but also the dermis and all intrinsic cell types and lipids that make up the milieu of the tissue environment (Mathes et al., 2014). Thus, HESC can prove invaluable in early drug screening, formulation development, and in vitro penetration testing to save time and money in early drug development.

5.2 Current thesis research

To demonstrate the ability of the HESC model to adequately recapitulate the in vivo human skin state, viability and integrity of HESC by histology, trans-epithelial electrical

resistance (TEER), and cellular gene expression over time in culture were investigated in Chapter 2 of this study. With the characterisation of the HESC model under normal conditions showing sustainable tissue integrity and cellular viability up to and beyond 9 days, the next step was validating the model to capture donor demographic and variability in an inflammatory state. This characterisation involved histology over time in culture as well as gene expression of influential biomarkers in dermatological inflammatory states. Tissue integrity with inflammation was evaluated by histology, while optimization of the stimulatory cocktail was achieved by RT-qPCR of gene expression. Three different stimulation cocktails were utilized to elicit a Th1, Th2, and Th17-mediated inflammatory disease state. Subsequent gene expression evaluated T cell activation, chemokine activation, macrophage and dendritic cell activation, epidermal cell activation, and monocyte and neutrophil activation under the three stimulatory conditions. With the data presented in Chapter 3; demographic comparisons were made of age, race, and compared to published lesional data. Also intra and inter-donor variability was calculated to determine means of gene expression in the model. Armed with these factors, investigations were performed to target specific inhibition, enlisting known anti-inflammatory drugs targeting human dermatoses to measure anti-inflammatory activity. It was determined that Th1, Th2, and Th17-specific genes, for example IL-12, IL-13, and IL-17 respectively, can be upregulated with exogenous stimulation in the HESC, and subsequently modulated by known anti-inflammatory therapeutics including; clobetasol, calcitriol, crisaborole, pimecrolimus, and tofacitinib, targeting dermatoses such as atopic dermatitis and psoriasis consistent with published clinical biopsy data. Jardet et al., 2020 developed a similar model for the evaluation of a psoriatic disease state by injecting CD3 and CD28 antibodies directly into the plated tissue to prime resident T cells for activation, and then adding a stimulatory cytokine cocktail basolaterally to induce an inflammatory reaction. Jardet showed tissue viability was maintained up to 7 days, with a time-dependent induction of different gene expression, and subsequent tissue degeneration accelerated by inflammation. While Th17 specific genes were readily upregulated, including IL-17, IL-22 and S100A7, off target Th2 specific genes IL-4 and IL-13 were not significantly detected indicating pathway specificity. The Jardet model also showed target specific inhibition with the steroid betamethasone and a PDE4 inhibitor (Jardet et al., 2020). While this data further substantiates the conclusions drawn from the HESC model described in this thesis, that disease specific signaling can be achieved in an ex vivo tissue state and modulated by drug treatment similar to an in vivo scenario, it stops short of translation to a clinical outcome due to lack of direct correlation with clinical samples. With the HESC model characterized herein using exogenous inflammatory stimulation to elicit an inflammatory dermatoses disease state similar to a clinical scenario for the purpose of early therapeutic research and development, it was timely to

characterize the translational capability to clinical outcome. A publication by Guttman-Yassky (Guttman-Yassky et al., 2017), involving clinical biopsy data from dermatitis patients treated with two commercially available topical therapeutics, Elidel (pimecrolimus) and Dermovate (clobetasol) provided this opportunity, allowing direct translation of the HESC model to clinical data by comparing gene expression data readouts. The effect of topical dosing on tissue penetration and gene expression showed correlations such as tissue penetration profile and inflammatory gene expression reduction as well as limitations of the model compared to real-world observation, for example barrier gene expression such as Filaggrin where an inverse modulation was shown between in vivo and ex vivo treatment. An example of pharmacodynamic translation is when IFNg expression was reduced by 20% in vivo and 22% ex vivo in the HESC system by pimecrolimus and by 95% in vivo and 97% in HESC by clobetasol. With the evidence of translational ability between the HESC model developed here and clinical trial outcome in Chapter 4, the future implications for novel therapeutic investigation prior to human application is apparent. These include early drug screening for lead molecules based on efficacious target engagement in a complex tissue environment, formulation optimisation based on permeation and penetration, antibacterial properties of infected tissue, healing capabilities on damaged skin, off target effects of drugs to alternate signaling pathways, and bioequivalence or superiority based on delivery and disease-specific activity. Additional adaptations to the HESC model, including vasculature, neuronal innervation, and lymphatic cell infiltration, could broaden the therapeutic repertoire of ex vivo preclinical investigation.

5.3 Future work

5.3.1 Vasculature

The vascular network permeating the skin strata is composed of intercommunicating plexuses mainly within the dermal layer. This blood flow supplied by vessels and branching into capillaries supplies needed oxygen, nutrients, hormones, and circulating lymphatic cells, as well as controlling thermal stress via increased blood flow and vasodilation (Kolarsick et al., 2011). The earlier models of vascularization focused on skin-graft applications in burn victims and used seeded endothelial cells grown into full thickness skin equivalents on scaffolds (Abaci et al., 2016)(Groeber et al., 2016). Endothelial cells combined with keratinocytes and fibroblasts creates a 3D skin equivalent model capable of forming a functional vascular system with physiological perfusion (Matei et al., 2019). As angiogenesis is limited in excised human skin tissue in culture, the ability to use this scaffold approach on HESC to create an engineered vasculature for the study of vessel-born components both from the circulation to the tissue, and

from the surrounding tissue into the vessel, is an exciting avenue of research to overcome this model limitation. Overlay of scaffolded endothelial cell seeding in combination with angiogenesis-promoting growth factors in HESC will allow studies of infiltrating signaling molecules, hormones, systemically applied drugs, and circulating immune cells, as well as provide an avenue to explore drug clearance from the surrounding tissue; Figure 15a.

5.3.2 Nerve innervation

While nerve endings exist in excised human skin, the severed nerves provide limited input to affect signalling capabilities in vivo. The nervous system of the skin is a complex network controlling not only direct sensory stimuli from touch and temperature, but also contributing to pathophysiological functions such as inflammation, immune defence, wound healing and cellular proliferation. Conducive to dermatoses research, the recognition of allergens and microbiological agents by neurons result in an increase of regulatory neurotransmitters necessary for understanding the full disease state such as pruritis and pain. Sensory neurons in the skin, known as nociceptors, are activated in the presence of chronic inflammation or pruritus associated with dermatoses, and increased neuronal innervation has been observed in inflammatory skin diseases, wound repair, and skin cancer (Roosterman et al., 2006). Activation of neurons by inflammation leads to release of a potent neuropeptide, calcitonin gene-related peptide (CGRP), capable of inducing mast cell degranulation and acetylcholine recognized by receptors on T cells, macrophages, and dendritic cells. Immune cells within the skin also release molecules capable of crosstalk with the neuronal network. Macrophages are capable of secreting neurotrophin growth factors to facilitate increased innervation and neuronal survival. While neuronal CGRP can induce keratinocyte proliferation associated with plaque psoriasis, keratinocytes can also release nerve growth factor facilitating innervation (Blake et al., 2019). The ability to utilize neuronal stimuli in HESC is limited by the abbreviated time in culture resulting in lack of angiogenesis and is further complicated by suitable detection methods in the complex tissue model. As such, the focus of a published ex vivo study has been with reconstructed human skin models employing human induced neuronal stem cells supplemented with NGF to facilitate growth. While these models provide a multicellular approach to study the neurological impact on skin physiology, they lack the fully stratified epidermis of in vivo human skin for barrier function (Vidal Yucha et al., 2019). Also, the use of these induced neuronal stem cells do not produce itch-specific sensory neurons, indispensable in the study of pruritic dermatoses. Following the research of Vidal Yucha et al., 2019, the use of induced pluripotent stem cells has been shown to be differentiated into sensory neurons which do respond to itch-specific stimuli under well controlled defined media, that was further facilitated by keratinocyte

coculture, and observed to innervate into a multicellular 3D reconstructed skin model (Guo et al., 2022). While this level of neuronal modelling in reconstructed human skin opens many doors to in vitro study of skin neuronal function, it still lacks the full environment of cell types and physiology contained in ex vivo tissue. Combining this progressive understanding in skin neuronal culture with the HESC model developed in this study could result in an advanced human skin model capable of utilizing induced pluripotent stem cells in combination with ex vivo skin to create a functional innervated model for the study of inflammation induced neuronal signaling and pruritic disease. One hypothetical design includes seeding pluripotent stem cells onto a permeable membrane with defined media to support neuronal differentiation, then layering on top with HESC to encourage innervation into the excised tissue similar to the keratinocyte coculture model; Figure 15b.

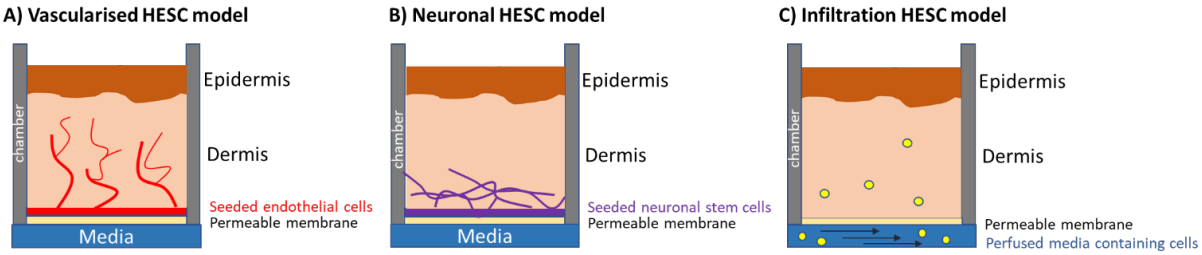
5.3.3 Lymphatic cell infiltration

Lymphatic cell infiltration is removed once the skin is excised from the body for ex vivo culture. These circulating immune cells are known to comprise T cells expressing cutaneous lymphocyte-associated antigen (CLA) enabling migration into the skin, that when activated by either autoimmune recognition or exogenous stimuli, may become over-excited resulting in chronic inflammatory dermatoses such as dermatitis (Czarnowicki et al., 2017). The lack of these circulating T cells means the HESC model is not a complete depiction of the disease state. A relatively new model has been developed called organs-on-chips, that incorporates cocultured cell types in perfusion chambers to better recapitulate vascular physiology (Bhatia & Ingber, 2014). Using this technology, skin-on-a-chip has been explored using human umbilical vein endothelial cells to create the epidermal layer and activated T cells introduced via perfusion. It was shown that T cell migration into the endothelial cell layer could be controlled by chemokine gradients (Ren et al., 2021). Expanding on that concept, Kwak, et al. engineered a bi-layered skin model including both epidermis and dermis on top and an endothelial layer on the bottom separated by a porous membrane to allow for the perfusion of neutrophil-like cells through the tissue to recapitulate leukocyte migration. This design showed the cells would migrate from the fluidic channel into the tissue upon ultraviolet insult (Kwak et al., 2020). The combination of the micro perfusion devise with an HESC model could allow for the introduction of known circulating immune cells into an ex vivo tissue to more closely mimic the disease state, thus compensating for the lack of lymphatic cell infiltrate experienced in vivo. By placing the dermal side of the HESC onto the porous membrane, perfusing circulating immune cells through the membrane, and introducing a chemokine specific media; immune cells may be prompted to migrate from the membrane to the dermal tissue, recapitulating the cell infiltration expected in

vivo; Figure 15c. By monitoring cellular infiltration with immunohistochemistry, an optimized time and defined media paradigm may be established. Once characterized, introduced T cell and B cell populations from the perfusion media may be evaluated for the effect on surrounding inflammatory state in concert with resident immune cells, and the effectiveness of therapeutics on the induced inflammation from either environmentally induced dermatoses or autoimmune dysregulation.

Introduction of these adaptations to the HESC model will allow for the preclinical investigation of systemically supplied drugs and vascular-associated dermatoses presenting with erythema such as rosacea (Figure 15a), pain and itch presenting dermatoses such as dermatitis (Figure 15b), and effect of lymphatic infiltrating immune cells and drug clearance on topical formulation application (Figure 15c).

Figure 15. **Proposed adaptations of the HESC model** (MedPharm Ltd.)



The HESC model developed and described herein is currently being employed in preclinical pharmaceutical development at MedPharm, Ltd to provide risk mitigation for novel topical drugs and formulations presented by emerging and established pharmaceutical companies. The data obtained provides critical information that provides context in MedPharm's clients regulatory filings for clinical trial applications and marketing authorisations by quantifying the pharmacokinetic actions of the drug in formulation in an ex vivo skin culture as well as the pharmacodynamic capabilities of the drug to engage and modulate cellular gene and protein expression. Focus areas currently being contracted include atopic dermatitis and psoriasis, evaluating anti-inflammatory drugs such as steroids, small peptides, JAK inhibitors, PDE4 inhibitors, and calcineurin inhibitors.

5.3.4 Model optimisation

The future of this HESC model is to fully explore model optimization, biomarker readout processes and profiles, and different dermatoses to create an ex vivo model that fully recapitulates the disease state. With this model we show tissue integrity and cellular metabolic activity beyond 7 days, but deteriorating after 9 days. While Frade et al (Frade et al., 2015)

reported viable ex vivo tissue out to 75 days in culture, the histology shows thinning of epidermal layer after Day 7, and keratinocyte proliferation makers at Day 75 with no controls. No additional representation of skin integrity or viability was presented. It is possible with exploration of different culture methods including mechanical stretching, optimized media, or perfusion that the viable time in culture of the HESC could be extended beyond the 7-9 days developed here by customizing the nutrient content of the media and rate of media exchange. This additional time in culture could allow for further evaluation of treatments and initiation of pathological change with stimulation unseen in the current timeframe. The degeneration of the tissue after 3 days of inflammatory stimulation could also be mitigated by further optimization of the stimulation cocktail to introduce lower levels of inflammatory mediators which deteriorate skin longevity, thus extending time in culture to allow for therapeutic investigation.

Method optimization beyond media exploration will determine the many additional opportunities afforded by the HESC model. Assessing the optimal time from tissue excision to stimulation to best recapitulate the in vivo inflammatory response, as well as expanding the time-dependent biomarker gene expression better delineates the experimental design to ensure reproducibility and translation capability. Readouts to include protein secretion as well as gene expression further clarifies the model, as gene transcription does not always equate to protein secretion due to translation constraints, nor does it accurately determine the lag time between initial gene expression and protein secretion in the tissue. Prostaglandin levels are known to increase with several inflammatory dermatoses (Chan et al., 1993), and quantification of these levels would both validate the disease model compared to in vivo scenarios and provide additional therapeutic investigation with drugs targeting the PGE pathway (Pustisek & Lipozencić, 2001). Specifically in atopic dermatitis, both prostaglandin D2 (PGD2) and prostaglandin E2 (PGE2) are present at high concentrations in the skin, created by the enzymatic cleavage of phospholipids by cytosolic phospholipase A2 (CPLA2) into arachidonic acid that is further metabolized into prostaglandins (Yanes & Mosser-Goldfarb, 2018). The metabolizing component of prostaglandin synthesis is cyclooxygenase (COX), thus by targeting the activity of COX can directly impact the level of prostaglandin produced in inflamed tissue, as demonstrated by the use of non-steroidal anti-inflammatory drugs (NSAIDs), to reduce pain, redness, and swelling (Ricciotti & FitzGerald, 2011). While direct evidence of pain, redness, and swelling cannot be observed in the HESC model, the ability to create increased prostaglandin secretion with stimulation and the resulting decrease with treatment is an indirect quantification of the pathological outcome. Inducible nitric oxide is another inflammatory mediator that serves a beneficial signalling function at normal levels such as wound healing and microbial protection, but when increased can lead to tissue damage and autoimmune disease (Sharma et al., 2007).

Nitric oxide expression is upregulated with stress such as ultraviolet B (UVB) irradiation and cytokine secretion. The most obvious use of nitric oxide production in ex vivo therapeutic evaluation is for UVB protection from skin damage and erythema caused by NO release, as well as melanogenesis resulting from UV exposure (Cals-Grierson & Ormerod, 2004). This avenue of experimentation can be achieved by solar simulation of the HESC culture to quantitate nitric oxide release, cytokine production, and levels of melanin in the tissue.

In addition to protein secretion and reactive oxygen species generation, exploration beyond prophylactic treatment of the HESC will better mimic clinical settings. In the characterisation of the HESC presented here, treatments were applied prior to stimulation of the tissue with the stimulatory cocktails. While this approach is designed to allow for penetration of the drug for efficacy due to abbreviated time in culture, it is not an accurate portrayal of a clinical use. Further studies involving cytokine cocktail strength and effect on tissue integrity to elongate time in culture after stimulation would allow for treatment post-stimulation to better compare to clinical outcome. The HESC model could also be used to explore drug efficacy over time in skin to determine treatment paradigms. Certain topical therapeutics are susceptible to enzymatic degradation. Common enzymes in the skin that can cause drug degradation into less active degradants include elastase, monooxygenase, cyclooxygenase, reductase, hydrolase, esterase, and transferase (Oesch et al., 2007). To research drug viability in skin, wash-out assays can be performed to determine drug efficacy over time. To achieve this, drug can be applied to the tissue at zero time point, followed by increasing increments of time before stimulation and quantitation to determine how long the drug retains anti-inflammatory activity after application.

5.3.5 Dry skin

Beyond method optimization, multiple exciting avenues could be explored with the modifications of the HESC model environment. Dry skin is a common grievance and a large cosmetic market trademark. Over 20 billion dollars are spent in the United States market alone for skin care products, with over 40% of that revenue being creams and moisturizers (Grand View Research, 2021). While recreating dry skin may be a challenge, control of the humidity of the incubators used for culture, as well as the addition of desiccants to the skin surface and use of transepithelial water loss quantitation could provide a HESC dry skin model capable of experimentally testing the outcome of these cosmetics prior to release.

5.3.6 Wound healing

Wound healing, specifically from burns, has only been extensively studied in animal models. Historically, the comb burn model, employed by heating a rectangular brass bar with

transverse notches in boiling water and applied to the skin (Regas & Ehrlich, 1992), has been used the majority of the time in both murine and porcine investigation (Salibian et al., 2016). While rats provide a controlled test subject group for wound healing, pigs are more closely related to humans in skin similarities. Even so, ethics against the use of animals in research and the high costs make the pig model less than ideal (Liu et al., 2021). The HESC model could be used to investigate inflammation, re-epithelialization, fibroblast migration and collagen deposition using a similar burn method and a combination of gene expression, protein secretion and histological analysis of the tissue.

5.3.7 Skin infection

Skin infection is a common factor in topical therapeutics, whether from wounding or autoimmune disruption such as dermatitis, and accounts for over $7 billion dollars in treatment globally (PPE News Hubb, 2022). Skin infection models have long used pigs (Rubinchik & Pasetka, 2010), however given the previously stated differences in animal and human tissue, an ex vivo skin model would facilitate the understanding of human infection. Prior assays utilizing reconstructed epidermis consisting of solely keratinocytes have been used to evaluate *Staphylococcus aureus and Escherichia coli* infection on the skin to quantify epidermal cytotoxicity and inflammatory response (Kohda et al., 2021)(Jahanshahi et al., 2020). While these models are helpful in understanding the pathogenesis of different dermal infections, they lack the complexity of the HESC in cell type, tissue structure, and signalling crosstalk that is present in human skin. By using the HESC model to investigate infection, scientists can not only explore all of these facets but combine that knowledge with therapeutic delivery to better understand drug effect on the microorganism (Brown & Williams, 2019). In combination with wounding, exploration of the effectiveness of microorganism colonization in the wound as well as effect on healing efficacy could contribute valuable information to understanding opportunistic infections, diabetic lesions, burn sepsis, and atopic dermatitis colonization.

5.4 Bioequivalence

Bioequivalence of a formulation is defined by the FDA as showing it can deliver the same amount of the same drug to the intended site of therapeutic action at the same rate as the already approved product (U.S. Food and Drug Administration, 2017). These studies can be performed in the laboratory in the absence of clinical studies, saving generic drug development companies millions of dollars on clinical trials. This method is well established for oral medications, relying on the pharmacokinetic evaluation of blood levels, however topical dermal formulations pose difficulties in sampling of the skin tissue. Vasoconstriction has been evaluated

as a bioequivalence readout in the skin, however this is specific to glucocorticoid steroids. The other option is a costly and time-consuming clinical population study. In an effort to circumvent these clinical studies, researchers are eager for an in vitro or ex vivo approach to better justify bioequivalence for generic or hybrid applications, especially when the ability to produce a generic is limited by the poor quality of the reference product or when excipients within it are no longer available or considered unsafe. Pharmaceutical development of topicals and transdermals, though meticulously planned and scrutinized, is fraught with pitfalls. Dermatological development accounts for approximately $50 million dollars in clinical trial expense alone. This expense is heavily dependent upon the later stage clinical trial aspect depending on number of patients needed to establish treatment effects and number of pivotal trials required to support marketing approval (Moore et al., 2020).

5.5 Conclusion

Through thorough characterisation of the HESC model including tissue integrity, viability, cellular activity, inflammatory stimulation, demographic variability, and topical therapeutic action, a comprehensive road map has been established regarding the capabilities of the model as a preclinical ex vivo drug development tool. The scientific process has also unveiled exciting new avenues of model optimisation and modification to fit multiple dermatoses that has yet to be explored. Overall, 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 characterisation and comparison of novel drugs and formulations with those already marketed has the potential of de-risking costly and time-consuming clinical trials.

CHAPTER 6 : References

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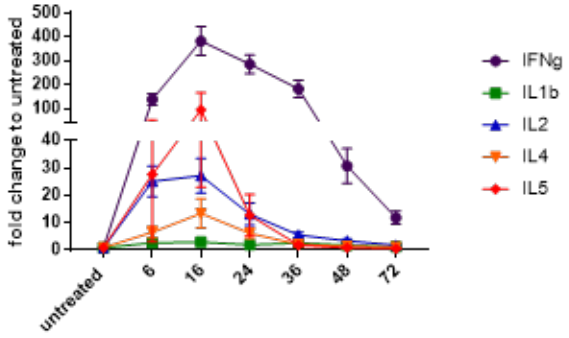
CHAPTER 6 : Appendix

Chapter 3. 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).

Th1


fold change to untreated
500
400
300
200
100
40
30
20
10
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untreated
6
16
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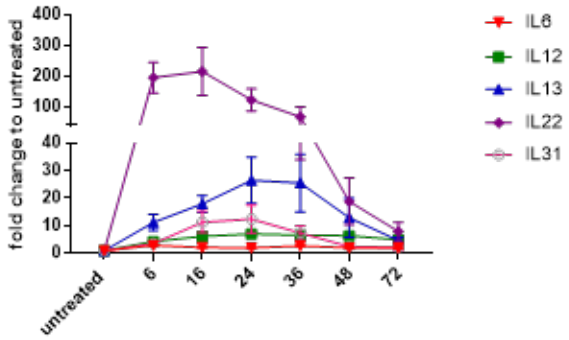
36
48
72
IFNg
IL1b
IL2
IL4
IL5


fold change to untreated
400
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IL6
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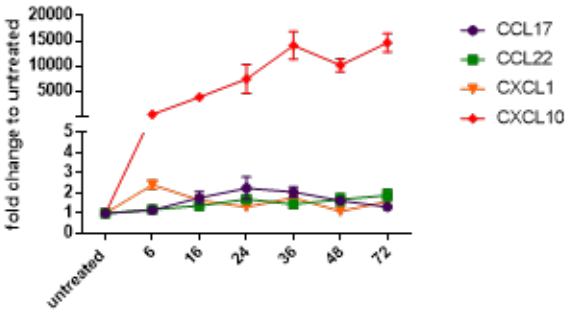
IL13
IL22
IL31


fold change to untreated
20000
15000
10000
5000
5
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1
0
untreated
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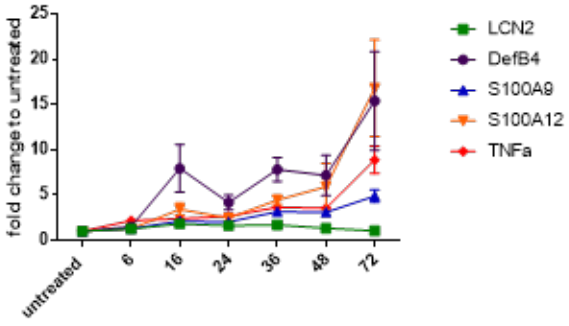
36
48
72
CCL17
CCL22
CXCL1
CXCL10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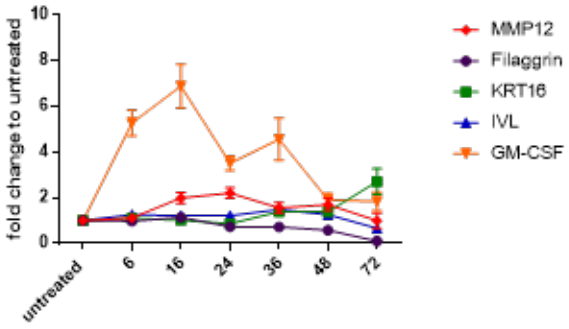
S100A12
TNFa


fold change to untreated
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16
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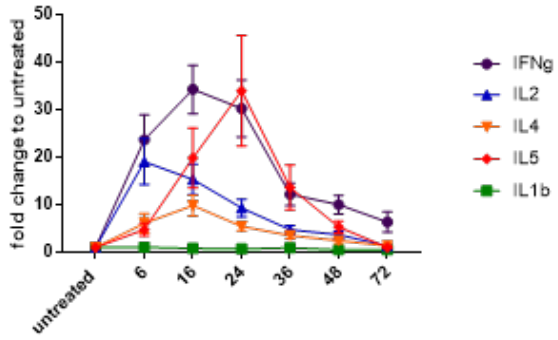
72
MMP12
Filaggrin
KRT16
IVL
GM-CSF

Th2


fold change to untreated
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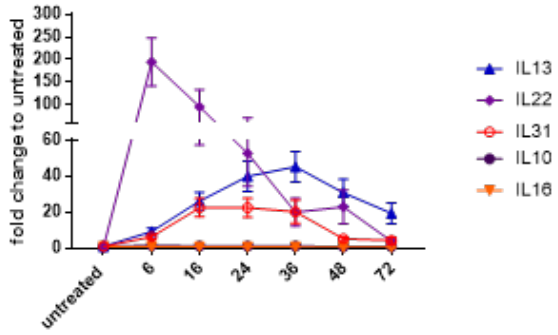
fold change to untreated
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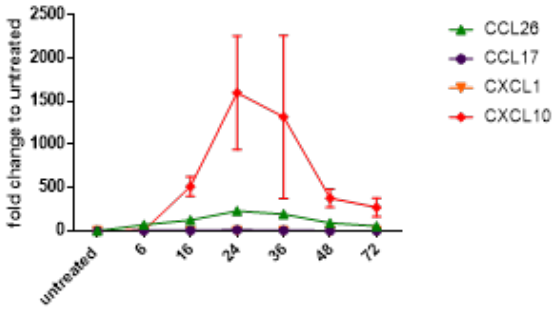
20
0
untreated
6
16
24
36
48
72
IL13
IL22
IL31
IL10
IL16


fold change to untreated
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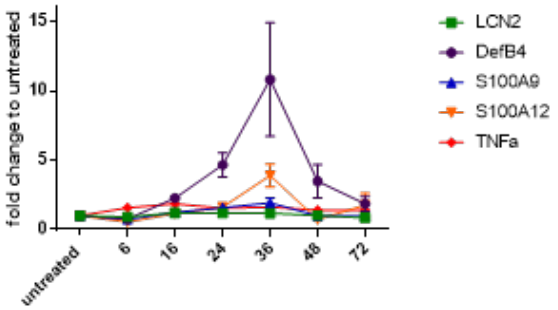
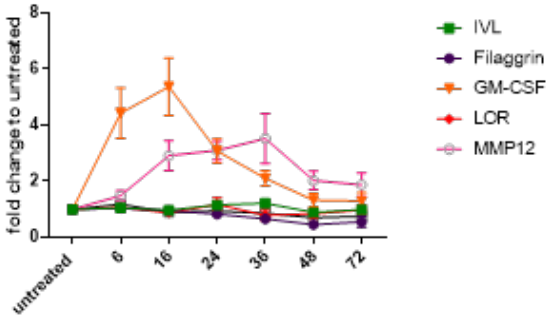
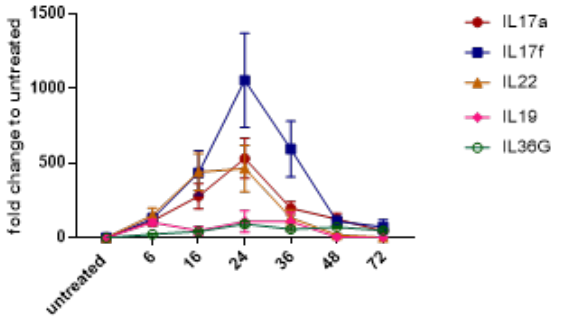
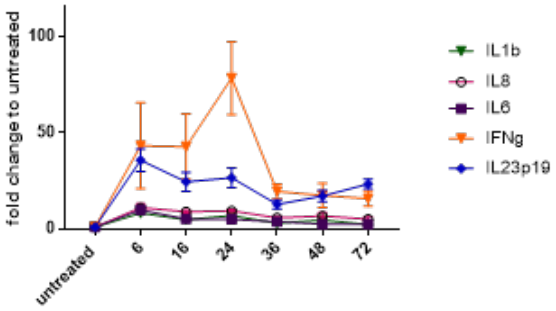
2000
1500
1000
500
0
untreated
6
16
24
36
48
72
CCL26
CCL17
CXCL1
CXCL10

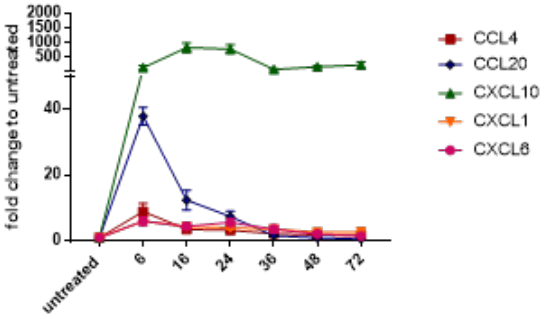

fold change to untreated
15
10
5
0
untreated
6
16
24
36
48
72
LCN2
DefB4
S100A9
S100A12
TNFa

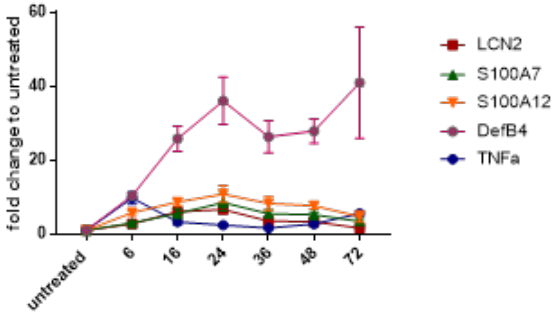

fold change to untreated
8
6
4
2
0
untreated
6
16
24
36
48
72
IVL
Filaggrin
GM-CSF
LCR
MMP12

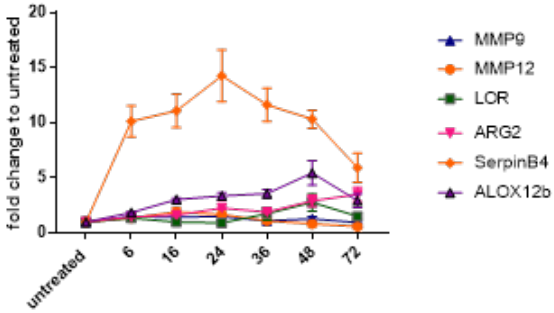
Th17


fold change to untreated
1500
1000
500
0
untreated
6
16
24
36
48
72
IL17a
IL17f
IL22
IL19
IL36G


fold change to untreated
100
50
0
untreated
6
16
24
36
48
72
IL1b
IL8
IL6
IFNg
IL23p19

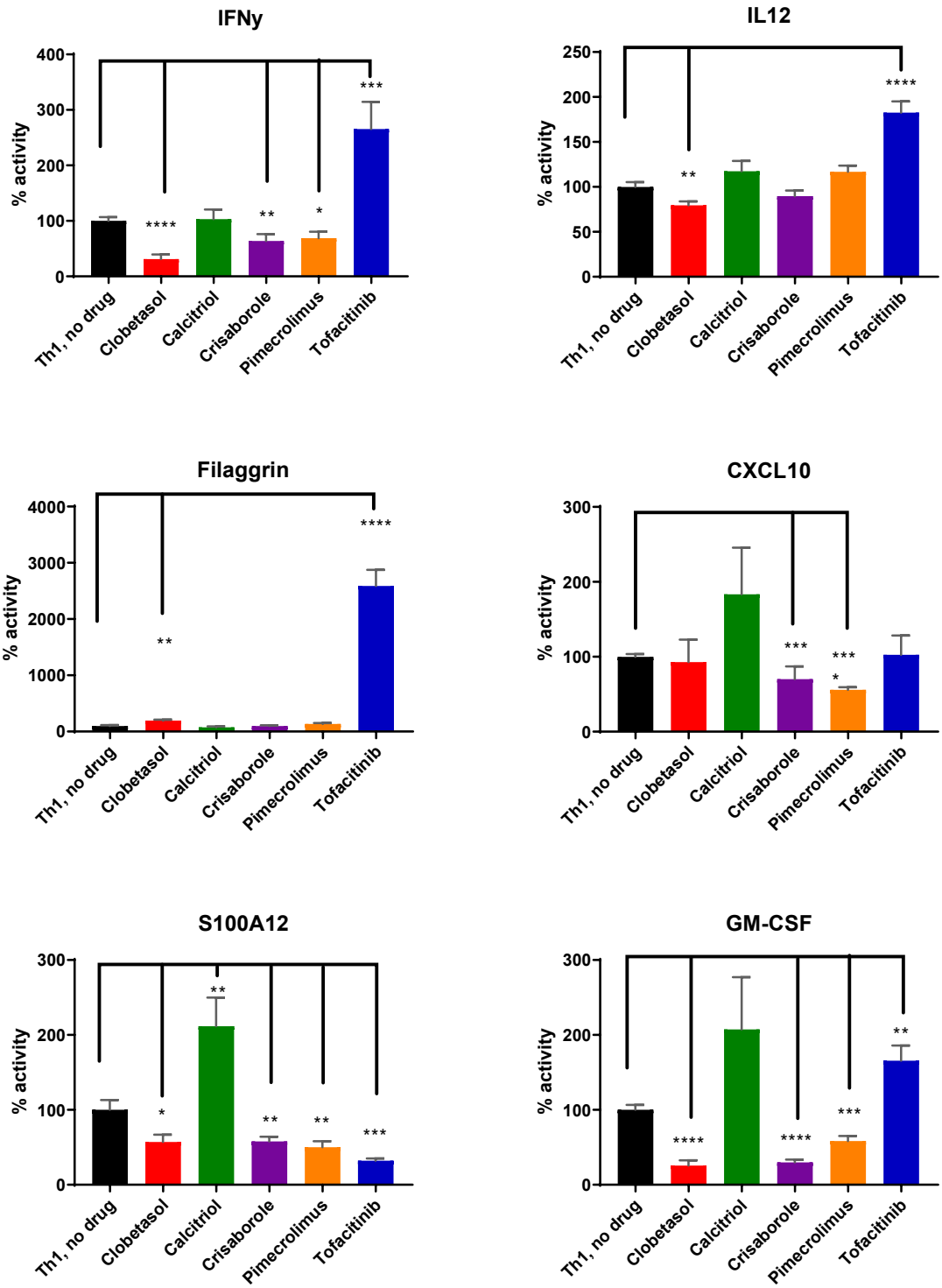

fold change to untreated
2000
1500
1000
500
40
20
0
untreated
6
16
24
36
48
72
CCL4
CCL20
CXCL10
CXCL1
CXCL8


fold change to untreated
60
40
20
0
untreated
6
16
24
36
48
72
LCN2
S100A7
S100A12
DefB4
TNFa

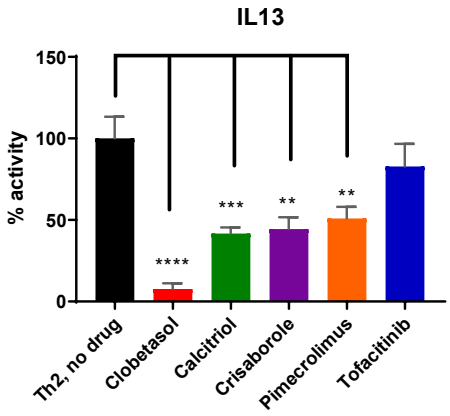

fold change to untreated
20
15
10
5
0
untreated
6
16
24
36
48
72
MMP9
MMP12
LCR
ARG2
SerpinB4
ALOX12b

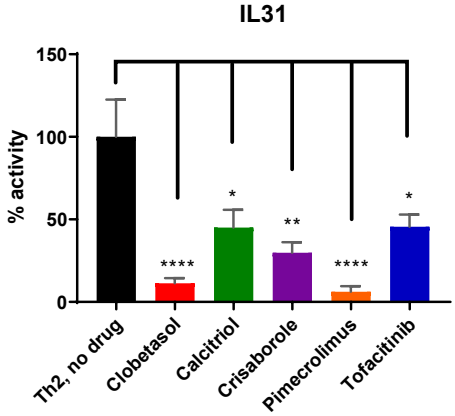
Chapter 3. Figure 7. **Target specific inhibition of inflammatory gene expression with Th1, Th2 or Th17 stimulation.** Average percent activity of three donors; n=4 replicates per treatment per donor; stimulated with Th1, Th2, or Th17 cytokine cocktail and target specific inhibitors. Error bars represented 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.

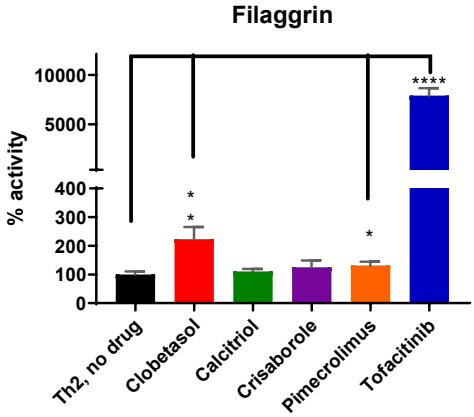
Th1

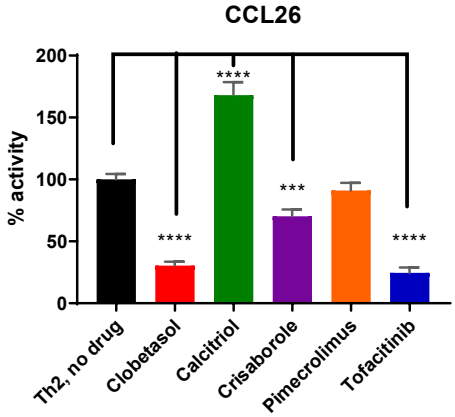


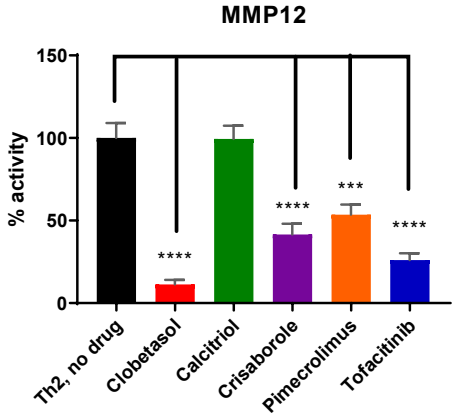
Th2

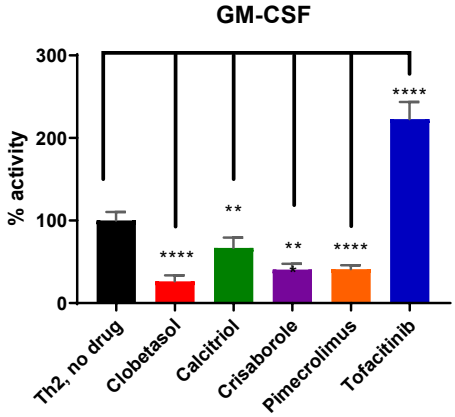

IL13
% activity
150
100
50
0
****
***
**
**
Th2, no drug
Clobetasol
Calcitriol
Crisaborole
Pimecrolimus
Tofacitinib


IL31
% activity
150
100
50
0
****
*
**
****
*
Th2, no drug
Clobetasol
Calcitriol
Crisaborole
Pimecrolimus
Tofacitinib

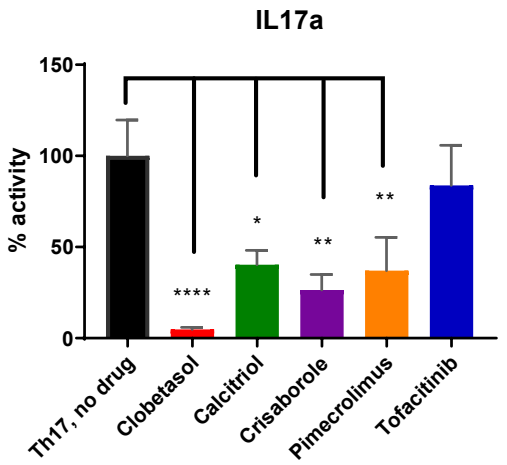

Filaggrin
% activity
10000
5000
400
300
200
100
0
*
*
*
****
Th2, no drug
Clobetasol
Calcitriol
Crisaborole
Pimecrolimus
Tofacitinib

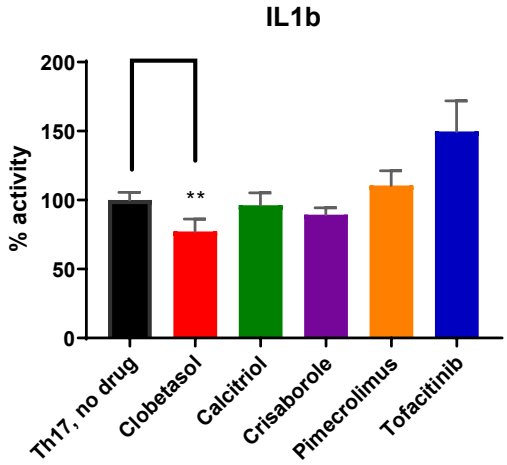

CCL26
% activity
200
150
100
50
0
****
****
***
****
Th2, no drug
Clobetasol
Calcitriol
Crisaborole
Pimecrolimus
Tofacitinib

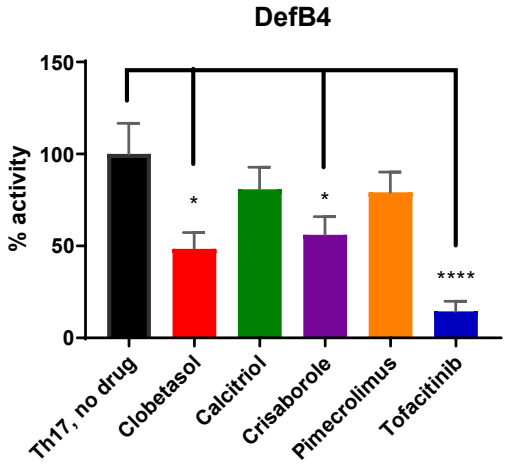

MMP12
% activity
150
100
50
0
****
****
***
****
Th2, no drug
Clobetasol
Calcitriol
Crisaborole
Pimecrolimus
Tofacitinib

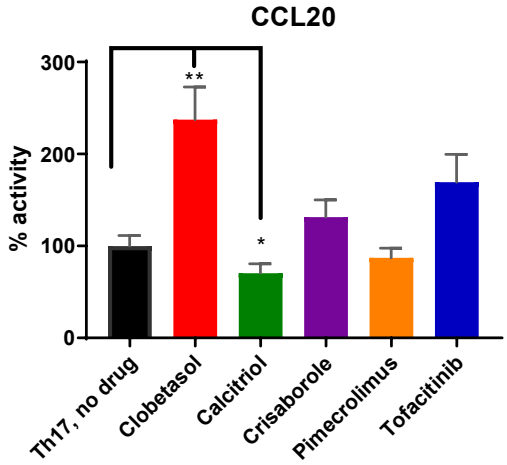

GM-CSF
% activity
300
200
100
0
****
**
****
**
****
Th2, no drug
Clobetasol
Calcitriol
Crisaborole
Pimecrolimus
Tofacitinib

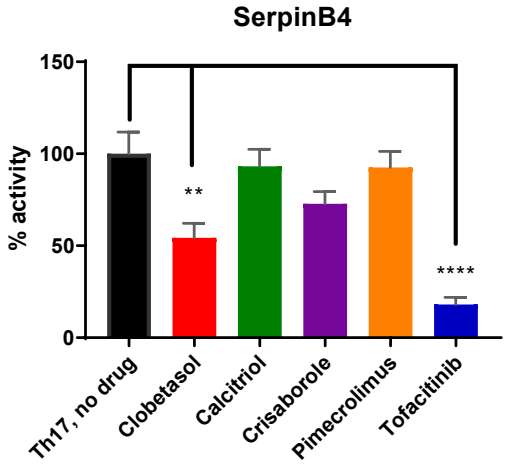
Th17

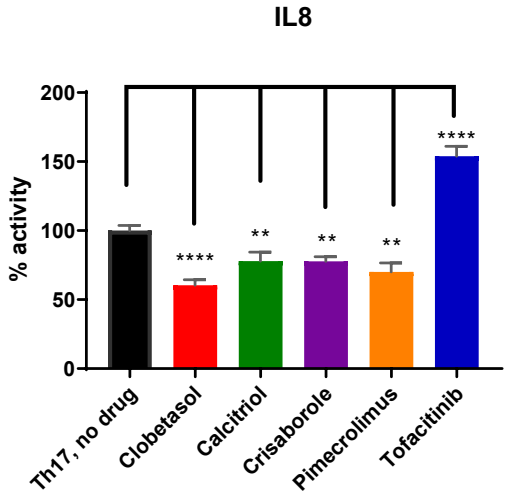

IL17a
% activity
150
100
50
0
****
*
**
**
Th17, no drug
Clobetasol
Calcitriol
Crisaborole
Pimecrolimus
Tofacitinib


IL1b
% activity
200
150
100
50
0
**
Th17, no drug
Clobetasol
Calcitriol
Crisaborole
Pimecrolimus
Tofacitinib

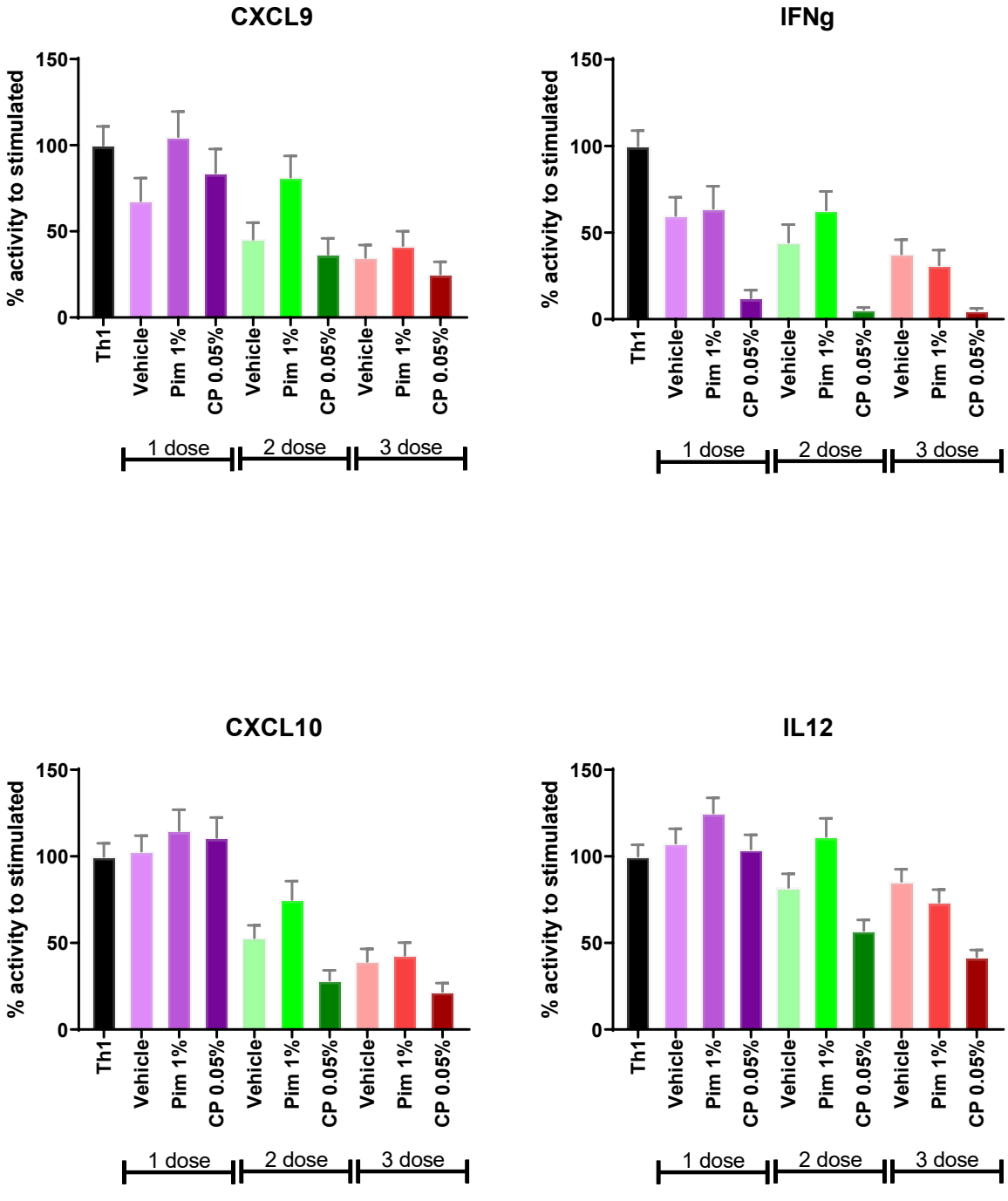

DefB4
% activity
150
100
50
0
*
*
****
Th17, no drug
Clobetasol
Calcitriol
Crisaborole
Pimecrolimus
Tofacitinib

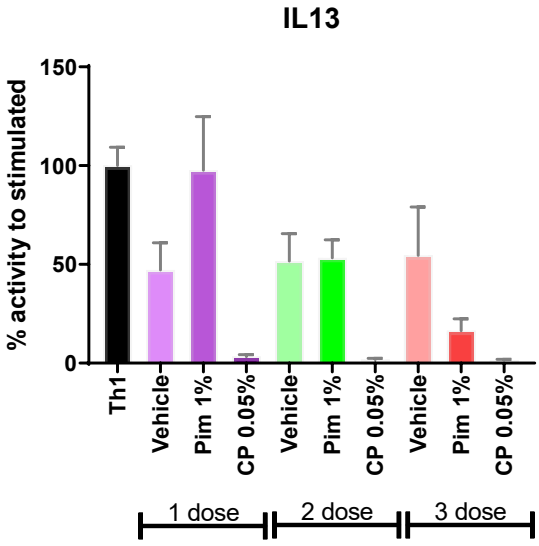

CCL20
% activity
300
200
100
0
**
*
Th17, no drug
Clobetasol
Calcitriol
Crisaborole
Pimecrolimus
Tofacitinib

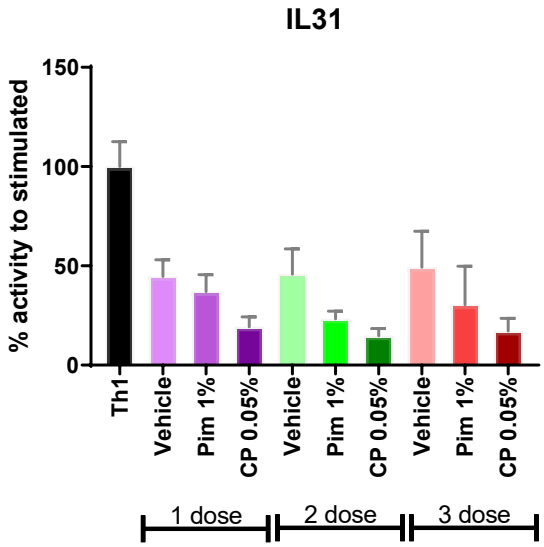

SerpinB4
% activity
150
100
50
0
**
****
Th17, no drug
Clobetasol
Calcitriol
Crisaborole
Pimecrolimus
Tofacitinib

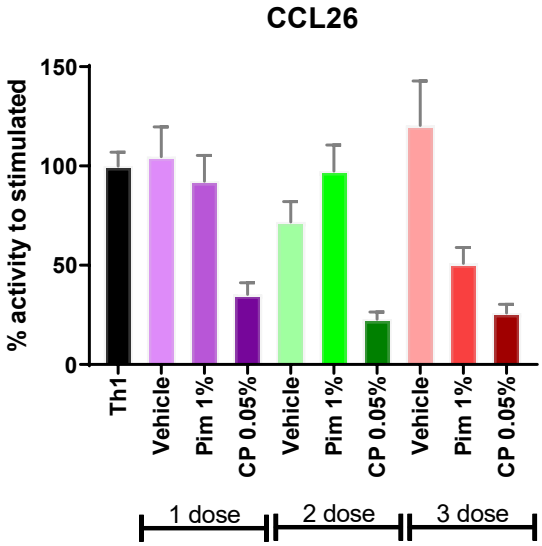

IL8
% activity
200
150
100
50
0
****
**
**
**
****
Th17, no drug
Clobetasol
Calcitriol
Crisaborole
Pimecrolimus
Tofacitinib

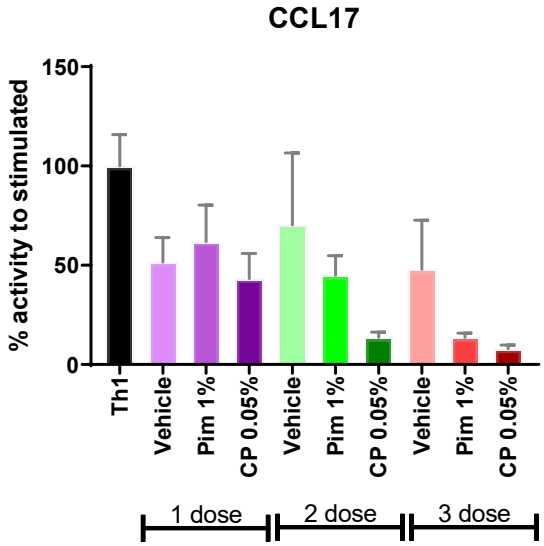
Chapter 4. Figure 2. **Percent activity of gene expression with topical application of Elidel and Dermovate compared to Th1-mediated stimulation with one, two and three daily doses.** Average fold change of four combined donors stimulated by Th1 cytokine cocktail. N=4 samples per treatment per donor. 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.

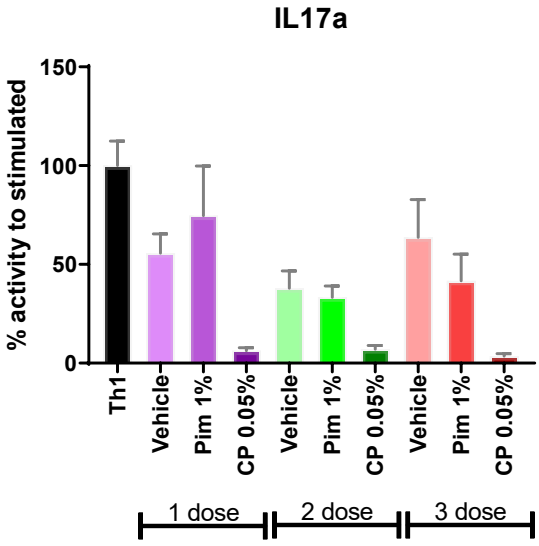


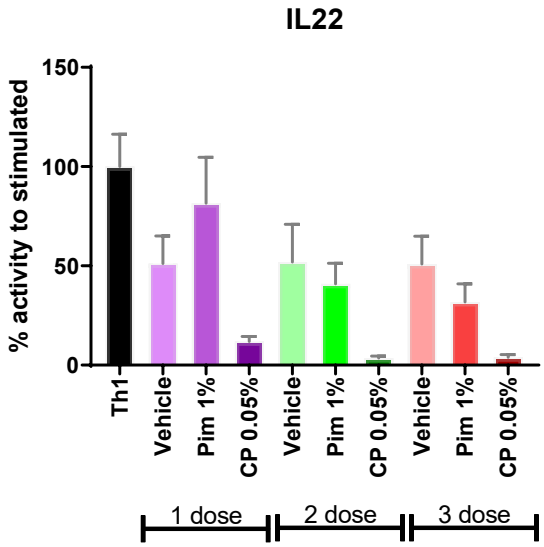

IL13
% activity to stimulated
150
100
50
0
Th1
Vehicle
Pim 1%
CP 0.05%
Vehicle
Pim 1%
CP 0.05%
Vehicle
Pim 1%
CP 0.05%
1 dose
2 dose
3 dose

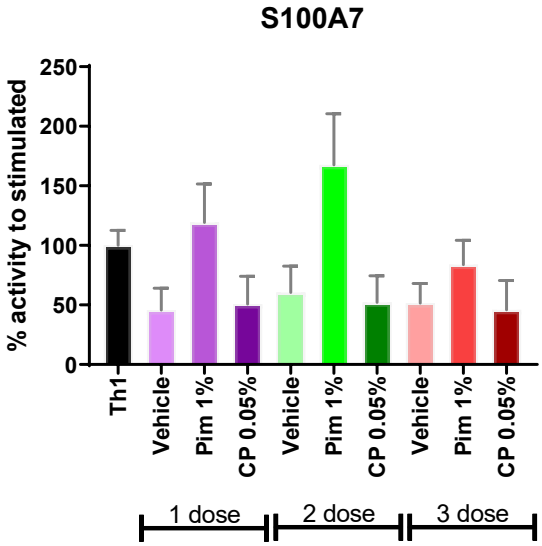

IL31
% activity to stimulated
150
100
50
0
Th1
Vehicle
Pim 1%
CP 0.05%
Vehicle
Pim 1%
CP 0.05%
Vehicle
Pim 1%
CP 0.05%
1 dose
2 dose
3 dose

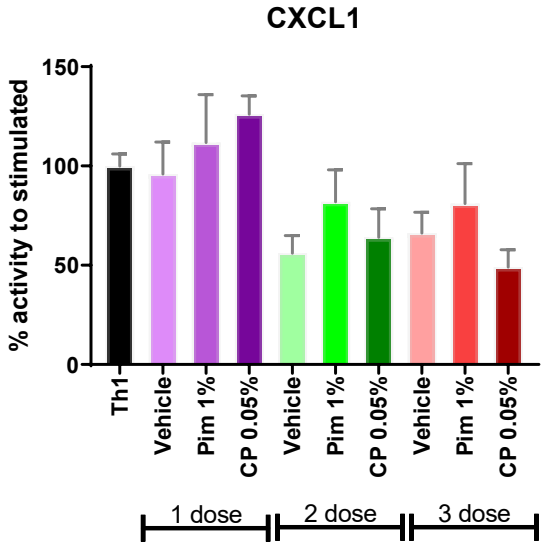

CCL26
% activity to stimulated
150
100
50
0
Th1
Vehicle
Pim 1%
CP 0.05%
Vehicle
Pim 1%
CP 0.05%
Vehicle
Pim 1%
CP 0.05%
1 dose
2 dose
3 dose

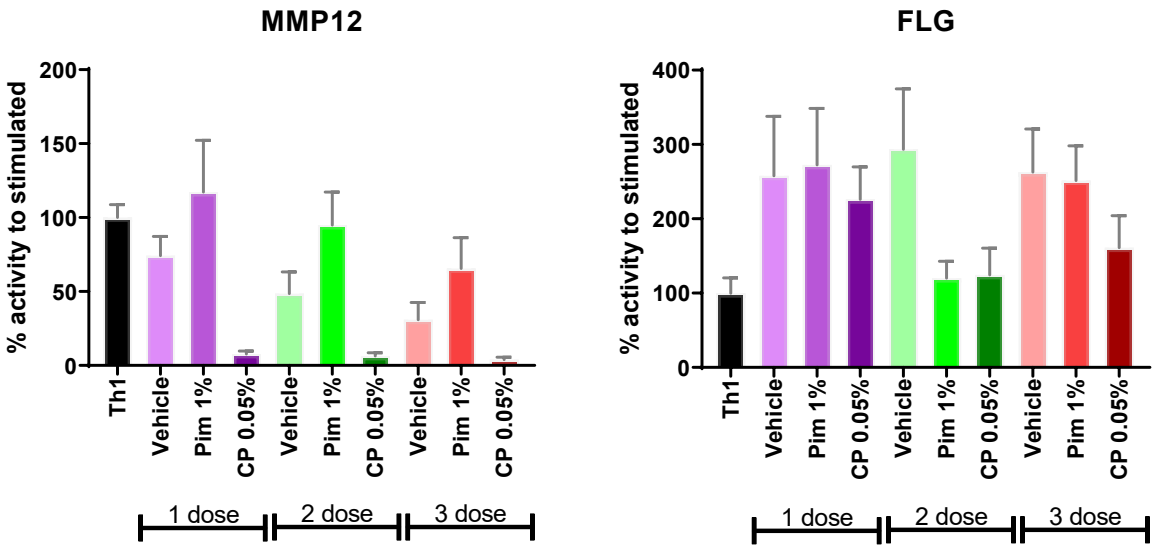

CCL17
% activity to stimulated
150
100
50
0
Th1
Vehicle
Pim 1%
CP 0.05%
Vehicle
Pim 1%
CP 0.05%
Vehicle
Pim 1%
CP 0.05%
1 dose
2 dose
3 dose


IL17a
% activity to stimulated
150
100
50
0
Th1
Vehicle
Pim 1%
CP 0.05%
Vehicle
Pim 1%
CP 0.05%
Vehicle
Pim 1%
CP 0.05%
1 dose
2 dose
3 dose


IL22
% activity to stimulated
150
100
50
0
Th1
Vehicle
Pim 1%
CP 0.05%
Vehicle
Pim 1%
CP 0.05%
Vehicle
Pim 1%
CP 0.05%
1 dose
2 dose
3 dose

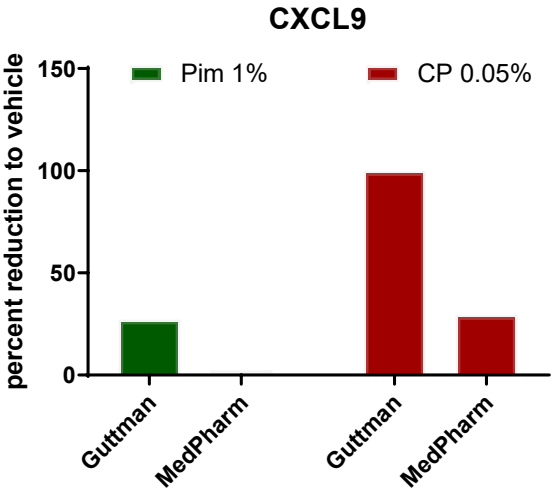

S100A7
% activity to stimulated
250
200
150
100
50
0
Th1
Vehicle
Pim 1%
CP 0.05%
Vehicle
Pim 1%
CP 0.05%
Vehicle
Pim 1%
CP 0.05%
1 dose
2 dose
3 dose

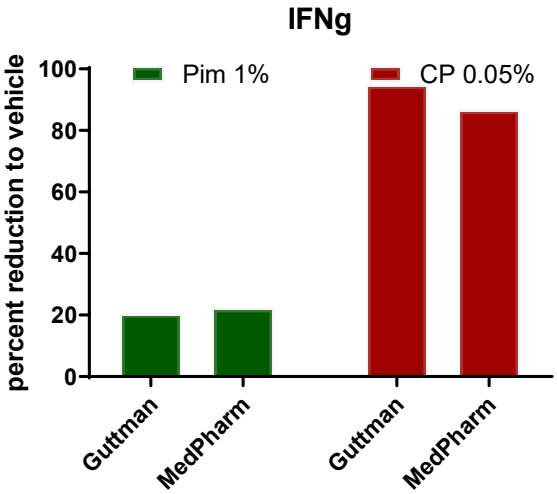

CXCL1
% activity to stimulated
150
100
50
0
Th1
Vehicle
Pim 1%
CP 0.05%
Vehicle
Pim 1%
CP 0.05%
Vehicle
Pim 1%
CP 0.05%
1 dose
2 dose
3 dose

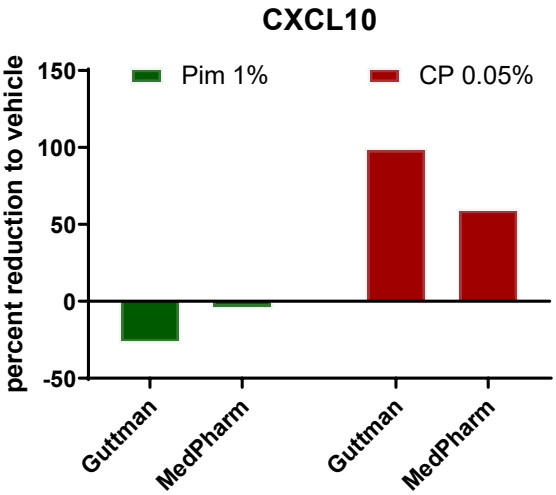


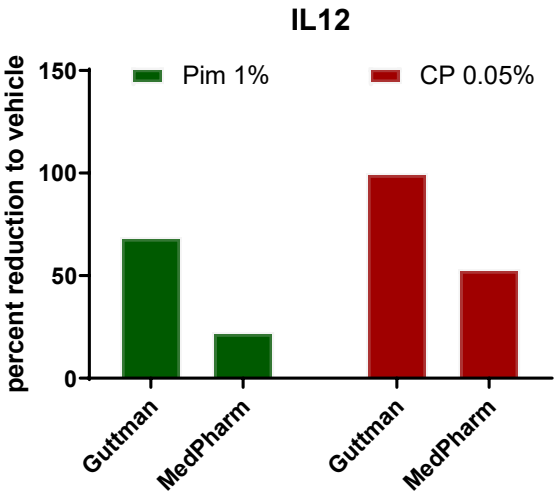
Chapter 4. Figure 4. **Percent reduction in vivo and ex vivo in gene expression calculated from fold change with 3 daily doses of Elidel and Dermovate compared to Th1-mediated stimulation**. In Vivo (Guttman); n=30 donor clinical trial, single replicate; cream applied daily for 14 days. Approximate values 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.

Th1-associated

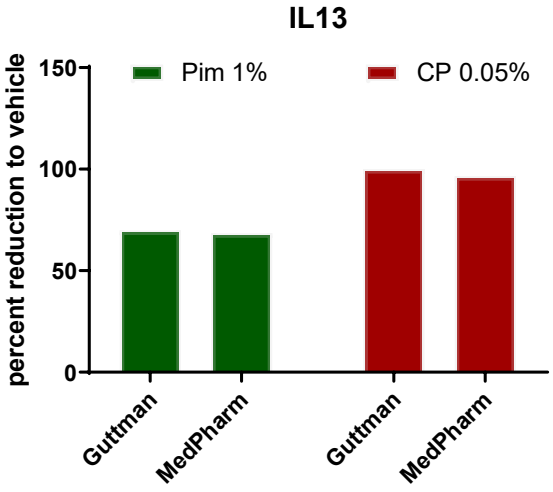


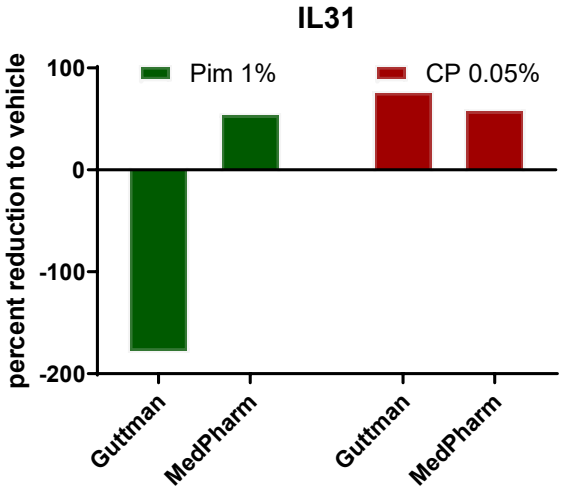


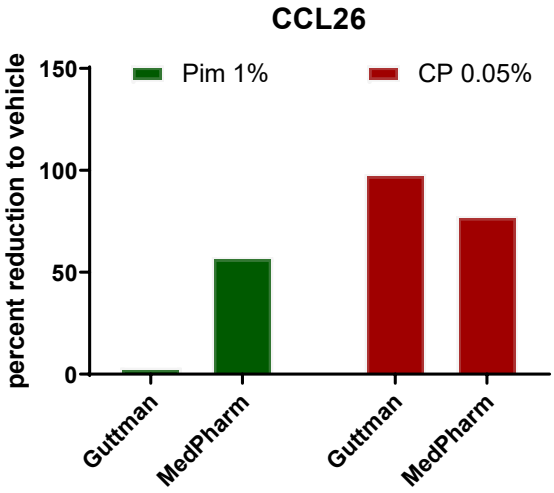


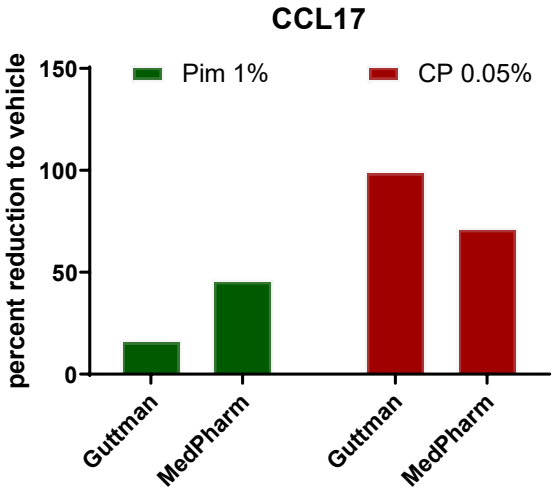


Th2-associated

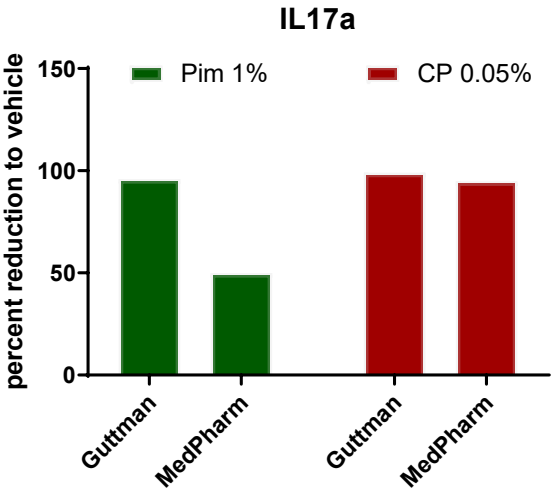


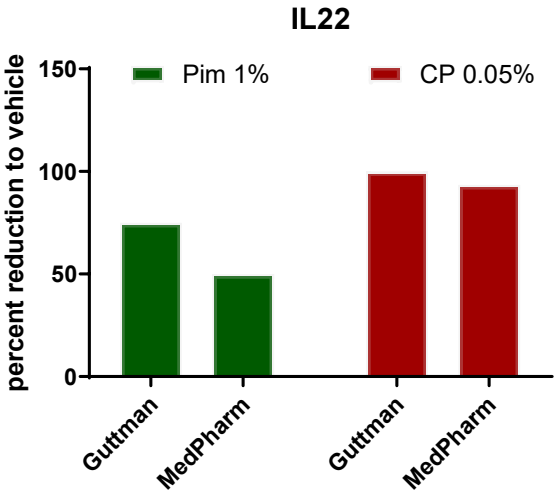


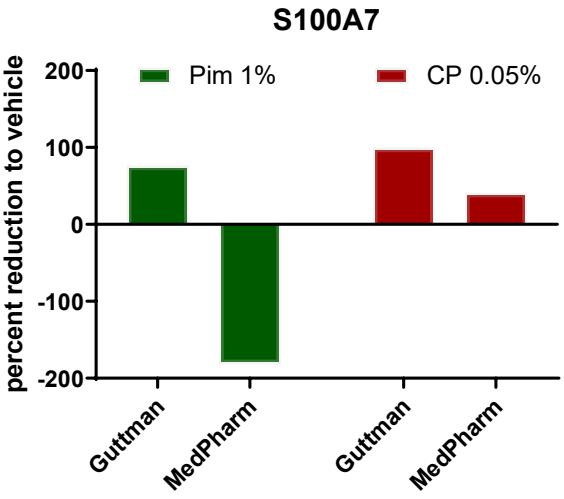


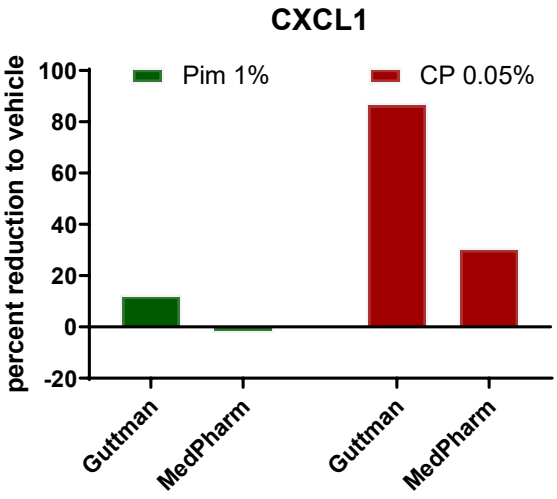


Th17-associated









MMP and differentiation