Role of PKCα in Psoriasis

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Abstract

Psoriasis is a chronic disease that affects the skin and joints, affecting over 125 million people worldwide, and is associated with a number of comorbidities. Psoriasis is predominantly a skin disease that manifests as raised, erythematous plaques with adherent silvery scales. It is characterised by hyperkeratosis, parakeratosis, acanthosis with regular elongation of the rete ridges, scaling and epidermal inflammatory infiltrate. Currently, there is no cure for psoriasis and the disease has a great negative impact on patient quality of life.

PKC\(\alpha\) is a ubiquitous molecule expressed in the epidermis and plays a role in numerous cellular signalling pathways. By utilising three mouse models, CA-PKC\(\alpha\), Aldara and TPA, alongside global PKC\(\alpha\) deficient mice, we have identified several contributions made by PKC\(\alpha\) to keratinocyte differentiation and basal proliferation.

By analysing keratinisation of the skin following the induction of a psoriasis-like phenotype, we have shown that PKC\(\alpha\) plays an important role in proliferation. In Chapter 3, we show that keratinocyte-specific overexpression of PKC\(\alpha\) in an inducible bitransgenic mouse induces a psoriasis-like phenotype, resulting in increased proliferating keratinocytes and decreased keratinocyte differentiation. In Chapter 4, we complement these data by demonstrating that in the absence of PKC\(\alpha\) in the TPA mouse model, keratinocyte proliferation and differentiation is depleted, highlighting the contribution of PKC\(\alpha\). Finally in Chapter 5, we use a specific PKC\(\alpha/\beta\) inhibitor and a clinically relevant pan PKC inhibitor to provide initial pre-clinical data to show that PKC\(\alpha\) can be inhibited topically. Importantly, we have shown that inhibition of PKCs can reverse keratinocyte proliferation and differentiation induced by TPA. These data provide a strong case for potential development of a specific PKC\(\alpha\) inhibitor as a therapeutic target for patients with psoriasis.
Declaration

I declare that no portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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Jeya
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<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AEB071</td>
<td>Sotrastaurin</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>AMP</td>
<td>antimicrobial peptide</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>BrdU</td>
<td>BromodeoxyUridine</td>
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<td>Ca2+</td>
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<td>CA-PKCα</td>
<td>Constitutively Active PKCα</td>
</tr>
<tr>
<td>cm</td>
<td>Centimetres</td>
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<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DEJ</td>
<td>dermal-epidermal-junction</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ESB</td>
<td>epidermolysis bullosa simplex</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
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<tr>
<td>Gö6976</td>
<td>PKCa/β inhibitor</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haemotoxylin and Eosin</td>
</tr>
<tr>
<td>IFNγ</td>
<td>interferon-gamma</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IMQ</td>
<td>Imiquimod</td>
</tr>
<tr>
<td>K(1-14)</td>
<td>Keratin</td>
</tr>
<tr>
<td>ml</td>
<td>Millilitre</td>
</tr>
<tr>
<td>mm</td>
<td>Millimeter</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>PASI</td>
<td>Psoriasis Area and Severity Index</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
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<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>PKCaPKCα</td>
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<tr>
<td>PKCβ</td>
<td>Protein Kinase C beta</td>
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<tr>
<td>PMA</td>
<td>phorbol-12-myristate-13-acetate</td>
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<tr>
<td>PS</td>
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<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
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</tr>
<tr>
<td>Ru486</td>
<td>Mifepristone</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe combined immunodeficiency</td>
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<tr>
<td>TAE</td>
<td>Tris-acetate buffe</td>
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<tr>
<td>TGFβ</td>
<td>Transforming Growth Factor-β</td>
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<td>Toll-like Receptor</td>
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<td>TNFa</td>
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<td>UV</td>
<td>Ultraviolet</td>
</tr>
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<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
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<tr>
<td>μg</td>
<td>Micrograms</td>
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1. Introduction

1.1 Human Skin Structure

Skin is our largest external organ and acts as a physical barrier between the external environment and the tissue beneath. It serves as a primary defence system to protect the body from dehydration, mechanical, chemical and thermal damage, UV exposure and pathogenic microorganisms. It also plays a significant role in homeostasis and thermoregulatory processes, as well as in a number of biochemical, metabolic and immune functions. Human skin occupies around 15% of the adult body weight and has a surface area between 16,000 and 18,000 cm$^2$. The thickness of the skin ranges across the body, from 40$\mu$m in upper eyelids to 1mm on the palmar (palms of hands) and plantar (soles of feet) (Morganti et al., 2001) (Ha et al., 2005).

The skin is comprised of three principle layers: the epidermis, the dermis and the subcutaneous layer, with the epidermis and dermis connected by a basement membrane (Morganti et al., 2001).

1.1.1. The epidermis

The epidermis is derived from the embryonic ectoderm and subdivided into four layers: stratum corneum, stratum granulosum, stratum spinosum and the stratum basal (Elias, 2005; Hardman et al., 1999) (Madison, 2003) (Figure 1.1). The topmost layer is the stratum corneum, consisting of keratin rich, cornified, dead keratinocytes. The stratum corneum acts as a physical barrier to prevent water loss and microorganism invasion (Elias, 2005). The second layer is the stratum granulosum containing between one to three layers of angular, flat shaped keratinocytes. The layer below is the stratum spinosum made of rows of spinous cells, which express keratins 1 and 10 - characteristic of terminally differentiating cells. Finally, the last layer, stratum basale, is connected to the basement membrane by hemidesmosomes and is made up of columnar cells with elongated nuclei which express K14 and K5, providing useful biochemical markers of basal cells (Menon, 2002). The stem cells situated in the basal layer terminally differentiate to move outwards to the top layer, undergoing keratinization, a highly orchestrated process to form the insoluble protective keratinized layer (Travis and Potempa, 2000). The epidermis contains four different types of cells: stratified
keratinocytes, which are primary epidermal cells; melanocytes which produce pigment granules and protect against UV; Langerhans cells, which contribute to immune defence; and Merkel cells, which are involved in sensation (Baroni et al., 2012). Keratinocytes cells synthesise keratin proteins. A number of keratin proteins are expressed in the epidermis, some of which are K1, K5, K10 and K14 (Lane and McLean, 2004). Keratinocytes produce chemical mediators called cytokines, which have a key role in inflammation and repair (Baliwag et al., 2015).

![Image of skin epidermis structure](image)

**Figure 1.1: Overall structure of the skin epidermis.** The skin comprises of the epidermis and dermis, separated by a basement membrane; only the epidermis is shown here. The epidermis contains several layers of squamous epithelia (stratum corneum, granular, spinous and basal layers). The basal layer is attached to the basement membrane and contains stem cells which terminally differentiate and move upwards to form the upper layers (Fuchs and Raghavan, 2002).

### 1.1.2. The basement membrane

The basement membrane or dermal-epidermal-junction (DEJ) regulates chemical signals between the epidermis and dermis, as well as operating as a physical barrier. The interactions between hemidesmosomes, anchoring filaments and fibrils allow for tight
anchoring of the epidermis and dermis. Hemidesmosomes are specialised proteins that attach to basal keratinocyte cells, and consist of two different classes: type I which is mainly found in stratified epithelium and type II which is found in the intestines (Litjens et al., 2006).

1.1.3. The dermis

The dermis accounts for majority of the skin and is derived from the embryonic mesoderm. It consists of strong fibres made up of collagens I (roughly 10%) and II (roughly 90%) (Tobin, 2006), elastin providing the skin with elastic qualities, polysaccharides, and some cells such as fibroblasts, masts cells, macrophages and lymphocytes (Richmond and Harris, 2014). The dermis starts at the lamina densa of the basement membrane and extends down to the subcutaneous layer. It contains a number of skin appendages such as sweat glands, sebaceous glands and hair follicles (Baroni et al., 2012). The dermis is made of two layers: upper papillary containing the DEJ and lower reticular providing anchoring for skin appendages, nerve endings and blood vessels.

1.1.4. The subcutaneous layer

The deepest layer of the skin is the subcutaneous layer, also known as the hypodermis. It is mostly composed of adipose tissue derived from the ectoderm that acts as a sponge between the skin muscles and bones (Baroni et al., 2012). The subcutaneous layer consists of two layers: panniculus adiposus, composed of adipose tissue and panniculus carnosus, composed of striated muscle fibre (Stewart et al., 2013).

1.2. Keratin and diseases

The major proteins found in keratinocytes are keratins. These proteins are intermediate filaments (10-12nm in diameter) that form the cytoskeleton of keratinocytes along with microtubules and microfilaments (Moll et al., 2008). Keratin expression changes as cells differentiate and move outwards to populate the stratum corneum. To maintain normal skin regulation, the balance between the keratinocyte stem cell population and the cells destined to become terminally differentiated must be maintained (Ramms et al., 2013).
1.2.1. Keratin function in the epidermis

Keratins fundamentally influence the structure and mitotic activity of epithelial cells (Deo and Deshmukh, 2018). They provide a framework for epithelial cells and tissue to maintain structural integrity, tolerate mechanical stress and establish cell polarity (Shetty and S, 2012). Keratins are involved in cell transport, signalling, compartmentalization and differentiation (Vaidya and Kanojia, 2007) as well as participating in wound healing.

Keratinocytes undergo a maturation process whereby cells in the basal layer produced by mitotic division migrate to the surface of the epidermis. They are then shed and replaced by a new maturing cell population. This process consists of two paths: keratinization and non-keratinization. At the basal layer, there are two types of cell populations: a progenitor population and a maturing population (Ramms et al., 2013). The progenitor population comprises of stem cells and amplifying cells, and as each cell divides, the daughter cells enter the progenitor population again or start maturing. Once the cells enter the maturing population, the keratinocytes differentiate and undergo several biochemical and morphologic changes as they migrate from the basal cell layer to the surface; this process is called maturation (Rittié, 2016). Finally, a dead cell with a tough cell membrane containing densely packed protein is formed with loss of nuclei and organelles. Once it reaches the surface, desquamation of the cell occurs where the cell is shed. The turnover time for a cell to divide and complete the maturation process is roughly 30 days in the skin (Benhadou et al., 2019; Min et al., 2017).

The cytoskeleton of epithelial cells, made of cytokeratins, microtubules and microfilaments form the structural framework of the cell. The keratinocytes of the stratum basale are the least differentiated, synthesise DNA and undergo mitosis to produce new daughter cells (Windoffer et al., 2011). Adjacent keratinocytes are linked to each other by specialized intracellular junctions called desmosomes (Kowalczyk and Green, 2013). Desmosomes contain two types of proteins called transmembrane proteins (the desmogleins and desmocollins) and attachment plaque proteins (desmoplakin, plakoglobin, plakophilin, envoplakin and periplakin). The next layer above the basal cell layer consists of polyhedral shaped stratum spinosum cells in which more protein-synthesizing activity takes place, indicating biochemical changes and
commitment to keratinization (Shetty and S, 2012). Above this layer lies the stratum granulosum layer where the cells are flatter and wider, and contain basophilic keratohyalin granules, which discharge their content at the granular and cornified cell layer junction to form a permeability barrier. The stratum granulosum cells also start to show degeneration of the nuclei and undergo pyknosis, as well as the formation of lamellar granules in the upper spinous and granular layers. Simultaneously, the inside of the cell membrane starts to thicken to form a cornified cell envelope through the participation of several proteins such as involucrin and loricrin (Deo and Deshmukh, 2018). The next layer is made up of cells larger and flatter than granular cells called keratinized squame, in which nuclei and organelles such as mitochondria and ribosomes are absent, as well as keratohyalin granules. The cells in the cornified layer are coated by filaggrin, a basic protein of keratohyalin granules. These keratinized cells are dehydrated, compacted and finally they desquamate. The rate and degree of keratinization of cells depends on various processes during differentiation such as synthesis, breakdown and dehydration which determines whether the epithelium takes the keratinization or non-keratinization path (Shetty and S, 2012).

1.2.2. Keratinocyte cell journey from basal layer to stratum corneum

Keratins form heterodimers consisting of keratin type 1 (keratin 9 – 20) and type 2 (keratin 1 – 8) (Steinert 1990). Type 1 keratins are acidic and are found on chromosome 17, whilst type 2 keratins are basic and found on chromosome 18. Type 1 and type 2 keratins are expressed in specific pairs depending on the tissue type, where keratin type 2 is expressed in cells first and induces type 1 synthesis. For instance K5/K14 are expressed in the basal cell layer as they proliferate, and K1/K10 in the suprabasal compartment during differentiation (Figure 1.2) (Sandilands et al., 2009). K6/K16 are normally found the palmoplantar epidermis, nail bed, hair follicles, sebaceous glands and sweat glands, but are induced by injury, wounds, UV radiation and diseases such as psoriasis.

Keratinocytes proliferate in the basal layer through, and as the cells move away from the basal layer, they lose their ability to proliferate and become terminally differentiating cells. Keratinocytes of the basal epidermal layer express K5, K14 and K15. Once the basal keratinocytes differentiate and exit the basal layer and migrate upwards
into the suprabasal layers, these keratins are replaced by K1 and K10 (Wang et al., 2016). In thicker epidermis such as palmer/plantar, K2 and K9 are additionally expressed to complement the K1/K10. K2 is more extensively expressed, where as in mice, K2 expression is limited to tails, ear and footpad epidermis only (Rentrop et al., 1987). Keratins K6, K16 and K17 are normally expressed in hair follicles but injured and hyperproliferative conditions upregulate a strong expression in the interfollicular epidermis (Kirfel et al., 2003). Normal skin thickness is maintained by a balance between the processes of proliferation and desquamation that results in the complete renewal of the skin roughly every 50-72 days.

**Figure 1.2: Keratin expression in the epidermal layer.** As keratinocytes differentiate, they migrate upwards through the epidermal layers (the spinous layer, granular layer and cornified layer or stratum corneum). They become anucleated and compacted, before desquamation. Each stage of epidermal differentiation is characterised by specific keratin expression. Adapted from (Sandilands et al., 2009).
1.2.3. Pathology of keratinization

An imbalance or abnormalities in the process of keratinization and non-keratinization whilst the cells migrate from the basal layer to the surface of the skin can cause disease (Haftek, 2015). Pathological changes in keratinization can be caused by defects in a gene or can be acquired (McLean and Irvine, 2007).

One of the defects seen in epithelial cells is hyperkeratinisation. In normal circumstances, the epithelial cells in cornified layer will desquamate at regular intervals, but during hyperkeratinisation an excess of keratin accumulation occurs due to lack of desquamation. Hyperkeratinization occurs due to higher rate of proliferation of the keratinocyte cells. On the other hand, decreased keratinization can also occur because of the lack of keratinocyte production and lack of cells undergoing complete differentiation and maturation (Shetty and S, 2012).

Premature keratinization of cells in a different strata before they reach the stratum corneum is called dyskeratosis. These cells become separated from their adjacent cells.

1.2.4. Examples of keratin disorders

- **K5/14 Disorders**

Mutations in keratin 5 and keratin 14, where there is reduced expression can cause various subtypes of epidermolysis bullosa simplex (EBS), a disorder where basal cells are mechanically fragile and blister with injury (Table 1.1) (Knöbel et al., 2015).

- **K1/K10/K2 Disorders**

Autosomal dominant mutations in keratin 1 or keratin 10 in the suprabasal cells leads to epidermolytic ichthyosis (EI), also known as epidermolytic hyperkeratosis (EHK). K1 and K10 proteins act as partners and are responsible for skin stability. It is characterised by hyperkeratosis, erythoderma and blistering of the upper skin layer (Oji et al, 2010). Increased proliferation results in ichthyosiform lesions in the flexural areas.
• **K6, K16 and K17 Disorders**

Pachyonychia congenita (PC) is a group of autosomal dominant genodermatoses caused by mutations in keratin 6A/B/C, keratin 16 or keratin 17 (Wilson et al. 2014). It is characterised by painful and extensive plantar keratoderma due to deep blister formation underneath the callus (McLean et al. 2011), hypertrophic nail dystrophy and epidermal cysts. Studies with keratin 16 knockout mice show that deletion of the gene produced oral lesions early after birth, followed by spontaneous palmoplantar keratoderma (PPK) if they survive into adulthood, suggesting loss of function phenotype in PC.

• **K9 Disorders**

Keratin 9 is expressed in the suprabasal cells in the palm and soles, playing a role in re-enforcing the skin during stress. Mutations in keratin 9 causes epidermolytic palmoplantar keratoderma (EPPK) (Lane and McLean 2004). EPPK is an inherited disease and manifests shortly after birth with hyperkeratosis of the palms and soles (Chamcheu et al. 2011).

1.2.5. **Keratin expression in psoriasis**

Terminal differentiation of keratinocytes is incomplete in psoriatic skin, which results in preferential activation and deactivation of cells that do not mature appropriately. These changes are largely related to altered expression of keratin (Fuchs and Green, 1980; Fuchs and Raghavan, 2002). In psoriasis, K1 and K10 expression is reduced and the hyperproliferation-associated K6, K16 and K17 expression are induced (Elango et al., 2015; Körver et al., 2006; Yang et al., 2017), as well as an increase in recruitment of cycling epidermal cells (Ki67 positive nuclei) (Körver et al., 2006). Additionally, a down regulation of loricrin expression is found in psoriatic skin (Nithya et al., 2015), along with K5/14 levels showing altered expression in the basal layer (Elango et al., 2018). These changes suggest that keratin pairs hold a functional role in epidermal keratinocyte differentiation.
Table 1.1: Table of keratin-associated diseases.

<table>
<thead>
<tr>
<th>Expression</th>
<th>Disorder</th>
<th>Type 2</th>
<th>Type 1</th>
<th>Clinical features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal keratinocytes of epidermis and stratified epithelia</td>
<td>EBS generalised severe (EBS Dowling-Meara) and intermediate (EBS Koebner) and localised (Weber-Cockayne)</td>
<td>K5</td>
<td>K14</td>
<td>Herpetiform blisters on any site with PPK, nail dystrophy and oral ulceration</td>
</tr>
<tr>
<td></td>
<td>EBS with mottled pigmentation</td>
<td>K5</td>
<td></td>
<td>Blistering, pigmentation of trunk and limbs, punctate hyperkeratosis of palms and soles, dystrophic nails.</td>
</tr>
<tr>
<td></td>
<td>Dowling-Degos disease</td>
<td>K5</td>
<td></td>
<td>Reticulate hyperpigmentation with hyperkeratotic papules in flexures</td>
</tr>
<tr>
<td></td>
<td>Recessive EBS</td>
<td></td>
<td>K14</td>
<td>Generalised blistering</td>
</tr>
<tr>
<td></td>
<td>Naegeli-Franceschetti-Jadassohn syndrome</td>
<td></td>
<td>K14</td>
<td>Reticulate hyperpigmentation, hypohidrosis, dental defects</td>
</tr>
<tr>
<td>Suprabasal cells of stratified, cornified epithelia</td>
<td>Epidermolytic ichthyosis</td>
<td>K1</td>
<td>K10</td>
<td>Erythroderma, blistering, hyperkeratosis.</td>
</tr>
<tr>
<td></td>
<td>Epidermolytic PPK</td>
<td>K1</td>
<td>K9, K16</td>
<td>Diffuse PPK with erythematous border</td>
</tr>
<tr>
<td></td>
<td>Ichthyosis hystrix, Curth-Mackin type</td>
<td>K1</td>
<td></td>
<td>Localised spiky keratoderma</td>
</tr>
<tr>
<td></td>
<td>Cyclic ichthyosis with EHK</td>
<td>K1</td>
<td>K10</td>
<td>PPK with erythematous round scaly patches.</td>
</tr>
<tr>
<td></td>
<td>Congenital reticular ichthyosiform erythroderma</td>
<td></td>
<td>K10</td>
<td>Development of small patches of normal skin within erythroderma and PPK</td>
</tr>
<tr>
<td>Palmoplantar epidermis, epidermal appendages, mucosa</td>
<td>Pachyonychia congenita</td>
<td>K6a, K6b, K6c</td>
<td></td>
<td>PPK, hypertrophic nail dystrophy, oral leukokeratosis, follicular keratosis, epidermal cysts.</td>
</tr>
</tbody>
</table>

K: Keratin  
EBS: Epidermolysis bullosa  
PPK: Palmoplantar keratoderma  
EHK: Epidermolytic hyperkeratosis
1.3. Psoriasis

Psoriasis is a chronic inflammatory disease affecting approximately 2-3% of the world’s population. More specifically, epidemiological studies have found global prevalence of psoriasis to be between 0.6 and 4.8% (Gelfand et al., 2005). Interestingly, 30% of patients suffering with psoriasis are known to have a relative also afflicted by the disease which suggests in part, some degree of genetic susceptibilities (Griffiths and Barker, 2007). In fact, evidence of this genetic susceptibility has also been found in twin studies in which concordance of psoriasis in monozygotic twins is 67% but only 18% for dizygotic twins (Krueger and Ellis, 2005). Psoriasis is associated with systemic disorders such as Crohn’s disease, diabetes mellitus, metabolic syndrome, depression and cancer (Griffiths and Barker, 2007). An increasing concern is the association of psoriasis with cardiovascular diseases (Jindal and Jindal, 2018).

1.3.1. Clinical features

Several different forms of psoriasis have been distinguished (Figure 1.3), with psoriasis vulgaris accounting for 90% of all psoriasis cases, whereby papulosquamous plaques (Figure 1.3a) are well defined from normal skin (Griffiths and Barker, 2007). Plaques are red in colour and covered in silvery white scales, are distributed symmetrically and predominantly occur on elbows, knees, scalp, lumbosacral region and umbilicus. Active inflammatory psoriasis (Figure 1.3b) is characterised by the Koebner phenomenon where lesions develop at the sites of pressure or injury. Flexural (inverse) psoriasis is site specific and develops in intertriginous sites (for example, axilla of the arm) and is shiny, red and black scales. Sebopsoriasis (Figure 1.3c) occurs in eyebrows, nasolabial folds, behind the ear and presternal sites (Boehncke and Schön, 2015).

Guttate psoriasis (Figure 1.3d) is an acute form of psoriasis and can develop in children and adolescents following β-haemolytic streptococcal infections such as tonsillitis or pharyngitis, or viral infections. It occurs as 10mm diameter papules on the trunk roughly 2 weeks after infection, but is able to self-resolves itself within 3 to 4 months of onset (Griffiths and Barker, 2007).

One of the clinical subtypes of psoriasis includes the rare and extreme pustular variant, where they are subdivided to generalised and localised forms. One of the generalised
forms of psoriasis is pustular psoriasis (Figure 1.3e), which is characterised by erythematous and painful skin studded with white sterile pustules (Benjegerdes et al., 2016). It affects all ages and races and can occur alongside or independent of other forms of psoriasis, and is usually triggered due to medication or infection (Hoegler et al., 2018). A localised form of psoriasis is palmoplantar pustular psoriasis (Figure 1.3f), which causes sterile pustules with erythema and scaling on the palms of hands and soles of the feet (Benjegerdes et al., 2016).

Around 80-90% of patients with plaque psoriasis also suffer from nail psoriasis (Figure 1.3g), and even more widespread in patients with psoriatic arthritis. Nail psoriasis manifestation includes pitting, leukonychia (white spots on nail plate), Beau’s lines (transverse grooves) and crumbling of the nail plate (Pasch, 2016). Nail psoriasis can also be an early indicator for patients at risk for psoriatic arthritis in the future (Langenbruch et al., 2014).
Figure 1.3: Different forms of psoriasis. (a) Psoriasis vulgaris (b) Flexural (inverse) psoriasis (c) Sebopsoriasis (d) Guttate psoriasis (e) Generalised pustular psoriasis (f) Palmoplantar pustular psoriasis (g) Nail psoriasis (Roberson and Bowcock, 2010)
1.3.2. Histopathology of psoriasis

Psoriatic skin differs histologically from normal skin (Figure 1.4A/B) (Wagner et al., 2010). Hyperproliferation is a hallmark of psoriasis where significant expansion is seen of both basal and suprabasal epidermal layers (Christophers and Mrowietz, 1995). Expression of specific keratins K5 and K14 are expressed in the basal layer, and as they differentiate, they are replaced with K1 and K10 in normal skin (Figure 1.5) (Mommers et al., 2000). By contrast, in psoriatic skin, the K1 and K10 keratins are reduced, whilst K6 and K16 levels are increased (Figure 1.5). Abnormal keratinocyte differentiation is also seen leading to parakeratosis (cell nuclei present in the cornified layer) (Figure 1.5). Hyperkeratosis (the thickening of the cornified layer) and elongated epidermal rete ridges (thickenings that extend down between dermal papillae) Figure 1.4 – arrows) (Hawkes et al., 2017a). Another hallmark of psoriatic skin is the increased inflammatory infiltrate of leucocytes, macrophages and neutrophils in the epidermis and dermis; these accumulate to form micropustules of Kogoj (neutrophil accumulation upper part of the epidermis) and Munro’s microabscesses (neutrophil accumulation in the stratum corneum) (Boehncke and Schön, 2015).

Studies show that IL-23, a cytokine that drives the development of IL-17 and IL-22 producing Th17 cells, is functionally involved in the pathogenesis of psoriasis. Expression of IL-23 is increased in psoriasis lesional skin and increased numbers of Th17 cells are present (Eberle et al., 2016; Fitch et al., 2007). Van der Fits et al showed that intradermal injection of IL-23 in mouse skin resulted in erythema, a mixed inflammatory infiltrate and epidermal hyperplasia. Data show that IMQ-induced dermatitis in mice closely resembles human psoriasis lesions in terms of phenotypic and histological characteristics, and that lesion development is critically dependent on IL-23 and IL-17 (van der Fits et al., 2009).
Figure 1.4: Histological features of human and mouse psoriatic skin. (A) H&E staining of human normal and psoriatic skin showing hyperproliferation, acanthosis, elongated rete ridges, hyperkeratosis, parakeratosis with increased levels of inflammatory cells. (B) H&E staining of mouse normal and psoriasis-like skin evident of similar features to human psoriasis such as acanthosis, elongated rete-like ridges, hyperkeratosis, microabscesses, parakeratosis with increased levels of inflammatory cells. Adapted from (Wagner et al., 2010)
Figure 1.5: Basal keratinocytes differentiate through spinous and granular layers of the epidermis to become corneocytes. In psoriatic epidermis, there is increased proliferation and altered differentiation of keratinocytes in both the granular and cornified layers. Adapted from (Bowcock and Krueger, 2005).
1.4. Mouse versus human skin

1.4.1. Murine skin structure

Human and murine skin are made up of the same layers described above, but with some key structural differences (Figure 1.6). Human skin contains a far thicker epidermis than mouse skin. Mouse skin lacks melanocytes and sweat glands, exhibiting synchronised hair cycle and rapid epidermal regeneration (Pasparakis et al., 2014). Importantly, T cell population in murine and human skin vary - where human epithelial cells contain αβ T cells, and murine skin contains γδ T cells. In spite of these differences, mouse models have been utilised to mimic human diseases and unravel multiple disease pathogenesis (Wagner et al., 2010).

The stratification of mouse skin and human skin is similar, although distinct differences do exist (Table 1.2). Despite the differences between mouse skin and human skin, mouse models have been successfully employed to mimic human skin disease.
Figure 1.6: Illustrating skin stratification and different cell types in human and mouse skin
(Wagner et al., 2010)

Table 1.2: Similarities and differences between human and mouse skin.
(Mestas and Hughes, 2004; Wong et al., 2011)

<table>
<thead>
<tr>
<th></th>
<th>Human Skin</th>
<th>Mouse Skin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hair cycle</td>
<td>Highly variable, region dependent</td>
<td>Approximately 3 weeks</td>
</tr>
<tr>
<td>Biomechanical properties</td>
<td>Thick, relatively stiff, adherent to underlying tissue</td>
<td>Thin, loose</td>
</tr>
<tr>
<td>Hypodermis thickness</td>
<td>Less variable</td>
<td>Hair cycle dependent</td>
</tr>
<tr>
<td>Method of wound healing</td>
<td>Re-epithelization</td>
<td>Contraction</td>
</tr>
<tr>
<td>Sweat glands</td>
<td>Present</td>
<td>Not present</td>
</tr>
<tr>
<td>Epithelial structure</td>
<td>Thicker epidermis</td>
<td>Thinner epidermis</td>
</tr>
<tr>
<td></td>
<td>Rete ridges present</td>
<td>Rete ridges not present</td>
</tr>
<tr>
<td>CD4 on macrophage</td>
<td>Present</td>
<td>Absent</td>
</tr>
<tr>
<td>Immune cells</td>
<td>High levels of Langerhans cells and CD8+ T cells</td>
<td>High levels of DETCs</td>
</tr>
<tr>
<td>Populated with</td>
<td>Macrophages, mast cells, conventional αβ T cells</td>
<td>Macrophages, mast cells, conventional αβ T cells</td>
</tr>
</tbody>
</table>

DETCs: dendritic epidermal T cells
αβ T cells: alpha/beta T cells
1.4.2. Mouse models of human psoriasis

Numerous mouse models have been used for research on psoriasis (Table 1.3) including spontaneous psoriasis models, genetically tissue engineered (transgenic and knockout) and induced (immune cell transfer and xenotransplantation) models (Danilenko, 2008). A mouse model of human psoriasis that presents all aspects of the disease is not yet established, including chronicity, although many of the models develop psoriasis-like skin disease exhibit some aspects of the human phenotype (Swindell et al., 2011). One such model this review will discuss is the imiquimod (IMQ)-induced mouse model which has been shown to exhibit similar psoriasis-like inflammation to humans (Patel et al., 2011) as well as being mediated by the IL-23/IL-17 axis (van der Fits et al., 2009). It has been shown that the application of topical treatment of Aldara, which contains IMQ, in patients and mice has led to the development of psoriasis (Fanti et al., 2006). This model will be utilised to manipulate PKC levels to address my aims in my projects.
### Table 1.3: Mouse models of psoriasis compared with direct phenotype of human psoriasis

<table>
<thead>
<tr>
<th>Class</th>
<th>Mouse model</th>
<th>Hyperkeratosis</th>
<th>Keratinocyte differentiation</th>
<th>Epidermal T cells</th>
<th>Neutrophil cell infiltration</th>
<th>Intraepidermal microabscesses</th>
<th>Increased vascularization</th>
<th>Psoriasis-like cytokine profile</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epidermal</td>
<td>K14/VEGF</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>K5/STAT3C</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>ND</td>
<td>(Boehncke and Schön, 2007)</td>
</tr>
<tr>
<td>Spontaneous</td>
<td>Asebia</td>
<td>Y</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>Y</td>
<td>ND</td>
<td>(Boehncke and Schön, 2007)</td>
</tr>
<tr>
<td></td>
<td>Flaky skin</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>ND</td>
<td>(Boehncke and Schön, 2007)</td>
</tr>
<tr>
<td>Inflammatory</td>
<td>Imiquimod</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>(Gilliet et al., 2004)</td>
</tr>
<tr>
<td>Xeno-transplant</td>
<td>SCID</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>ND</td>
<td>(Nestle and Nickoloff, 2005)</td>
</tr>
<tr>
<td></td>
<td>Nude</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>ND</td>
<td>(Nestle and Nickoloff, 2005)</td>
</tr>
<tr>
<td>Knock-out</td>
<td>CD18</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>KS-JunB/c-Jun</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>(Wagner et al., 2010)</td>
</tr>
<tr>
<td>Humanized</td>
<td>Hu-PBL</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>(Griffin et al., 2015)</td>
</tr>
</tbody>
</table>

Y: yes  
N: no  
ND: not disclosed
1.5. Imiquimod mouse model of psoriasis

Imiquimod (IMQ) or R837 (1-(2-methylpropyl)-1H-imidazo[4,5-c]quinolin-4-amine) is an imidazoquinoline derivative (Flutter and Nestle, 2013). IMQ acts as a ligand for TLR7 and TLR8 (in humans) and is a potent immune activator (Figure 1.7). It is widely used as a topical treatment for genital and perianal warts caused by human papilloma virus (van der Fits et al., 2009), treatment of actinic keratosis and superficial basal cell carcinomas (Gilliet et al., 2004). Daily topical application of 5% imiquimod cream (Aldara™) (3M Health Care Limited) induces inflammation and psoriasis-like skin lesions (Figure 1.7) (Flutter and Nestle, 2013). It was found that IMQ induced psoriasis in areas that were treated with the cream, and interestingly also in distant untreated areas (Wu et al., 2004) (Rajan and Langtry, 2006).

The benefit of this model is that it is quick and cheap, however the major disadvantage is the lack of chronicity when compared to human disease. It does not exhibit certain features such as arthritis (Nestle et al., 2009b), which can be found in some transgenic mice such as CD18 hypomorphic mice and in JunB/c-Jun double knock out mice (Wang et al., 2008).

Psoriasiform is manifested on a number of different mouse strains, however, there are some key differences in the local and systemic responses to Aldara treatment. Aldara treatment on BALB/c mice results in the development of flaky and inflamed skin more rapidly than in C57BL/6 mice (Nestle et al., 2009a). Likewise in 129/Sv mice, epidermal hyperplasia and parakeratosis is more pronounced than in C57BL/6 mice (Walter et al., 2013). However, in contrast, Aldara treatment in C57BL/6 mice presents a more severe systemic effect, where the mice suffer from dehydration (Nestle et al., 2009a).

The IMQ-induced psoriasis model is acknowledged as a clinically relevant model of psoriasis vulgaris and like human psoriasis, it is largely mediated by the IL-23/IL-17 axis (van der Fits et al., 2009). Below is a schematic of possible mechanisms by which IMQ induces a skin lesion in mice, showing that Aldara is able to induce a systemic effect through various pathways (Figure 1.7). The principal mechanism action of imiquimod is via the ligation of TLR7 in mice (Figure 1.7i) (Hemmi et al., 2002) and TLR7/8 in humans (Jurk et al., 2002). Ligation of TLR7 by imiquimod or R848 activates NF-κB signalling pathway in peritoneal cells in a MyD88 dependant manner (Hemmi et al., 2002). Murine
cell populations such as macrophages and dendritic cells express high levels of TLR7, which when stimulated activate the c-Jun and IRAK pathway that results in the production of many proinflammatory cytokines. Downstream of NF-κB signalling following activation by imiquimod also include activated STAT1 and STAT3 pathways, which is required for cytokine production by dendritic cells (Larangé et al., 2009). In figure 1.7ii, another possible mechanism of action is illustrated that is independent of TLR7 and MyD88 signalling. Imiquimod could activate inflammasomes through the NALP3 pathway, resulting in caspase 1 to be activated and thereby resulting in the production of IL-1β and IL-18 (Kanneganti et al., 2006). Additionally, imiquimod has also been shown to act as an antagonist ligand for adenosine receptors (Figure 1.7iii) (Flutter and Nestle, 2013). High concentrations of imiquimod leads to altered expression of Bcl-2 family members and caspase activity which leads to increased apoptosis and downstream inflammation (Schön and Schön, 2004). The final mechanism of action of imiquimod illustrated by Flutter and Nestle, is that the isostearic acid, a component in the vehicle of Aldara has some biological activity (Walter et al., 2013). Isostearic acid has been shown to stimulate inflammasome activation in keratinocytes, leading to production of proinflammatory cytokines and increased keratinocyte proliferation and cell death.

Although there are a number of different possible mechanisms of action as to how imiquimod treatment results in psoriasisiform, it is understood that a full psoriasis phenotype by Aldara treatment is primarily through MyD88 signalling as MyD88 deficient mice are highly resistant to the disease (Wohn et al., 2013).
Figure 1.7: Possible mechanisms of action of Imiquimod to activate immune pathways.

Possible mechanisms by which IMQ induces a skin lesion in mice: (i) TLR7-dependent activation of MyD88 pathway in immune cells, (ii) NALP3 activation of inflammasome, (iii) antagonistic signalling of adenosine receptor resulting in reduced levels of cyclic antimicrobial peptide (AMP), (iv) direct activation of inflammasomes or (v) cytotoxic effect of IMQ/vehicle leading to the release of preformed IL-1α and cell debris (Flutter and Nestle, 2013).
1.6. Protein Kinase C

Research on the role of Protein Kinase C (PKC) in psoriasis dates back to the late 1980’s (Fisher et al., 1993, Rasmussen and Celis, 1993). Changes in PKC isotype expressions and increased levels of DAG occur in psoriatic human epidermis suggesting an important role in the pathogenesis of psoriasis (Reynolds et al., 1993). Since then, a PKC pan inhibitor, sotrastaurin, has been tested as a monotherapy in a clinical trial in patients with moderate to severe plaque psoriasis. The study found sotrastaurin improved severity of psoriasis significantly in patients (He et al., 2014a; Skvara et al., 2008).

The serine/threonine PKC family comprises around 2% of the human kinome and is broadly conserved throughout all eukaryotes (Dempsey et al., 2000) ranging in complexity from a single isoform in yeast S. cerevisiae, five isoforms in D. melanogaster and twelve in mammals (Mellor and Parker, 1998). They were first discovered in the early 1980s as receptors for the tumor-promoting phorbol esters and became important in research into signal transduction (Akamine et al., 2003). By the mid-1980s, a family of kinases were discovered (Balendran et al., 2000). PKCs are involved in a number of important biological processes such as proliferation, differentiation, apoptosis, adhesion and migration (Poole et al., 2004), where each PKC isoform differ in their structure, cofactor requirements and substrate specificity, and carry out a variety of cellular functions depending on the proteins they phosphorylate (Takai et al., 1979).

1.6.1. The PKC family

PKC isoforms in mammals are classified into three subgroups: classical or conventional, novel or non-classic, and atypical isoforms (Table 1.4). Conventional PKCs include PKCα (the first isozyme to be cloned) (Parker et al., 1986), PKCβI and PKCβII and PKCγ; they are activated by diacylglycerol (DAG) and phosphatidylserine (PS) in a calcium dependent manner (Steinberg, 2008). Novel PKCs include PKCδ, PKCε, PKCη and PKCθ, and are calcium independent, whilst being activated by DAG and PS. Atypical PKCs consist of PKCζ, PKCλ and PKCι, and are also calcium independent, unresponsive to DAG but can be sensitive PS.
Table 1.4: Activation cofactors for PKC subgroups.

<table>
<thead>
<tr>
<th>PKC Isoforms</th>
<th>Diacyglycerol (DAG)</th>
<th>Phosphatidylserine (PS)</th>
<th>Calcium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conventional: PKCα, βI, βII, γ</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Novel: PKCδ, ε, η, θ</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Atypical: PKCζ, λ/ι</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
</tbody>
</table>

All PKC members consist of a highly conserved regulatory domain and kinase domain (Figure 1.6). The regulatory domain is located in the amino terminal, which also carries the auto-inhibitory pseudosubstrate domain that maintains the PKC in an inactive conformation, and membrane targeting modules C1 and/or C2 that regulate the subcellular localisation of PKC. The amino terminal is linked by a flexible hinge segment to the carboxy-terminal, which contains the kinase domain. The kinase domain contains motifs that are required for ATP/substrate binding and catalysis (Newton, 2003) (Parker and Murray-Rust, 2004). Conventional PKCs encompass domains C1a/C1b and C2, where C1a/b, binds to diacyglycerol (DAG), and phorbol esters such as 12-O-tetradecanoylphorbol-13-acetate (PMA/TPA) (Hurley, 2006) to become activated. The C2 motif confers sensitivity to anionic lipid phosphatidylserine (PS) in a calcium dependent manner (Blumberg et al., 2008). Novel PKCs also contain C1a/C1b and C2 domains, however the locations are exchanged (relative to conventional PKCs) at the amino terminal. Furthermore the C2 domain is non-functional and lacks a calcium binding site. Finally, the atypical PKCs also lack functional C2 motifs, but contain an atypical C1 domain that confers sensitivity to PS (Steinberg, 2012).
Figure 1.8: Structure of PKC isoforms. Pseudosubstrate motif (pink), C1 domain (blue), C2 domain (red), kinase domain (green).

Table 1.5: PKC isoform tissue expression and function

<table>
<thead>
<tr>
<th>Group</th>
<th>PKC Isoforms</th>
<th>Tissue Expression</th>
<th>Knockout mouse phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conventional</td>
<td>PKCα</td>
<td>Ubiquitous</td>
<td>Reduced proliferation, defective IgG switching, reduced IFNγ production</td>
<td>(Pfeifhofer et al., 2006; Tibudan et al., 2002)</td>
</tr>
<tr>
<td></td>
<td>PKCβ</td>
<td>Ubiquitous</td>
<td>Mast cell defect</td>
<td>(Nechushtan and Razin, 2001)</td>
</tr>
<tr>
<td></td>
<td>PKCγ</td>
<td>Brain</td>
<td>ND</td>
<td>(Saito and Shirai, 2002)</td>
</tr>
<tr>
<td></td>
<td>PKCδ</td>
<td>Ubiquitous</td>
<td>Enhanced IL-2 secretion, proliferation, proapoptotic</td>
<td>(Gruber et al., 2005)</td>
</tr>
<tr>
<td></td>
<td>PKCe</td>
<td>Ubiquitous</td>
<td>Macrophage defect, influence on nervous system, faulty bacterial clearance</td>
<td>(Castrillo et al., 2001; Kumar et al., 2002)</td>
</tr>
<tr>
<td></td>
<td>PKCη</td>
<td>Ubiquitous</td>
<td>Impairment of epithelial regeneration in wound healing, increased susceptibility to tumour development in SCC, defective homeostatic proliferation</td>
<td>(Chida et al., 2003; Fu et al., 2011)</td>
</tr>
<tr>
<td></td>
<td>PKCB</td>
<td>T cells, platelets, monocytes</td>
<td>Reduced proliferation and IL-2 production, abrogated AP-1 and NF-κB, impaired Th2 immunity against <em>N. brasiliensis</em></td>
<td>(Marsland et al., 2004; Salek-Ardakani et al., 2004; Sun et al., 2000)</td>
</tr>
<tr>
<td>Atypical</td>
<td>PKCζ</td>
<td>Ubiquitous</td>
<td>Impaired Th2 cytokine secretion</td>
<td>(Martin et al., 2005)</td>
</tr>
<tr>
<td></td>
<td>PKCζ/λ/ι</td>
<td>Ubiquitous</td>
<td>Lethal phenotype</td>
<td>(Bandyopadhyay et al., 2004; Farese et al., 2007)</td>
</tr>
</tbody>
</table>

ND: not disclosed
SCC: squamous cell carcinoma
1.6.2. PKC mechanisms of activation

In an inactive state, the regulatory domain is bound to the kinase domain, inhibiting the activity of the PKC. PKCα activity is regulated through the phosphorylation of three residues in the kinase domain: the activation loop site Thr-497, the autophosphorylation site Thr-638 and the hydrophobic C-terminal site Ser-657 (Nakashima, 2002). The PKCα is released into the cell cytosol once all three sites are phosphorylated (Newton, 2001). PKCα then interacts with specific cofactors DAG and PS, where the intermolecular bond between them will dissociate and a conformational change will be initiated leading to its translocation to the cell membrane (Figure 1.8).

1.6.3. PKCα

PKCα is involved in a number of roles in the mammalian system, including processes that are linked to wound healing, suggesting that PKCα may also have a key role in skin homeostasis. PKCα is a potent regulator of the cell cycle with epidermal over-expression leading to keratinocyte hyperproliferation suggesting its involvement in repopulation of keratinocytes in the epidermis after injury. PKCα also regulates inflammation as overexpression results in increased levels of cytokines (CCL3, CXCL2, Cox-2) (Cataisson et al., 2003). Research shows that activators and inhibitors of conventional PKCs regulate the switch for calcium independency and dependency (Wallis et al., 2000).

Differential expression of PKC isoforms in the epidermis illustrates diverse roles in keratinocyte biology (Breitkreutz et al., 2007). Keratinocyte terminal differentiation may be tightly regulated by PKCα. When PKCα is activated by TPA, it localises to the membrane of suprabasal keratinocytes in the spinous and granular layers of the epidermis coincident with the initiation of terminal differentiation (Tibudan et al., 2002).

The induction of keratinocytes to proliferate may result in abnormal regulation, demonstrated in basal and squamous cell carcinoma, chronic wounds and in psoriasis. PKC is a known trigger of hyperproliferation; treatment with phorbol esters such as TPA can activate PKC to increase proliferation (Palazzo et al., 2017).
The most widely used active phorbol esters are: TPA (4β-12-O-tetradecanoylphorbol-13-acetate) and PDBu (4β-phorbol-12,13-dibutyrate). They differ only by their substitutions at positions 12 and 13 of ring B. TPA, also called phorbol-12-myristate-13-acetate (PMA), is a phorbol ester and a potent tumour promoter used to activate the signal transduction enzyme PKC. The effects of TPA on PKC result from its similarity to one of the natural activators of classic PKC isoforms, diacylglycerol (DAG). TPA acts as an analogue for DAG (Segal, van Duuren, and Mate 1975). The regulation of PKC by DAG and TPA occur by the same mechanism, with some differences in the strength of interaction (Silinsky and Searl 2003), with TPA being stronger. TPA hyperactivates PKC and triggers cell proliferation, thereby amplifying the effects of carcinogens. TPA is widely used as a biomedical research tool in models of carcinogenesis.

TPA is an oil drawn from seeds of the skin-irritating shrub, the croton plant, a shrub found in Southeast Asia (Pagani et al., 2017). The interaction of TPA with PKC affects activities of several enzymes, biosynthesis of protein, DNA, polyamines, cell differentiation processes, and gene expression. During normal signal transduction, the enzyme is activated by DAG, which is then rapidly hydrolysed. DAG is responsible for activating PKC function by increasing its affinity for phosphatidylserine (PS)-containing membranes. Upon activation, PKC enzymes are translocated to the plasma membrane by RACK (receptor for activated C kinase) proteins to carry out various other signal transduction pathways.

The regulatory domain on PKCα serves as the binding region for TPA. The region contains two β sheets, which separate a water-filled cavity. After displacing a molecule of water, TPA fits into this opening, replacing lost hydrogens by oxygens at the C3, C4 and C20 positions. The binding of the TPA in the C1 domain allows for a hydrophobic cover that
aids the insertion and anchoring of the complex into membranes with little conformational change. Once insertion into the membrane has been completed, the catalytic domain of PKC is activated and, phosphorylation begins with the appropriate substrates bound to the C4 domain (Silinsky and Searl, 2003).

1.6.5. Inhibitors of PKC

In our experiments, we used a pharmacological compound, Gö6976, which has previously been demonstrated to inhibit PKC isoenzymes α and βI. It is a derivative of indolocarbazoles (ICZs) which are a class of compounds that studied due to their potential as anti-cancer drugs. It is also known to have a three-fold greater specificity to PKCα than PKCβ. It acts as an ATP-competitive inhibitor.

Sotrastaurin (AEB071) is an investigational immunosuppressant that blocks T-lymphocyte activation through protein kinase C inhibition. It was tested as a monotherapy in a clinical trial in patients with moderate to severe plaque psoriasis. Dosages of 200mg to 300mg were administered twice a day for two weeks which improved the psoriasis area severity index significantly from baseline compared to placebo (Skvara et al., 2008) This study provided evidence that sotrastaurin is a potential treatment of T-cell driven diseases, and served as a foundation study to enter Phase II clinical trials for renal transplantation (Friman et al., 2011; Wagner et al., 2011). Phase II clinical trials in both psoriasis and renal transplantation showed evidence of efficacy of sotrastaurin (He et al., 2014a) Sotrastaurin is a low-molecular-weight, synthetic compound that strongly and reversibly inhibits PKC-theta, PKCα, and PKCβ with lesser activity on PKC-delta (Wagner et al., 2009). Sotrastaurin therefore provides a new mechanism of action when compared to current treatments.

1.6.6. PKCα involvement in psoriasis

Keratinocytes secrete cytokines including IL-1, IL-6, IL-8, IL-17, IL-23 and interferon-gamma (IFNγ) (Pietrzak et al., 2008). A number of cutaneous inflammatory diseases are associated with alterations in cytokines, including psoriasis. Out of the PKC isotypes, six are expressed in human and mouse epidermis in vivo (Wang and Smart, 1999), with each one playing a specific role in keratinocyte function. Changes in PKC isotype expressions
and increased levels of DAG occur in psoriatic human epidermis suggesting an important role in the pathogenesis of psoriasis (Reynolds et al., 1993). PKCα is a major conventional PKC isotype expressed in the epidermis (Cataisson et al., 2003). It is involved in a number of roles in the mammalian system, including processes that are linked to wound healing, suggesting that PKCα may also have an active role in wound healing itself (Thomason et al., 2012). PKCα is a potent regulator of the cell cycle with epidermal overexpression leading to keratinocyte hyperproliferation suggesting its involvement in repopulation of keratinocytes in the epidermis after injury. PKCα also regulates inflammation as overexpression has resulted in increased levels of cytokines such as TNF-α, IL-8, IL-6, and IFNγ (Cataisson et al., 2003). Research also shows that activators and inhibitors of conventional PKCs regulate the switch for calcium independency and dependency (Wallis et al., 2000).

PKCα was initially detected in the early 1990’s in keratinocytes in vitro and in vivo (Dlugosz and Yuspa, 1993; Wang et al., 1993), mainly restricted to the suprabasal layer of the epidermis (Denning, 2004). It has also been shown an increase in TPA-induced PKC activation (Passos et al., 2013). To date, there is no literature that specifically looks at PKCα expression levels in psoriatic patients, however, we know that sotrastaurin, a PKC inhibitor, acts by predominantly blocking PKCα, PKCθ, and PKCβ isoforms in T cells, which resulted in an improved phenotype of psoriasis in patients (Skvara et al., 2008).

**1.7. Current therapy for psoriasis**

Psoriasis is considered as a Th1/Th17 disease as it plays a key role in the cytokine network in the pathogenesis of psoriasis, in keratinocyte hyperproliferation and inflammation. Psoriasis, until recently has been considered as a classical Type 1 autoimmune disease, with a strong IFNγ Th1 signal. However, a subset of T cells, Th17 cells, have been found to be involved in murine models of autoimmune inflammation, and in psoriasis. Th17 cells produce IL-17, IL-22, and have other important downstream pro-inflammatory effects in skin (Zaba et al., 2009). Due to this, a number of treatments, targeting products in this pathway, are being used in patient suffering from psoriasis. Current psoriasis treatments that are reducing the severity of the disease are targeted at: TNF-α (etanercept, adalimumab, infliximab) (Iskandar et al., 2017; Kircik and Del Rosso, 2009), IL-12/23p40 (ustekinumab) (Samuel and Reynolds, 2017), IL-17A
(secukinumab, ixekinumab), and IL-17 receptor (brodalumab) (Chiricozzi and Krueger, 2013). Treatments involving the inhibition of IL-1α/β, IL-6, IFNγ have so far been unsuccessful, however other products such as IL-19, IL-20, IL-22 (fezakinumab) (Tsai and Tsai, 2017), IL-23p19 (CNOT1959) and IL-36 cytokines and their respective receptors are being investigated into as potential therapeutic targets (Baliwag et al., 2015). Although psoriasis is considered as an inflammatory disease, the role of the epidermal keratinocytes in psoriasis is undetermined. A number of studies outline altered epidermal differentiation in psoriasis; however there is much debate of whether epidermal defects are caused by the inflammatory response or vice versa.

1.7.1. Clinical studies linking PKCs to psoriasis

A clinical study showed oral administration of PKC inhibitor sotrastaurin AEB071 in a dose dependent manner over 2 weeks inhibited activation of peripheral blood T cells in human volunteers, with a reduction in clinical symptoms of psoriasis. AEB071 inhibited a broad range of PKC isoforms and was therefore suggested to be a potential therapeutic agent for autoimmune diseases. However, further studies and analysis were needed to establish long-term safety, clinical efficacy and tolerability (Skvara et al., 2008). Phase II clinical trials in both psoriasis and renal transplantation showed evidence of efficacy of sotrastaurin (He et al., 2014a).

Since 2008, Phase II clinical trials in both psoriasis and renal transplantation showed evidence of efficacy of sotrastaurin (He et al., 2014a). They showed that the AEB071 inhibited effector T cell response (CD28-induced T-cell) and pro-inflammatory cytokines. However, an enhanced regulatory response was present with high levels of T regulatory cell markers such as Foxp3 and CD25 expression and lacked of IL-17A and IFNγ production. Even when stimulated by Th17 (IL-1b/IL-23), AEB071 enhanced Foxp3 expression and prevented IL-17A. This study concluded that pharmacological inhibition of PKCs can be instrumental in inhibiting effector T cells and facilitate regulatory T cells, and could therefore be a potential therapy the treatment of psoriasis (He et al., 2014a).
1.7.2. Research on PKCα mice psoriasis

K14Glp65 and TK-CA-PKCα mice were crossed to produce a bitransgenic mouse TK-CA-PKCα mouse. This bitransgenic mouse was developed by Thomason et al in the Hardman laboratory to study the role of PKCα in wound healing (Thomason et al., 2012) (Figure 1.10). They found that overexpression of PKCα in the CA-PKCα mouse model PKCα promotes re-epithelialization. However, as a side effect, the observed the CA-PKCα mice to exhibit similar histological features to human psoriatic skin phenotype. RU486 was topically applied for five days on CA-PKCα mice to activate the overexpression of PKCα. Without the RU486 treatment, the mouse exhibited normal activity. Ru486 binds to mutant progesterone receptor in the K14Glp65 construct resulting in a conformational change which exposes DNA binding domains GAL4 on the TK-CA-PKCα construct. The K14Glp65 construct can then bind to the Gal4 sites to stimulate the expression of CA-PKCα construct. Compared to RU486 treated wild-type mice, CA-PKCα mice show typical psoriasis-like phenotype. These pilot studies suggest that CA-PKCα mice could be a potential model of human psoriasis, exhibiting similar key characteristics of human psoriasis such as abnormal proliferation and intraepidermal inflammation.

The TPA-induced psoriasis mouse model has been utilised by many researchers to study psoriasis, since TPA acts as analogue for DAG, thereby activating PKCs (Nakajima and Sano, 2018). Inflammation is induced by topically applying or by subcutaneous injections to either on the dorsal skin or the back of the ear daily (Hvid et al., 2008; Madsen et al., 2016). In our experiments, we have used a global PKCα knockout mouse. This mouse does not exhibit any unusual symptoms or activity in comparison to wildtype mice. TPA is well known for its tumour promoting activity (Hennings et al., 1983) through the induction of clonal expansion of cells carrying activated ras oncogenes (Dotto et al., 1985). The PKC family has been recognised as the main target for TPA, but their role in tumour promotion remains debateable (Antal et al., 2015). PKC activation by TPA also results in enhanced Ras/Raf-1/MAP kinase (Wen-Sheng, 2006) and Wnt/β-catenin signalling cascade (Su et al., 2018). Moreover, TPA induces the activation of NF-κB and AP-1 transcription factors in mice skin, leading to an effect on NH2- terminal kinase, Akt, ERK and c-Jun (Kundu et al., 2006).
Figure 1.9: Figure 5: TK-CA-PKCα construct. K14Glp65 and TK-CA-PKCα mice are crossed to produce a bitransgenic mouse consisting of both constructs with inducible CA-PKCα expressed in the epidermis. The K14Glp65 construct is under keratin 14 promoter and is therefore expressed in the epidermal keratinocytes. Ru486 is topically applied and binds to mutant progesterone receptor in the K14Glp65 construct resulting in a conformational change which exposes DNA binding domains GAL4 on the TK-CA-PKCα construct. The K14Glp65 construct can then bind to the Gal4 sites to stimulate the expression of CA-PKCα construct. This novel mouse model will be used to carry out immunohistochemistry techniques to identify specific markers in normal and psoriatic skin.
1.8. Aims and Hypothesis

The development of new targeted therapeutic approaches for psoriasis requires a detailed understanding of the mechanism by which psoriasis is initiated and maintained.

Whilst there are systemic therapies available that act on immune cells that have been successful in managing psoriasis, it is also likely that cross talk from keratinocytes both initiate and drive pathogenesis of the disease. There is a wealth of evidence to suggest that keratinocytes in the epidermis are altered during psoriasis, affecting cell migration and subsequent differentiation from basal layer to the stratum corneum. PKCα is known to be involved in a multitude of signalling pathways, including proliferation and differentiation of keratinocytes. Although PKCα has been shown to play a role in a number of diseases, it is as yet unclear how it contributes to the pathogenicity of psoriasis.

I hypothesise that PKCα plays a role in psoriasis, particularly in the enhanced keratinocyte proliferation, migration and differentiation seen in disease. Here I will investigate a potential link between PKCα and psoriasis using in vivo models to achieve the following two primary aims:

1. To evaluate the psoriasis-like phenotype in mice overexpressing PKCα in the epidermis only, and to determine changes in specific keratinocyte markers in the epidermis. This will be achieved by:
   a. Macroscopic evaluation of the model after PKCα activation using RU486.
   b. Microscopic evaluation using BrdU and immunohistochemistry for key differentiation markers K14, K6, K10 and loricrin.

2. To determine the role of PKCα in two established mouse models of psoriasis: Aldara- and TPA-induced psoriasis. This will be achieved by:
   a. Use of PKCα globally knockout mice to study skin responses to either Aldara or TPA topical application, in the absence of PKCα. Macroscopic and microscopic evaluation will be carried out as described in Aim 1.
b. Use of topical inhibitors Gö6976 and sotrastaurin to determine efficacy of PKCα inhibition in the TPA model of psoriasis. Macroscopic and microscopic evaluation will be carried out as described in Aim 1.

c. To compare any additive effects of pan PKC inhibition with data from PKCα knockout mice to determine whether PKCα inhibition is sufficient as a primary target to reverse the psoriatic phenotype. Macroscopic and microscopic evaluation will be carried out as described in Aim 1.
2. **Materials and Methods**

2.1. **In vivo procedures and treatments**

All animal studies were carried out in accordance with regulations set out in the Animals Scientific Procedures Act (1986, updated January 2013) and approved by the UK Government Home Office (Epistem Project licence 40/3713) following local ethics committee approval. Wild type C57BL/6 and PKCα<sup>−/−</sup> mice were anaesthetised using the small rodent inhaled anaesthesia method, (2% isoflurane in oxygen (2L/min) and nitrous oxide (2L/min)). Hair was removed from the back of the mice using electric clippers (a patch of approximately 2cm x 3cm). Whilst under anaesthesia, topical application of treatments was administered over the shaven area on a daily basis for 6 days as detailed in the text.

**Table 2.1: Concentrations of chemical components for in vivo experiments**

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Weight/Volume/Concentration</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>AldaraTM</td>
<td>50g</td>
<td>Unidrug Distribution Group</td>
</tr>
<tr>
<td>Vaseline (ingredients: cetearyl alcohol, sodium cetearyl sulfate, sodium lauryl sulfate, sorbitol, sorbic acid, sodium hydroxide)</td>
<td>50g</td>
<td>Fagron</td>
</tr>
<tr>
<td>TPA</td>
<td>0.1mM</td>
<td>Sigma</td>
</tr>
<tr>
<td>Acetone</td>
<td>200ul</td>
<td></td>
</tr>
<tr>
<td>Gö6976</td>
<td>0.1mM, 0.001mM, 0.001mM</td>
<td>Stratech Scientific Ltd</td>
</tr>
<tr>
<td>Sotrastaurin</td>
<td>0.5mM</td>
<td>ApexBio</td>
</tr>
</tbody>
</table>

Daily observations along with photo of each mouse included weight and behaviour following each treatment. PASI scoring was employed by myself only for all mice which included a score out of 4 for redness, thickness of skin and scaling (range: 0 = no changes in phenotype to 4 = severe phenotype).
2.2. Genotyping (from ear punches)

Ear punch biopsies were taken from the mice to confirm mice were wildtype or PKCα−/−.

2.3. DNA Extraction

Skin was disaggregated in 300 μL of Cell Lysis Solution (Qiagen, UK) containing 1.5 μL of Proteinase K (5Prime) and incubated at 55°C overnight. The samples were centrifuged at 13,000 rpm for 5 min to separate out debris and fur, and supernatant transferred into a clean eppendorf. 100 μL of Protein Precipitation Solution (Qiagen) was added and vortexed vigorously for 20 seconds then placed on ice for 10-30 minutes to allow protein to precipitate. The samples were spun at 13,000 rpm for 5 min. Supernatant was transferred to a clean eppendorf containing 300 μL of Isopropanol (Propan-2-ol), and the tube was inverted several times to maximise precipitation. Samples were incubated at 4°C for up to 1 hr, then centrifuged at 13,000 rpm for 5 min and supernatant was discarded taking care not to dislodge the pellet. The pellet was washed with 300 μL of ice cold 70% ethanol and centrifuged again at 13,000 rpm for 5 min. Supernatant was discarded and tubes were inverted on absorbent paper for 15-20 min, then left to air dry before the pellet was resuspended in 50 μL of nuclease-free water. DNA was allowed to re-dissolve overnight at 4°C.

2.4. Polymerase Chain Reaction

PCR was carried out using a Hotstar Taq Master Mix kit (Qiagen). PCR primers (Table 2.1) were obtained as lyophilised powder and resuspended in nuclease free water at 100pmoles/μl. 1 μl of DNA was added to 0.5 μl of forward primer and 0.5μl of reverse primer. Each reaction contained: 1 μl DNA, 12.5 μl Hotstar Taq Master Mix, 0.5 μl forward primer, 0.5 μl reverse primer, 10 μl nuclease free water. The PCR reaction (Table 2.2) was carried out on PTC-100 thermocycle PCR machine (MJ Research), with the following program below before storing the samples at 4°C.
### Table 2.2: Primer sequence for genotyping

<table>
<thead>
<tr>
<th>Primer type</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKCα common</td>
<td>CTTGCTACAATGTGGTCTCGG</td>
</tr>
<tr>
<td>PKCα wildtype reverse</td>
<td>CTTACCTTGCCACTGGAAGC</td>
</tr>
<tr>
<td>PKCα mutant reverse</td>
<td>GATGCCTGCTTGCCGAATATC</td>
</tr>
</tbody>
</table>

### Table 2.3: qPCR program for genotyping

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
<th>Description</th>
<th>Cycle Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>95°C</td>
<td>1 min</td>
<td>Initial denature</td>
<td></td>
</tr>
<tr>
<td>95 °C</td>
<td>15 sec</td>
<td>Denature of nucleic acids</td>
<td>35 cycles</td>
</tr>
<tr>
<td>55 °C</td>
<td>15 sec</td>
<td>Primer annealing</td>
<td></td>
</tr>
<tr>
<td>72 °C</td>
<td>15 sec</td>
<td>Extension</td>
<td></td>
</tr>
<tr>
<td>72 °C</td>
<td>10 min</td>
<td>Final extension</td>
<td></td>
</tr>
</tbody>
</table>

#### 2.5. Agarose Gel Electrophoresis

A 2% agarose gel was prepared (Melford Laboratories) in Tris Acetate EDTA (TAE buffer) (per litre 50x TAE Buffer: 50x Tris Acetate EDTA (TAE Buffer): 242g Tris Base (Sigma-Aldrich, UK), 57.1ml Glacial Acetic Acid (Fisher Scientific), 100mL 0.5M EDTA pH8.0 (VWR), make up to 1L with dH2O). 5 μl of Safeview nucleic acid stain (NBS Biologicals) was added to the gel on cooling. 15μl of samples were loaded into the wells alongside 5μl of 1kb DNA ladder (Bio-Rad, UK). Gels were run at 80V for 60 – 90 minutes before visualising the DNA fragments in the gels using UV transilluminator (UVB Bioimaging System).

#### 2.6. Animal tissue collection

After completing the treatment, the mice were sacrificed using rising carbon dioxide concentration. Blood samples were collected from the thoracic cavity, followed with cervical dislocation. Full thickness skin from each dorsal treatment area, including underlying muscle was excised. Sections of skin samples were formaldehyde fixed (2.9% formaldehyde, 0.16M sodium chloride, 2% glacial acetic acid and 1.5 nM centrimide) for histology. Blood samples were allowed to clot and centrifuged at 13000 rpm for 10 minutes and serum removed. Samples were stored at -80°C.
Figure 2.1: 2cm by 3cm of full thickness skin from the dorsal area was excised after each treatment.

2.7. Generation of constitutively active PKCα (CA-PKCα) x K14Glp65 mice

PKCα mice were generated at The University of Manchester by Helen Thomason et al (Thomason et al., 2012). Constitutively active form of PKCα (donated by Dr Peter Parker, Decock et al 1994) were subcloned into pcDNA3.1Hygro vector upstream of a PolyA tail and downstream of a series of Gal4 binding sites (x17) and a thymidine kinase (TK) promoter. The PKCα gene was made constitutively active through a single point mutation Alanine (A25) to Glutamic Acid (E). The construct was then microinjected into fertilised 129/sv eggs in a linearized form before being implanted into wildtype pseudo pregnant females at The University of Manchester. Founder mice were backcrossed onto a wild type background, which were then crossed with K14-Glp65 mice (provided by Dr John DiGiovanni) (Matsumoto et al., 2004). Double positive mice were identified as bi-transgenic mouse that overexpresses CA-PKCα in the epidermis under the control of a K14 promoter when induced with Ru486

2.8. Histological analysis

2.8.1. Tissue processing

Skin samples for histology were submerged in formalin fixative solution (Chapter 2.2) for 24 hours at room temperature and were transferred to 70% ethanol thereafter. Tissue processing was carried out using the Tissue-TEK Vacuum Infiltration Processor (Miles Scientific, Illinois, USA) to embed the tissue in paraffin wax via graduated aqueous alcohol and citroclear.
2.8.2. Vectabond coating of slides

Glass slides were submerged in 100% acetone for 5 minutes and transferred to acetone (350ml) containing one vial of Vectabond solution (Vector Laboratories, Peterborough, UK). Slides were washed in water and left to dry overnight at room temperature.

2.8.3. Tissue embedding and sectioning

The paraffin wax embedded skin samples were sectioned to 6µM using a RM2235 microtome (Leica, Manchester, UK). Sections were floated on glass microscope slides (Scientific Laboratory Supplies Ltd, Nottingham, UK) that were pre-treated with Vectabond reagent (Vector Laboratories, Peterborough, UK) in a water bath at 45°C. Slides were allowed to dry overnight in a drying oven (45°C) before being stored at room temperature.

2.8.4. Dewaxing and rehydration

Skin sections were dewaxed (100% xylene, 15 minutes) and rehydrated using 1 minute incubations in decreasing ethanol concentrations (100%, 100%, 90%, 70% and 50%), finally rinsing in distilled water. Following staining, the sections were dehydrated by 1 minute incubations in increasing ethanol concentrations (50%, 70%, 90% 100% and 100%) and 15 minutes in xylene. The slides were mounted with Pertex mounting medium (CellPath, Newtown Powys, UK) with glass coverslips (Scientific Laboratory Supplies Ltd.).

2.8.5. Haematoxylin and Eosin staining

Slides were dewaxed and rehydrated as above, immersed in Gill’s Haematoxylin (Vector Laboratories, Peterborough, UK) for 5 minutes and rinsed in running tap water (10 minutes). To counterstain, slides were immersed in 1% Eosin (Vector Laboratories, Peterborough, UK) for 15 seconds and rinsed under running tap water (2 minutes or more). Slides were then dehydrated and mounted as described above.
2.9. Peroxidase Immunohistochemistry

Slides were dewaxed and rehydrated as described above (Chapter 2.7), and antigen was unmasked by immersing the sections in citrate buffer (Chapter 2.2) and heated in a microwave until boiling point was reached. The slides were cooled for 15-20 minutes before being washed in distilled water. A hydrophobic barrier was drawn around each section (ImmEdge pen, Vector) and slides placed in a humidity chamber. The sections were then incubated in 0.3% H₂O₂ (Chapter 2.2) for 30 minutes at room temperature. Paired primary/secondary antibodies and appropriate blocking serum (Vector) (Chapter 2.2) are shown in Table 2.3. The slides were then washed in Phosphate Buffered Saline (PBS) (Chapter 2.2) for 15 minutes followed by incubation in blocking serum (20 minutes, room temperature) (Chapter 2.2). Excess solution was wiped away carefully and sections were incubated in primary antibody, isotype control and PBS for 30 minutes at room temperature. Slides were washed in PBS for 15 minutes and incubated with biotinylated secondary antibody (30 minutes, room temperature) (Chapter 2.2). The slides were washed again in PBS for 15 minutes and incubated in ABC reagent (Vectastain ABC kit, Vector) (Chapter 2.2) for 30 minutes at room temperature. After washing the slides in PBS for 15 minutes, they were incubated in NovaRed solution (Vector) (Chapter 2.2) until uniform colour change was achieved. Slides were rinsed in running water, before counterstaining with Gill’s haematoxylin for 20 seconds. Slides were rinsed in running water and dehydrated in graded ethanol and citrusclear, mounted and cover-slipped with Pertex mounting media (CellPath, UK).
### Table 2.4: Concentrations of antibodies

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Clones</th>
<th>Supplier</th>
<th>Working Concentration</th>
<th>Raised in</th>
</tr>
</thead>
<tbody>
<tr>
<td>K5</td>
<td>Poly19055</td>
<td>BioLegend</td>
<td>1.0 μg/ml</td>
<td>Rabbit</td>
</tr>
<tr>
<td>K6</td>
<td>SP87</td>
<td>Sigma</td>
<td>2μg/ml</td>
<td>Rabbit</td>
</tr>
<tr>
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<td>Rabbit</td>
</tr>
<tr>
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<td>Poly19053</td>
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<td>Rabbit</td>
</tr>
<tr>
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<td>Monoclonal SP6</td>
<td>Abcam</td>
<td>5μg/ml</td>
<td>Rabbit</td>
</tr>
<tr>
<td>Loricrin</td>
<td>Poly19051</td>
<td>1μg/ml</td>
<td>1μg/ml</td>
<td>Rabbit</td>
</tr>
<tr>
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<td>M3/84</td>
<td>BD Bioscience</td>
<td>10μg/ml</td>
<td>Rat</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>RB6-8C5</td>
<td>Thermo Fisher</td>
<td>1μg/ml</td>
<td>Rat</td>
</tr>
</tbody>
</table>

### 2.10. Immunofluorescent Staining

Slides were dewaxed and rehydrated as described above (Chapter 2.7). Antigen retrieval was performed using citrate buffer as described above. A hydrophobic barrier was drawn around each section (ImmEdge pen, Vector) and slides placed in a humidity chamber. The sections were then incubated in 0.3% \( \text{H}_2\text{O}_2 \) for 30 minutes at room temperature and washed in PBS for 15 minutes. Paired primary/secondary antibodies and appropriate blocking serum (Vector) are shown in Table 2.3. The slides were then washed in PBS for 15 minutes followed by incubation in blocking serum (20 minutes, room temperature). Excess solution was wiped away carefully and sections were incubated in primary antibody, isotype control and PBS for 30 minutes at room temperature. Slides were washed in PBS for 15 minutes and incubated with secondary antibody conjugated to fluorescein in the dark (30 minutes, room temperature). The slides were washed again in PBS for 15 minutes and incubated in ABC reagent (Vectastain ABC kit, Vector) for 30 minutes at room temperature in darkness. Sections were then mounted with VECTASHIELD Antifade Mounting Medium (Vector) containing DAPI onto glass slides, cover slipped and left overnight to dry.
2.1. Quantification

Stained sections were photographed using a light microscope (Leica) with an attached camera or slides were imaged using the Slide Scanner in the Imaging Facility at The University of Manchester, and then imaged using Case Viewer. The area of concern was measured using Image Pro Premier software (Media Cybernetics). The positive stained area was calculated as a percentage of the area of concern.

Figure 2.2: Example of quantification method

2.2. Solutions

Citrate Buffer

- 2.1g Citric Acid (VWR)
- 900ml dH20
- pH to 6.0
- Make up to a total volume of 1L with dH20

Phosphate buffered saline

- 25ml Phosphate buffer (Sigma)
- 6g sodium chloride (Fisher Scientific)
- 975ml dH20
0.3% \( \text{H}_2\text{O}_2 \)
- 400\( \mu \)l 30% H2O2
- 40ml dH2O

Tissue Fixative
- 920ml dH2O
- 40ml Formaldehyde
- 20 ml Acetic Acid
- 0.5g Centrimide (Sigma)
- 9g sodium chloride (Fisher Scientific)

Blocking Serum
- drops of normal blocking serum to 10ml PBS (Vector)

Secondary antibody
- 2 drops of Biotinylated antibody to 10 ml PBS (Vector)

ABC Reagent
- To 10ml PBS add:
  - 4 drops of Reagent A (Vector)
  - 4 drops of Reagent B (Vector)

Novared
- To 10ml dH2O add:
  - 6 drops reagent 1 (Vector)
  - 4 drops reagent 2 (Vector)
  - 4 drops reagent 3 (Vector)
  - 4 drops hydrogen peroxide (Vector)
3. **CA-PKCα: a novel mouse model for psoriasis**

An inducible bitransgenic mouse model was generated whereby mice expressing a constitutively active (CA) PKCα were cross-bred with K14-GLP65 mice (CA-PKCα-K14-GLP65 or CA-PKCα) as described previously (Materials and Methods, Chapter 2). Topical application of RU486 in bitransgenic mice results in expression of CA-PKCα in K14 expressing cells only in the epidermis. Whist carrying out preliminary investigations into the role of PKCα in wound healing (Thomason et al., 2012), histological analysis of unwounded skin suggested a psoriasis phenotype (unpublished data). We therefore assessed the CA-PKCα mouse as a potential model for human psoriasis.

### 3.1 RU486 induced epidermal thickening and increased proliferation in CA-PKCα mice

Histology showed a normal epidermis in untreated wildtype and CA-PKCα mice. RU486 treated wildtype mice at days 1, 2, 3, and 5 did not show any histological changes in epidermal thickness. However, RU486 treated CA-PKCα mice showed a significant increase in epidermal thickness from day 3 (2 way ANOVA, p<0.0001; Figure 3.1A, 3.1C), with a further significant increase after 5 days of treatment (2 way ANOVA; p<0.0001). RU486 treatment on CA-PKCα resulted in the development of microabscesses (arrow).

Bromodeoxyuridine (BrdU), a proliferation marker showing DNA synthesis 2 hours before sacrifice, does not increase in expression in wildtype RU486 treated mice. However, CA-PKCα mice treated with RU486 for 3 days show a significant upsurge in BrdU expression (2 way ANOVA, p<0.0001), but this reduced with an additional 2 days of RU486 treatment (2way ANOVA, p=0.0003) (Figure 3.1B, 3.1D). Interestingly, the location of proliferation was diffuse at day 3, but then became localised to the basal layer by day 5.
Figure 3.1: Topical application of RU486 induced epidermal thickening and an increase in keratinocyte proliferation in CA-PKCα mice. Representative immunohistochemical images of paraffin-embedded skin sections (5μm) of C57BL/6 wildtype and CA-PKCα mice treated with topical RU486 for 5 days. Light microscope images stained positive for (A) H&E and (B) BrdU. Histogram showing (C) epidermal thickness and (D) BrdU expression following RU486 treatment. Data representative of 1 experiment (n=5). Data represented as standard error of mean (2 way ANOVA with unpaired t-tests: p-value: * p≤0.05, ** p≤ 0.01, *** p≤ 0.001, **** p≤ 0.0001). Scale bar: 50μm.
3.2. Abnormal differentiation in 5 day RU486 treated CA-PKCα mice

Immunohistochemistry was conducted to analyse keratinocyte differentiation in order to determine whether this was perturbed after RU486 treatment. K14 expression in wildtype mice after RU486 treatment was normal, but RU486 treatment in CA-PKCα mice after 3 days showed extended K14 expression throughout the epidermis (Figure 3.2A, 3.2C). Analysis of the differentiation marker K10, showed patchy expression in the upper epidermal layer. The percentage of K10 expressing cells was depleted with RU486 treatment compared to wildtype mice after 3 and 5 days of RU486 treatment (Figure 3.2B, 3.2D), suggesting that keratinocyte differentiation was not keeping pace with the increase in proliferation.

Histology of wildtype mice after RU486 treatment showed healthy K6 expression. However 3 days of treatment of CA-PKCα mice with RU486 induced a strong up regulation of K6 throughout the entire epidermis after 3 and 5 days of RU486 (Figure 3.3A, 3.3C). Immunofluorescent staining of the late differentiation marker loricrin showed inconsistent staining in the stratum granular layer in wildtype and CA-PKCα mice. However expression did increase in CA-PKCα mice after RU486 treatment (Figure 3.3B, 3.3D, p<0.01).
Figure 3.2: Abnormal keratinocyte differentiation after 3 and 5 day RU486 treatment of CA-PKCα mice. Representative immunohistochemical images of paraffin-embedded skin sections (5μm) of C57BL/6 wildtype and CA-PKCα mice treated with topical RU486 for 5 days. Light microscope images stained positive for (A) K14 and (B) K10. Histogram showing (C) K14 and (D) K10 expression following RU486 treatment. Data representative of 1 experiment. Data represented as standard error of mean (2 way ANOVA with unpaired t-tests: p-value: * p≤0.05, ** p≤ 0.01, *** p≤ 0.001, **** p≤ 0.0001). Scale bar: 50μm.
Figure 3.3: Increased inflammatory marker K6 and abnormal loricrin expression after 5 days of RU486 treatment in CA-PKCα mice. Representative immunohistochemical images of C57BL/6 wildtype and CA-PKCα mice treated with topical RU486 for 5 days. (A) Light microscope images of paraffin-embedded skin sections (5μm) stained positive for K6. (B) Immunofluorescence staining of paraffin-embedded skin sections (5μm) stained positive for loricrin. Data representative of 1 experiment. Data represented as standard error of mean (2 way ANOVA with unpaired t-tests: p-value: * p≤0.05, ** p≤ 0.01, *** p≤ 0.001, **** p≤ 0.0001). Scale bar: 50μm.
3.3. RU486 treatment induced macrophage and neutrophil infiltration in CA-PKCα mice

Monocytes are rapidly recruited to inflammatory sites of the skin, where they differentiate and release mediators which have local effects on cell populations. Persistent macrophage activity plays a part in the development of chronic inflammatory skin diseases such as psoriasis (Valledor et al., 2010). Treatment of CA-PKCα mice with RU486 showed an increase in macrophages per mm² proportionate to the number of days of treatment (Figure 3.4A). Macrophages significantly increased after 2 days of RU486 treatment (2way ANOVA, p=0.0038), increasing further on day 3 in RU486 treated CA-PKCα mice, before levelling off from day 3 to day 5 (2 way ANOVA, p=0.992) (Figure 3.4C).

Neutrophil accumulation in the skin is one of the histological characteristics of psoriasis plaques (Perera et al., 2012). In psoriasis, neutrophils first infiltrate into the dermis at an early stage and then later into the epidermis in chronic stages (Albanesi et al., 2010). Neutrophil marker Ly-6G was found to show a transient increase that peaked after 3 days of RU486 treatment in CA-PKCα mice, followed by a reduction by day 5 (Figure 3.4D). RU486 treated wildtype mice had very few neutrophils present through the duration of the treatments.

Prominent infiltration and microabscess formation by neutrophils is also a distinct hallmark of psoriasis (Wetzel et al., 2006), which is reflected in our data (Figure 3.4E).
Figure 3.4: RU486 induces macrophage and neutrophil infiltration. (A) Macrophage marker Mac3 and (B) neutrophil marker Ly-6G staining demonstrating immune infiltration at day 1, 2, 3 and 5 days of topical treatment of RU486 in C57BL/6 wildtype and CA-PKCa mice. Quantification of (C) macrophages and (D) neutrophil per mm². (E) H&E and neutrophil stained serial section illustrating an epidermal microabscess. Data representative of 1 experiment. Data represented as standard error of mean (2 way ANOVA with unpaired t-tests: p-value: * p≤0.05, ** p≤ 0.01, *** p≤ 0.001, **** p≤ 0.0001). Scale bar: 50μm.
3.4. Summary

Previous work carried out in the Hardman laboratory at the University of Manchester shows that RU486 treatment of CA-PKCα mice induced similar histological features to human psoriasis. RU486 was topically applied for five days to CA- PKCα mice. This pilot study suggested that CA-PKCα mice had potential as a model of psoriasis, exhibiting similar key characteristics of the disease such as abnormal proliferation and intraepidermal inflammation.

In wildtype mice, activation via RU486 did not stimulate a psoriasis-like phenotype, demonstrating that only low levels of endogenous activators of RU486 are present in keratinocytes. In transgenic mice, over-expression of PKCα was not spontaneous, but inducible only by topical application of RU486. Since activated PKCα regulates calcium dependence of desmosomal adhesion, a rigid management of this specific isotope is required for efficient cell-cell adhesion involved in the formation of skin barriers. With increased PKCα activation, cell-cell adhesion would be expected to decrease thus allowing keratinocytes to differentiate and migrate upwards through the epidermis (Cataisson et al., 2003).

Epidermal thickness in RU486 treated CA-PKCα increased 6.5 fold when compared to the control (Figure 3.1C) along with a surge in proliferation of basal keratinocytes after 3 days of treatment (Figure 3.1D). Immunostaining showed a strong up regulation of K6 (Figure 3.3A), extended K14 expression (Figure 3.2A), an increase in loricrin at the granular layer (Figure 3.3B) and patchy K10 expression (Figure 3.2B), indicating changes in epidermal differentiation.

We found that 5 day RU486 treated CA-PKCα mice skin produced a phenotype that mirrored aspects of psoriasis. Studies have shown that plaques of psoriasis have a specific clinical and histological morphology, they also show distinctive signs of altered epidermal differentiation. It has been evident in a number of studies that psoriatic epidermis and dermis is thicker than in normal skin (Lowes, Zhang). Several studies have also demonstrated changes in the percentage of keratinocyte markers in psoriasis such as loricrin, K6 and K14, where they are upregulated (Man et al., 2015), which is also seen in CA-PKCα mouse model (Figure 3.2C, 3.3C-D). These dysregulations are important to note as loricrin facilitates the formation of the skin barrier (Kim et al., 2008), K14 is a
prototypic marker of dividing basal keratinocytes (Alam et al., 2011) indicating a dysfunctional epidermis. K6, not normally present in healthy epidermis is increased our study, indicating an inflamed epidermis (Hattori et al., 2002). Immunohistochemical analyses of CA-PKCα mouse model showed similar aspects such as morphological changes in epidermis and dermis, altered epidermal markers and infiltration of immune cells.

Psoriasis has been shown to be a T-cell mediated disease, with a significant role for IL-17 in pathology (LOWES, CAI). However there is also evidence suggestive of a role for macrophages in the pathophysiology of plaque and pustular psoriasis (Nickoloff and Nestle, 2004). For instance, it has been shown that neutralization of TNFα, a cytokine released by macrophages, considerably improves human psoriasis (Antoni et al., 2008). Wang et al demonstrated skin lesions in a spontaneous mouse model, the CD18 hypomorphic mice, contained large numbers of macrophages (Wang et al., 2006). It was found that these macrophages were the source of TNFα in the skin. When TNFα signalling was blocked by etanercept, psoriasiform skin inflammation was improved, thereby decreasing the number of macrophages in skin lesions and reducing the production of TNFα. Furthermore, Wang et al depleted macrophages from skin lesions using clodronate liposomes, whilst maintaining normal levels of T cells, neutrophils, mast cells, and mature Langerhans cells. Depletion of macrophages improved inflammation and reduced levels of TNFα. There is also evidence that overexpression of PKCα in mouse epidermis aggravates inflammation and increases neutrophil infiltration and the expression of TNFα, and chemokines such as IL-12 (Cataisson et al., 2005, Wang and Smart, 1999), and IL-6 (Ma et al., 2015). These cytokines are also secreted by macrophages when exposed to an inflammatory stimuli. Our data show over-expression of PKCα induces an increased level of macrophages (Figure 3.4C) in line with reported literature discussed above.

Activation of PKCα in keratinocytes is an important event that mediates neutrophil responses in the skin (Cataisson et al., 2003; Ueno et al., 2005; Wang and Smart, 1999). A study found accelerated corneal epithelial healing in the absence of PKCα was linked to reduced neutrophil migration (Chen et al., 2010). They discovered that neutrophil recruitment during the inflammatory phase after wounding was significantly reduced in PKCα knockout mice. This suggests that perhaps an overexpression of PKCα would
induce enhanced migration of neutrophils, corroborating the data presented here. Massive intraepidermal influx of immune cells is unusual in healthy skin, hence it can be speculated that PKCα activation in plaque and pustular psoriasis may have a role to play in attracting neutrophils into the epidermis resulting in the formation of microabscesses as seen in Figure 3.4E, a key marker of psoriasis. Moreover, a study conducted by Toichi et al showed the severity of psoriasis reduces during a drug-induced agranulocytosis, with a return of psoriasis following the recovery of neutrophils in the blood. A recent study was conducted by Takeshi Fukumoto using granulocyte and monocyte adsorption apheresis (GMA), a therapy that removes pathogenic granulocytes and monocytes from peripheral blood (Matsuoka et al., 2018). They found that patients with pustular psoriasis and psoriatic arthritis reacted more positively to the treatment than patients with plaque psoriasis. This is probably due to the fact that plaque psoriasis is mediated by Th-17 cells, whereas Th-1 cells, which activate neutrophils and macrophages, are involved in the development of pustular psoriasis and psoriatic arthritis.

The CA-PKCα transgenic mouse model provides a novel insight into the immune pathogenesis of psoriasis, suggesting a key role for PKCα in disease. We have shown that keratinocyte-specific activation of PKCα via topical application of RU486 leads to a phenotype closely resembling human psoriasis. Although these data suggest that PKCα can drive a psoriatic phenotype, they do not demonstrate a specific role in human disease. However, the involvement of PKCs in psoriasis has been demonstrated in clinical trials (He et al., 2014), although the precise mechanisms of action was not shown.

In summary, this mouse model shows specific hallmarks of psoriasis following 5 days of Ru486 treatment, such as epidermal thickening and increased proliferation. We see an abnormal differentiation as well as increased hyperproliferation and an increase in the late differentiation marker. The CA-PKCα transgenic mouse therefore corroborates clinical data suggesting a role for PKCα in human disease, justifying further investigation of this molecule which may prove useful as a candidate for therapy.
4. **PKCα plays a role in mouse models of psoriasis**

4.1. **Introduction**

The Aldara mouse model is a well-documented model of psoriasis, driven by the topical application of Aldara™ cream directly to the skin. This model was used to investigate the role of PKCα in a psoriatic phenotype using PKCα-/- mice.

Aldara is widely used as a topical treatment for genital and perianal warts caused by human papilloma virus (van der Fits et al., 2009), and for actinic keratosis and superficial basal cell carcinomas (Gilliet et al., 2004). Daily topical application of Aldara induces inflammation and psoriasis-like skin lesions (Flutter and Nestle, 2013). Epicutaneous application of Aldara on mouse skin induces local psoriasis-like symptoms, such as inflammation, thickening and scaling of the skin (Walter et al., 2013). Skin thickening (hyperproliferation) is caused by an increase in keratinocyte proliferation. Treatment with Aldara induces a transient increase in proinflammatory cytokines in the IL-23/IL-17 axis similar to the immune phenotype observed during human psoriasis. The active ingredient in the Aldara cream, imiquimod (5%, IMQ), acts as a ligand and potent immune activator for TLR7 in mouse, and TLRs7/8 in humans. There is no published literature showing the importance or role of PKCα in response to Aldara, but show an indirect link between PKCs and TLR7 (Saitoh et al., 2017).

4.1 **Aldara treatment results in psoriasis-like phenotype**

In vivo models can vary in severity and clinical manifestation despite standardisation of protocols. It is now more clear that the housing conditions of animals can be major contributing factors to disease induction and manifestation (Nakajima and Sano, 2018). In order to confirm that the application of Aldara induces a psoriasis-like phenotype similar to previously reported (Walter et al., 2013), Aldara cream and control cream (vehicle) was applied on the shaved dorsal skin of 5 C57BL/6 mice for 6 consecutive days (Figure 4.19). As expected, macroscopic pictures at day 7 of mice treated daily with vehicle did not show any sign of disease illustrated (Figure 4.1A) by the PASI score (Chapter 2.1). PASI ranks severity of erythema (redness), thickness, and desquamation (scale) of the dorsal skin of mice. The mice treated daily with Aldara however, showed
signs of redness (Figure 4.1B), thickness (Figure 4.1C), Scaling (Figure 4.1D). The independent scores are depicted in Figure 4.1B-D. Between day 2 and 3 of treatment, the mice treated with Aldara started showing macroscopic signs of skin inflammation (Figure 4.1A/E), visible by redness of the skin. From day 4 onwards, skin thickness significantly worsened with each subsequent daily treatment (Figure 4.1E). Additionally, the PASI score for scaling, a hallmark of psoriasis, from day 4 increased with Aldara treatment compared to untreated mice. Scaling characterises defective keratinization and is caused by keratinocyte hyperproliferation. We also saw parakeratosis, which is characterised by corneocytes in the stratum corneum with retained nuclei, and usually signifies increased cell turnover. Parakeratosis in the epidermis is abnormal. Control and untreated mice did not display any signs of inflammation, increased skin thickness or scaling, which was supported by the PASI score (Figure 4.1F, unpaired t-test). After 6 days of treatment, the animals were killed using rising carbon dioxide concentration followed by cervical dislocation and their dorsal skin was excised and placed into fixative solution (Chapter 2.1). The skin was then embedded in wax and sectioned, and stained with H&E (Chapter 2.7). Analysis of H&E-stained skin sections from the daily Aldara-treated mouse cohort (n=5) showed significantly increased epidermal thickening in comparison to untreated mice (Figure 4.1 A/F). The overall epidermal thickness was measured from below the stratum corneum to the dermo-epidermal junction (DEJ) as described in Materials and Methods.
Figure 4.1: Aldara-induced skin inflammation in mice phenotypically resembles psoriasis. (A) Macroscopic presentation on day 7 of C57BL/6 mouse dorsal skin after 6 days of treatment (n=5). Light microscope images of paraffin-embedded skin sections (5μm) stained for H&E are shown in the right hand column. (B) Redness, (C) thickness, and (D) scaling of the back skin was scored daily on a scale from 0 to 4. (E) The cumulative PASI score (red, scaling and thickness) was calculated out of a possible total of 12. (F) Quantitative analysis of epidermal thickness after vehicle and Aldara topical application using Image Pro Premier. (G) Experimental design. Data representative of 2 independent experiments. Data represented as standard error of mean (unpaired t-tests: p-value: * p≤0.05, ** p≤ 0.01, *** p≤ 0.001, **** p≤ 0.0001). Scale bar: 50μm.
4.2 Daily Aldara treatment resulted in altered differentiation of keratinocytes

The PASI scores verified a psoriasis-like phenotype of mouse skin following 6 days of Aldara treatment. In order to investigate whether treatment affected specific keratinocyte markers in the epidermis, immunohistochemical staining was performed on treated and untreated skin (n=5) for Ki67, K14, K10, K6, loricrin.

Positive staining of nuclei by Ki67 monoclonal antibody (mAb), a basal proliferation marker, was observed in the epidermis (Figure 4.2A). In normal epidermis, Ki67 positive cells were sparse in the basal layer, while in Aldara treated epidermis, Ki67 positive cells were abundant in the basal layers as well as several layers above in the suprabasal layer (Doger et al., 2007; Henno et al., 2009), indicating abnormal proliferation. This is similar to other reported studies, where proliferating cells were found in the suprabasal layer after Aldara treatment. Increase in Ki67 positive cells in Aldara treated mice, correlated with the observed increase in epidermal thickness (Figure 4.1F), in accordance with published literature (Adişen et al., 2006; Jesionek-Kupnicka et al., 2013). Increased basal keratinocyte proliferation is a sign of epithelial hyperplasia (acanthosis), a common feature in human psoriasis.

Studies have shown that K14 and K6 expression increases in psoriatic skin, alongside dysregulation of loricrin, a marker for terminally differentiated keratinocytes, which is located in the granular layer (Hirabayashi et al., 2017). K14 is a marker for dividing basal keratinocytes, normally localised in the cytoplasmic regions of the basal layers of the skin. K14 was found evenly in the entire epidermal layer in both untreated and Aldara treated skin, as seen in human psoriasis (Man et al., 2015) (Figure 4.2B). We also show altered differentiation of keratinocytes where K10 was down-regulated in the psoriasis-like mouse epidermis (Figure 4.2C). However, K1/10 knockout mice do not exhibit any sever phenotype. Studies show that K1/10 knockout mice does not impair epidermal stratification, but does affect nuclear integrity and desmosomal structure (Wallace et al., 2012). In our study, we do not have a knockout of K1/10, but we do have reduced K10 expression. This may be the reason as to why we structural changes within the epidermis, where the keratinocytes are not as adhered to each other as they should be.

Analysis showed epidermal hyperproliferative marker K6 in normal skin to be restricted to the hair follicles only, but after Aldara treatment, K6 expression was also expressed
in the epidermis (Figure 4.2D), indicating hyperproliferation and inflammation. Loricrin, was expressed clearly in the control mice, but was very faint in the Aldara treated mice (Figure 4.2E).
Figure 4.2: Keratinocyte marker expression following Aldara treatment. (A-E) Light microscope images of paraffin-embedded skin sections (5μm) stained for (A) Ki67, (B) K14, (C) K10, (D) K6, (E) loricrin in C57BL/6 mouse dorsal skin after 6 days of topical Aldara treatment (n=5) with histogram showing quantified data. Data representative of 2 independent experiments. Data represented as standard error of mean (unpaired t-tests: p-value: * p≤0.05, ** p≤ 0.01, *** p≤ 0.001, **** p≤ 0.0001). Scale bar: 50μm.
4.3 Aldara treated skin contains inflammatory cell infiltrates

The main driver of the Aldara mouse model is TLR7 activation in mice, and TLR7/8 in humans. Murine immune cell populations express high levels of TLR7 include dendritic cells and macrophages which lead to downstream production of proinflammatory cytokines IFNγ and TNF-α (Flutter and Nestle, 2013).

The PASI score for redness indicated increased inflammation when treated with daily topical application of Aldara (Figure 4.1B), as well as the immunohistochemistry staining showing increased hyperproliferative keratinocyte cells (Figure 4.2D). Since daily topical Aldara treatment is characterised by immune infiltration (Nerurkar et al., 2017), we therefore decided to quantify Mac3 and Ly6G expression to look at macrophages and neutrophils respectively. We found that macrophage infiltration in the dermis (Figure 4.2A) was significantly upregulated after Aldara treatment (Figure 4.3A,D, p<0.05) compared to vehicle. Neutrophils were found in the dermis after daily Aldara treatment (Figure 4.2B), with a significant increase in comparison to vehicle treated mice (Figure 4.2E). Neutrophils were also identified in abscess-like structures in the epidermis similar to structures in psoriatic skin (Figure 4.2C). As expected, untreated/vehicle treated skin did not have any microabscesses or significant infiltration of neutrophils into the dermis or epidermis. Other general features of human psoriasis were also visible such as hyperkeratosis, which mirrored aspects of human psoriasis (Nestle et al., 2009b).
Figure 4.3: Keratinocyte marker expression following Aldara treatment. (A-E) Light microscope images of paraffin-embedded skin sections (5μm) stained for (A) Ki67, (B) K14, (C) K10, (D) K6, (E) loricrin in C57BL/6 mouse dorsal skin after 6 days of topical Aldara treatment (n=5) with histogram showing quantified data. Data representative of 2 independent experiments. Data represented as standard error of mean (unpaired t-tests: p-value: * p≤0.05, ** p≤ 0.01, *** p≤ 0.001, **** p≤ 0.0001). Scale bar: 50μm.
4.4 Treatment of PKCα knockout mice with Aldara results in less severe psoriatic phenotype

Aldara cream is thought to induce a psoriatic phenotype via CD4⁺ Th17 cells and intradermal γδ T cells in an IL-17/IL-23-dependent manner. Independently, Meisel et al found that PKCα knockout cells fail to elicit an appropriate IL-17A responses in vitro (Meisel et al., 2013), suggesting that PKCα plays a role in the dominant immune pathway induced by Aldara. We therefore treated PKCα knockout mice with Aldara, in order to determine whether PKCα plays a role in this psoriasis model.

Again, Aldara cream was applied to the shaved dorsal area of control wildtype and PKCα knockout mice (n=5) for 6 consecutive days (Figure 4.4G). At the outset, before treatment, PKCα knockout mice were no different physically or behaviourally compared to C57BL/6 wildtype mice (Figure 4.4A). Phenotypical presentation of dorsal skin at day 7 revealed that the inflammatory response to Aldara was dampened in mice lacking PKCα. The PASI score illustrated that the development of psoriasis-like skin inflammation upon Aldara treatment was less severe in PKCα knockout mice (Figure 4.4B-E).

Upon completion of treatments of all groups, dorsal skin was analysed by histology as described in Chapter 2.8. Our results showed a normal epidermis in untreated and vehicle treated wildtype and PKCα knockout mice (Figure 4.4A). However, as expected, Aldara treated control wildtype mice at day 7 displayed superficial erythema and scaling (Figure 4.4B-D). Thickness of the skin was measured from H&E stained sections using Image Pro Premier, which showed thickening of the skin treated with Aldara in both groups (Figure 4.4F), with PKCα knockout mice showing a significant reduction in overall PASI score (Figure 4.4E, p<0.05) and a statistically significant lower epidermal thickness (p<0.0001) in comparison to wildtype mice (Figure 4.4F). These data suggest that PKCα plays a role in driving epidermal thickness in the Aldara mouse model of psoriasis.
Figure 4.4: Aldara-induced psoriatic phenotype is significantly reduced in PKCα knockout mice.
(A) Macroscopic presentation of C57BL/6 wildtype and PKCα knockout mouse dorsal skin after 6 days of treatment (n=5). Below, light microscope images of paraffin-embedded skin sections (5μm) stained for H&E. (B) Redness, (C) thickness, and (D) scaling of the dorsal skin was scored daily on a scale from 0 to 4. (E) The cumulative PASI score (red, scaling and thickness) was calculated out of a possible total of 12. (F) Epidermal thickness was measured using Image Pro Premier after vehicle and Aldara topical application. n=5. (G) Experimental design. Data representative of 2 independent experiments. Significance calculated using 2 way ANOVA (p-value: * p≤0.05, ** p≤ 0.01, *** p≤ 0.001, **** p≤ 0.0001).
4.5 Absence of PKCα reduces proliferating basal keratinocytes following Aldara treatment

Following 6 days of consecutive treatment with Aldara, Ki67 on dorsal mouse skin (n=5) was analysed by immunohistochemistry in order to investigate whether PKCα is involved in epidermal proliferation. Absence of PKCα significantly reduced Ki67 positive keratinocyte cells with Aldara treatment compared to wild type, but did not completely abrogate treatment induced proliferation (Figure 4.5A/B). This suggests that PKCα contributes to keratinocyte proliferation in the epidermis, but is not the only player involved. It is also likely that this reduced proliferation contributed to the significant reduction in the epidermal thickening in the Aldara treated PKCα knockout mice in comparison to wildtype (Figure 4.4F).

As keratinocyte cells differentiate, K5/14 expression is downregulated and is replaced by other keratin pairs such as K1/10. K14, a prototypic marker for dividing basal keratinocytes aids in maintaining the epidermal cell shape, while providing resistance to mechanical stress (Alam et al., 2011). K14 and K5, normally expressed in the mitotically active basal cells in mouse skin were both expressed in all layers of the epidermis in the untreated and Aldara treated skin (Figure 4.5C/D). However, expression was patchy and less intense in treated skin resulting in significantly lower expression in both wildtype and PKCα knockout mice when compared to untreated mice (Figure 4.5 D/F). Additionally, K5 and K14 seemed to be more intense in the upper layer of the epidermis in the Aldara treated wildtype mice than the Aldara treated PKCα knockout mice (Figure 4.5 C/E). It is possible that the low staining in the sections is a technical artefact, although the staining of the sections from all treatments were carried out at the same time. K14 knockout mice have been shown to exhibit extensive blistering and die around 2 days after birth, indicating a function role in maintaining the integrity of epithelial cells. Alam et al have shown that K14 knockout mice have reduced cell proliferation, indicating a possible role in maintenance of cell proliferation. In our study, we have shown that Aldara reduces K14 expression in both wildtype mice and PKCα knockout mice, and parallel to that, PKCα knockout mice also show reduced keratinocyte proliferation.
Figure 4.5: Keratinocyte marker expression after Aldara treatment in wildtype and PKCα knockout mice. Light microscope images of paraffin-embedded skin sections (5μm) stained for (A) Ki67, (C) K14 and (E) K5 of C57BL/6 wildtype and PKCα knockout mouse dorsal skin after 6 days of Aldara treatment (n=5). Data quantified from immunohistochemistry using Image Pro Premier for (B) Ki67, (D) K14 and (F) K5 expression. Data representative of 2 independent experiments. Significance calculated using 2 way ANOVA (p-value: * p≤0.05, ** p≤ 0.01, *** p≤ 0.001, **** p≤ 0.0001). Scale bar: 50μm.
4.6 PKCα does not play a role in differentiation, hyperproliferation and keratinization in the Aldara mouse model

As keratinocyte cells enter the terminal differentiation program becoming postmitotic and suprabasal, keratins K5 and K14 are substituted by K10. Percentage epidermal expression of K10 was drastically reduced during disease state following Aldara treatment (Figure 4.6A/B), although the location was maintained in the upper layers of the epidermis. Keratin K6 which is normally absent from the interfollicular epidermis, is induced during disease state (Mommers et al., 2000). Our data also show that after topical Aldara treatment, K6 increases in expression, but with no significant differences between wildtype and PKCα knockout mice (Figure 4.6C/D). Loricrin was shown clearly in the control mice, but faint in the Aldara treated PKCα knockout and wild type mice (Figure 4.6E/F).

In summary, keratinocyte markers investigated ie: K5, K14, K10, and loricrin, showed no significant difference between wildtype and PKCα knockout mice when treated with Aldara. This suggest that PKCα does not play a role in differentiation, hyperproliferation and keratinization in the Aldara mouse model.
Figure 4.6: PKCα does not play a role in differentiation, hyperproliferation or loricrin expression in the Aldara mouse model. Light microscope images of paraffin-embedded skin sections (5μm) stained for (A) K10 (C) K6 and (E) loricrin of C57BL/6 wildtype and PKCα knockout mouse dorsal skin after 6 days of Aldara treatment (n=5). Data quantified from immunohistochemistry using Image Pro Premier for (B) K10, (D) K6 and (F) loricrin expression. Data representative of 2 independent experiments. Significance calculated using 2 way ANOVA (p-value: * p≤0.05, ** p≤ 0.01, *** p≤ 0.001, **** p≤ 0.0001). Scale bar: 50μm
4.7 TPA induces a psoriasis-like phenotype

Having found that PKCα may play a role in keratinocyte proliferation in the Aldara model, we wanted to investigate the role of PKCα in an alternative mouse model of psoriasis. We therefore topically applied 12-O-tetradecanoyl phorbol-13-acetate (TPA), an established driver of psoriatic pathology, directly to mouse skin daily for 6 days (Figure 4.7G). Since TPA drives global PKCs, we wanted to compare the phenotype in wildtype and PKCα knockout mice. We were interested to investigate whether a psoriatic phenotype could be achieved through application of TPA to PKCα knockout mice to highlight any specific role for PKCα.

Macroscopic presentation of mouse dorsal skin of 6 week old untreated wildtype and PKCα knockout mice at day 6 showed no disease development at gross level (Figure 4.7A), nor did either group have any changes in the PASI score throughout the 6 days (Figure 4.7B-E). However, mice that were treated with TPA daily showed changes in the phenotype of the skin from day 3 with evident redness, scaling, and thickened skin, supported by the PASI scores (Figure 4.7B-E). One of the indications, redness, appeared at day 2 in wildtype mice, and day 3 in PKCα knockout mice (Figure 4.7B). The skin thickness started increasing at day 2 in both wildtype mice and PKCα knockout mice (Figure 4.7C), while scaling started at day 3 (Figure 4.7D). The PASI scores show redness and scaling to be significantly higher in wildtype mice compared to PKCα knockout mice from day 4 (Figure 4.7B/D), from day 4 and 5 respectively, while skin thickening was not significant between strains (Figure 4.8C, p=0.0943). Combined components of the PASI score indicated that PKCα knockout mice had a less severe phenotype than wildtype mice (Figure 4.7E). Most importantly, scaling at day 7 was noticeably more severe in wildtype mice than PKCα knockout mice (Figure 4.7E). Histological examination of H&E stained sections revealed that TPA induced epidermal thickening, but interestingly there was no significant difference between wildtype and PKCα knockout mice (Figure 4.7F, p=0.4005), supporting the PASI score data.
Figure 4.7: TPA induces a psoriasis-like phenotype in C57BL/6 and PKCα knockout mice. (A) Macroscopic presentation on day 7 of C57BL/6 wildtype and PKCα knockout mouse dorsal skin after 6 days of treatment (n=5). Below, light microscope images of paraffin-embedded skin sections (5μm) stained for H&E day 7. (B) Redness, (C) thickness, and (D) scaling of the dorsal skin was scored daily on a scale from 0 to 4. (E) The cumulative PASI score (red, scaling and thickness) was calculated out of a possible total of 12. (F) Epidermal thickness was analysed day 7 using Image Pro Premier after acetone and TPA topical application. (G) Experimental design. Data representative of 2 independent experiments. Significance calculated using 2 way ANOVA (p-value: * p≤0.05, ** p≤ 0.01, *** p≤ 0.001, **** p≤ 0.0001). Scale bar: 50μm.
4.8 Keratinization is disrupted after TPA treatment in wildtype and PKCα knockout mice.

In order to investigate whether PKCα plays a role in keratinocyte differentiation, we analysed Ki67, K14, K10, K6 and loricrin expression using immunohistochemistry following 6 days of TPA treatment (n=5). As expected Ki67, a keratinocyte proliferation marker, increased in expression in both wildtype and PKCα knockout mice when treated with TPA (Figure 4.8A). However, PKCα knockout mice had significantly reduced expression when compared to wildtype mice (Figure 4.8B), demonstrating a role for PKCα in proliferation. K14 cell marker was present throughout in all cells of the epidermis regardless of treatment or strain (Figure 4.9A), but the intensity of the stain was less in PKCα knockout treated mice (Figure 4.9C).

K10, normally expressed in keratinocytes in the upper layer of the epidermis appears to be patchy in expression in TPA treated PKCα knockout mice (Figure 4.9B), resulting in significantly lower K10 expressing keratinocyte cells compared to wildtype mice (Figure 9D). Similarly, K6, a hyperproliferative marker, in TPA treated PKCα knockout mice was noticeably decreased in expression in comparison to TPA treated wildtype mice (Figure 4.10A/C). This suggests that PKCα contributes to keratinocyte differentiation and hyperproliferation.

Loricrin expression in untreated and vehicle treated groups (n=5) remained similar throughout for wildtype and PKCα knockout mice (Figure 4.10B). However, after 6 days of daily treatment of TPA, loricrin expression was significantly lower in PKCα knockout mice (Figure 4.10D). Loricrin expression in wildtype mice was present in at least two layers of keratinocytes from the stratum corneum, but appeared to be patchy or absent in PKCα knockout mice in comparison (Figure 4.10B). Our data indicates that PKCα plays a role in keratinization in the TPA mouse model.

In summary, TPA-induced psoriasis-like skin lesions express patchy loricrin and K10 expression in PKCα knockout mice. As expected, K6 expressing cells were elevated with TPA induced inflammation, but interestingly, K6 expression was lower in the absence of PKCα. This suggests that in this model, PKCα may have a role in both keratinocyte hyperproliferation and differentiation.
Figure 4.8: PKCα contributes to proliferation of basal keratinocytes. Light microscope images of paraffin-embedded skin sections (5μm) stained for (A) Ki67 of C57BL/6 wildtype and PKCα knockout mouse dorsal skin after 6 days of TPA treatment (n=5). Data from quantified from immunohistochemistry using Image Pro Premier (B). Data representative of 3 independent experiments. Significance calculated using 2 way ANOVA (p-value: * p≤0.05, ** p≤ 0.01, *** p≤ 0.001, **** p≤ 0.0001). Scale bar: 50μm
**Figure 4.9: PKCα is important in differentiation of keratinocytes.** Light microscope images of paraffin-embedded skin sections (5μm) stained for (A) K14 and (B) K10 of C57BL/6 wildtype and PKCα knockout mouse dorsal skin after 6 days of TPA treatment (n=5). Data from quantified from immunohistochemistry using Image Pro Premier for (C) K14 and (D) K10 expression. Data representative of 3 independent experiments. Significance calculated using 2 way ANOVA (p-value: * p≤0.05, ** p≤ 0.01, *** p≤ 0.001, **** p≤ 0.0001). Scale bar: 50μm
Figure 4.10: PKCα contributes to keratinization and hyperproliferation of keratinocytes. Light microscope images of paraffin-embedded skin sections (5μm) stained for (A) K6 and (B) loricrin of C57BL/6 wildtype and PKCα knockout mouse dorsal skin after 6 days of TPA treatment (n=5). Data quantified from immunohistochemistry using Image Pro Premier for (C) K6 and (D) loricrin expression. Data representative of 3 independent experiments. Significance calculated using 2 way ANOVA (p-value: * p≤0.05, ** p≤ 0.01, *** p≤ 0.001, **** p≤ 0.0001). Scale bar: 50μm.
4.9 Comparison of psoriatic mouse models in PKCα knockout mice

In order to assess which model is more suitable for the study of PKCα in psoriasis, we directly compared data from both the Aldara and TPA mouse models.

It is clear from the H&E images that untreated wildtype and PKCα knockout mice do not exhibit any physical differences before Aldara or TPA treatment (Figure 4.11A). In both models, treatment led to increased epidermal thickness and retention of nuclei in the stratum corneum, a hallmark of human psoriasis. Direct quantitative comparison of epidermal thickening showed that TPA treatment induced a more severe thickening than application of Aldara (Figure 4.11C). However loss of PKCα only influenced epidermal thickening after Aldara treatment (Figure 11C, p<0.0001). There was no significant difference in PASI scores for PKCα knockout mice between each of the models (Figure 4.11D), however, there was a trend towards a higher PASI score in TPA treated wildtype mice. PKCα knockout mice had a lower PASI score than wildtypes in both models.
Figure 4.11: Aldara and TPA mouse models both exhibit psoriasis-like features, but with significantly thicker epidermis in the TPA model. Light microscope images of paraffin-embedded skin sections (5μm) stained with H&E for (A) control groups (B) treatment groups of C57BL/6 wildtype and PKCa knockout mouse dorsal skin after 6 days of Aldara and TPA treatment (n=5). Data quantified from immunohistochemistry using Image Pro Premier for (C) epidermal thickness. (D) The cumulative PASI score (red, scaling and thickness) was calculated out of a possible total of 12 for Aldara and TPA models. Data representative of 2 independent experiments for Aldara treatment and 3 independent experiments for TPA treatment. Significance calculated using 2 way ANOVA (p-value: * p≤0.05, ** p≤ 0.01, *** p≤ 0.001, **** p≤ 0.0001). Scale bar: 50μm
4.10 Aldara mouse model presents with less severe disruption in keratinocyte proliferation and keratinization.

The percentage of Ki67 positive keratinocytes in the Aldara mouse model in both strains is significantly lower than that of the TPA treated mice, suggesting that TPA has a more direct effect on proliferation than Aldara (Figure 4.12A). In both mouse models, absence of PKCα resulted in depleted levels of proliferating cells in comparison to wildtype mice, confirming that PKCα plays a role in proliferation of keratinocytes in the epidermis. This is verified by a number of reports that demonstrate the involvement of PKC in regulating target proteins associated with proliferation (Musashi et al., 2000).

Interestingly, percentage of K14 expression was the same in treated wildtype and PKCα knockout mice in both models (Figure 4.12B). Although our data contradicts published data in respect of mRNA expression of K14 in psoriasis, this is the first time that K14 protein expression has been studied in psoriatic models. Our data show a down regulation of K14 protein expression after Aldara treatment. It is well known that down regulation of K14 leads to defects in keratin filament formation in basal keratinocytes which consequently causes blistering of the skin due to the fragility of the basal layer in human and mouse skin. Autosomal dominant mutations of K14 have been associated with skin blistering disease Epidermolysis Bullosa simplex (EBS) (Coulombe, 2016).

K10 expression after Aldara treatment was not significantly different between wildtype and PKCα knockout mice. However after TPA treatment, the absence of PKCα caused a significant reduction in K10 expression (Figure 4.12C). This suggests that after pan activation of PKCs in the TPA mouse model, PKCα plays a role in differentiation. During disease state or injury, keratinocytes down-regulate K10 expression (Wong and Coulombe, 2003).

As K10 is down regulated during disease state, K6 expression is up-regulated. TPA treatment induced higher levels of hyperproliferative keratinocytes than Aldara treated groups (figure 4.12D), which also correlated with increased epidermal thickness (Figure 4.11C). Data here is indicative of more intense hyperproliferation in the TPA mouse model than in the Aldara mouse model. However, observations show no differences between wildtype and PKCα knockout in both mouse models, suggesting PKCα does not contribute to keratinocyte hyperproliferation in either.
Loricrin expression in TPA treated wildtype mice was noticeably elevated in comparison with Aldara treated mice (Figure 4.12E). Interestingly, lack of PKCα in the TPA mouse reduced loricrin expression back to levels expressed in normal untreated mice, which as discussed above, suggests a role for PKCα in keratinization and desquamation. The same cannot be said for mice in the Aldara group, since no differences were seen between wildtype mice and PKCα knockout mice following Aldara treatment.

In summary, TPA treatment induces a more severe psoriasis-like phenotype in wildtype mice than Aldara treatment with respect to increased epidermal thickness, Ki67, K14, K10, K6 and loricrin expression. Lack of PKCα improves the psoriasis-like phenotype of both models in terms of reduced Ki67 expression, indicating a role for PKCα in proliferation only in both mouse models.
Figure 4.12: Dysregulation of keratinocyte differentiation in Aldara and TPA treated mice. Data quantified from immunohistochemistry of C57BL/6 wildtype and PKCα knockout mouse dorsal skin after 6 days of Aldara and TPA treatment (n=5). Quantified using Image Pro Premier for (A) Ki67, (B) K14, (C) K10, (D) K6 and (E) loricrin. Data representative of 2 independent experiments for Aldara treatment and 3 independent experiments for TPA treatment. Significance calculated using 2 way ANOVA (p-value: * p≤0.05, ** p≤ 0.01, *** p≤ 0.001, **** p≤ 0.0001).
4.11 Summary

This chapter has discussed the Aldara and TPA mouse models, both of which are established models of psoriasis. In our experiments, both mouse models employed topical application of treatments on dorsal skin of C57BL/6 wildtype and PKCα knockout mice daily over 6 days, with samples collected on the 7th day.

We observed a number of key similarities between the Aldara and TPA mouse models as described in the previous section. Our data show that both mouse models induce a thickening of the epidermis (Figure 4.11C), and increased proliferation as measured by Ki67. (Figure 4.12A) Immunohistochemical analysis revealed epidermal hyperproliferation, increased dermal thickness with infiltration of immune cells, presence of microabscesses, and parakeratosis/hyperkeratosis indicating altered epidermal differentiation.

However, PKCα deficiency induced more significant changes in the TPA model. Both wildtype and PKCα knockout mice had a higher percentage of Ki67 expressing cells when treated with TPA compared to Aldara treatment (Figure 4.12A) (p≤ 0.0001) and this was reduced in the absence of PKCα. Loricrin and K10 expression were both decreased in PKCα knockout mice compared to wildtype mice after TPA treatment (Figure 4.12D-E). Interestingly, epidermal thickness and basal proliferation did not correlate across the strains after TPA treatment: PKCα deficiency significantly reduced proliferation but not epidermal thickness. As the absence of PKCα affected keratinization in the granular layer and differentiation of cells in the basal layer, this may have had a knock on effect on the epidermal thickness, compensating for reduced proliferation.

In conclusion, using two independent mouse models of psoriasis, we have shown that PKCα contributes to keratinocyte proliferation using Ki67 expression. In addition, in the presence of pan PKC activation (using TPA), the absence of PKCα induced a dysregulation of keratinocyte differentiation and keratinization, with the outcome of decreased terminally differentiated cells in the stratum corneum of the granular layer, indicated by decreased loricrin expression. This correlated with reduced scaling at macro level. Taking into consideration studies that have reported the efficacy of pan PKC inhibitor sotrastaurin in patients suffering from psoriasis (He et al., 2014), these data suggest that
the TPA model would allow more insight into the mechanism of action of PKCα during psoriatic inflammation.
5. Inhibition of PKCα/β during TPA treatment

5.1. Introduction

Sotrastaurin, a pan PKC inhibitor, has been investigated in a proof-of-concept study in patients with moderate to severe plaque psoriasis (Skvara et al., 2008). Clinical data demonstrated an improvement in disease; the PASI score revealed a significant reduction in the baseline disease activity compared to placebo. This study demonstrated that PKC inhibition has clinical potential and served as a preliminary study to enter into Phase II trials. Phase II clinical trials in both psoriasis and renal transplantation showed evidence of efficacy of sotrastaurin (Friman et al., 2011; He et al., 2014b; Wagner et al., 2011). It is well known that TPA drives PKCs in mice (Cataisson et al., 2005) and my preliminary data in PKCα knockout mice suggested that PKCα plays a key role in this model. However, global loss of any gene can lead to developmental and cellular defects that may be undetectable in health. Furthermore, a topical PKCα inhibitor has the potential to be used as a therapeutic agent for treating patients with psoriasis. To understand the effect of a lack of PKCα in real time during psoriasis induction in mice, we used a PKCα/β inhibitor (Gö6976) in conjunction with TPA for 6 days as an alternative to PKCα knockout mice.

Gö6976, a PKCα/β inhibitor (0.1M) was applied topically to C57BL/6 wildtype and PKCα knockout mice along with TPA (0.1mM). Following 6 days of treatment, dorsal skin sections were stained for Ki67, K14, K10, K6 and loricrin. We observed significant alterations in keratinocyte differentiation from basal cell layer to stratum corneum after TPA and TPA/Gö6976 treatment in both mouse strains.
5.2. Inhibition of PKCα/β reduces proliferating basal keratinocytes in wildtypes, but remains at similar levels in PKCα knockout mice

Treatment with Gö6976 alone had no significant effect on Ki67 expression (Figure 5.1A) (no significant difference between untreated, acetone and Gö6976 control only) in both wildtypes and PKCα knockout mice (Figure 5.1B). As seen in previous experiments 6 days of TPA treatment on dorsal mouse skin increased the percentage of proliferating cells in the basal layer of the epidermis in comparison to untreated mice (Figure 4.8A).

In wildtype mice, daily Gö6976/TPA treatment significantly reversed induction of proliferation of suprabasal keratinocytes to levels seen in TPA treated PKCα knockout mice (Figure 5.1B) (p<0.0001), corroborating a role for PKCα in proliferation. Additional PKCα/β inhibition in PKCα knockout mice did not further reduce proliferation (Figure 5.1B) indicating other factors are involved in proliferation.

Epicutaneous application of TPA induces local psoriasis-like symptoms in susceptible humans and in mice, such as inflammation, thickening and scaling of the skin. Given the effect on proliferation, one might expect epidermal thickness to reduce after Gö6976 treatment. However there was no significant difference between TPA and TPA/Gö6976 treated wildtype or PKCα knockout mice, although there was a trend towards decreased epidermal thickness in both treated groups (Figure 5.1C).

It is important to note that although Gö6976 (EC50: 4000nM) is a PKCα/β inhibitor, it is also involved in inhibiting additional targets. Gö6976 was found to be a potent inhibitor of JAK2 and JAK3 at nanomolar concentrations, an important signalling cascade essential for embryonic development, tissue growth and innate and adaptive immunity (Grandage et al., 2006). Gö6976 is also known to inhibit other targets such as Chk2 and ATM which are involved in cell cycle checkpoint regulation (Bain et al., 2007), resulting in reduced proliferation and increased apoptosis (Grandage et al., 2006). It is therefore possible that Gö6976 has an indirect effect on keratinocyte differentiation.
Figure 5.1: Topical application of PKCα/β inhibitor reduces proliferating basal keratinocytes in wildtype mice. Light microscope images of paraffin-embedded skin sections (5μm) stained for (A) Ki67 at day 6 of TPA treatment in C57BL/6 wildtype and PKCα knockout mice. Histogram showing (B) Ki67 expression following 6 days of daily TPA treatment. (C) Epidermal thickness was measured using Image Pro Premier after TPA topical application. Significance calculated using 2 way ANOVA (p-value: * p≤0.05, ** p≤ 0.01, *** p≤ 0.001, **** p≤ 0.0001). Scale bar: 50μm
5.3. Chemical inhibition of PKCα/β with TPA treatment does not alter K14 expression or differentiation.

K14 was evenly expressed across the whole epidermis despite treatments (Figure 5.2A); Percentage of K14 expression remains the same across all treatment groups, as well as control groups, regardless of strain or treatment (Figure 5.2C). Our data suggests that PKCα and PKCβ do not play a role in K14 expression produced by stratified squamous keratinocytes, in support of our previous data Chapter 4.

Expression of K10 is located in the upper half of the epidermis, usually positive in only one cell layer since untreated mouse skin is one or two keratinocyte cells thick. Treatment with Gö6976 alone had no significant effect on K10 expression (no significant difference between untreated, acetone and Gö6976 control only) in both wildtypes and PKCα knockout mice (Figure 5.2A). During TPA treatment in both wildtype and PKCα knockout mice, K10 remains in the upper half of the epidermis, spanning 2 or 3 cell layers, reflecting the thickened epidermis (Figure 5.2B). K10 was unchanged in all treatment groups in wildtype mice epidermis. (Figure 5.2D) In line with previous experiments, TPA treatment in PKCα knockout mice significantly reduced expression of K10 compared to wildtype mice, suggesting PKCα plays a role in differentiation. However, Gö6976 treatment of wildtype mice did not phenocopy TPA treatment of PKCα knockout mice, which was unexpected, indicating that neither PKCα or PKCβ is contributing to differentiation. However, it could also mean that the concentration of Gö6976 is not high enough to inhibit all PKCα and PKCβ in the epidermis following TPA treatment or there is some compensation for the lack of PKCα/β. Interestingly, Gö6976 inhibition of PKCα/β with TPA in the PKCα knockout mice does not alter levels of K10 expression. This suggests that PKCβ is not playing a role in keratinocyte differentiation either, unless the concentrations are not high enough since Gö6976 inhibits PKCα three times more than PKCβ.
Figure 5.2: Chemical inhibition of PKCα/β during TPA treatment does not alter K14 or K10 expression. Light microscope images of paraffin-embedded skin sections (5μm) stained for (A) K14 and (B) K10 at day 6 of TPA treatment in C57BL/6 wildtype and PKCα knockout mice. Histogram showing (C) K14 and (D) K10 expression following 9 days and 6 days of daily TPA treatment. Significance calculated using 2 way ANOVA with follow up T test (p-value: * p≤0.05, ** p≤0.01, *** p≤0.001, **** p≤0.0001). Data representative of two independent experiments. Scale bar: 50μm.
5.4. Chemical inhibition of PKCα/β with TPA treatment reduces K6 expression to PKCα knockout levels in wildtype mice, but does not affect loricrin expression

Treatment with Gö6976 alone did not produce an inflammatory response across all control groups (no significant difference between untreated, acetone and Gö6976 control only) in both wildtypes and PKCα knockout mice (Figure 5.3C). As expected with TPA treatment, K6 was upregulated in the epidermis in both wildtype and PKCα knockout mice (Figure 5.3A/C), although loss of PKCα did induce a significant 0.25 fold reduction in K6 expression as shown in Chapter 4 (Figure 4.10C). Interestingly, Gö6976 treatment of wildtype mice reduced K6 expression to similar levels observed in TPA treated PKCα knockout mice, supporting a role for PKCα in hyperproliferation (Figure 5.3C). However, during TPA treatment, inhibition of PKCα/β in PKCα knockout mice restored K6 expression to similar levels exhibited by wildtype mice, suggesting PKCβ may play a compensatory role (Figure 5.3C). This may be because following high concentration of Gö6976 treatment, which has 3 times more specificity to inhibit PKCα than PKCβ, PKCα is inhibited, but there is an influx and upregulation of PKCβ. At low Gö6976 treatment, there is not enough inhibition of PKCα, so we do not see a compensatory role for PKCβ.

Treatment with Gö6976 alone had no significant effect on loricrin expression (no significant difference between untreated, acetone and Gö6976 control only) in both wildtypes and PKCα knockout mice (Figure 5.3D). As shown previously, loricrin expression decreased with TPA treatment in both wild type and PKCα knockout mice. However, treatment with PKCα/β inhibitor in both wildtype and PKCα knockout mice did not alter the expression of loricrin across groups (Figure 5.3 B/D), demonstrating that PKCα does not play a role in loss of loricrin after TPA treatment.
Figure 5.3: Chemical inhibition of PKCα/β with TPA treatment reduces K6 expression to PKCα knockout levels in wildtype mice, but has no impact on loricrin expression. Light microscope images of paraffin-embedded skin sections (5μm) stained for (A) K6 and (B) Loricrin at day 6 of TPA treatment in C57BL/6 wildtype and PKCα knockout mice. Histogram showing (C) K6 and (D) Loricrin expression following 6 days of daily TPA treatment. Significance calculated using 2 way ANOVA with follow up T test (p-value: * p≤0.05, ** p≤ 0.01, *** p≤ 0.001, **** p≤ 0.0001). Data representative of two independent experiments. Scale bar: 50μm
5.5. Summary

In this section, we have shown that PKCα/β inhibition in wildtype mice does not have an inhibitory effect on keratinocyte cell expression of loricrin, K10 or K14. However, PKCα/β inhibition in wildtype mice caused a significant reduction in K6, similar to that observed in TPA treated PKCα knockout mice. PKCα/β inhibition also significantly reduced Ki67 to equivalent levels seen in TPA treated PKCα knockout mice.

Additional PKCα/β inhibition in PKCα knockout mice showed slight reduction in epidermal thickness (p= 0.1148), but this was independent of Ki67 which did not change. This may be due to an insufficient dosage of the Gö6976 inhibitor or due to time course of the experiment; a longer study may have yielded more significant results in this respect. PKCα/β inhibition was able to rescue the reduced K6 phenotype seen in the PKCα knockout mice, suggesting that PKCα and PKCβ work together to regulate K6 expression.
5.6. Dose dependent inhibition does not affect wildtype or PKCα knockout mice macroscopically

The data observed in previous sections demonstrate that PKCα plays a role in keratinocyte proliferation and K6 expression. This was confirmed using Gö6976, a PKCα/β inhibitor together with TPA on dorsal mouse skin for 6 consecutive days. In order to confirm this observations, we carried out a dose dependent study of Gö6976 (0.1mM, 0.01mM, 0.001mM) with co-application of TPA within the same experimental parameters.

Macroscopic images showed the control groups of both mouse strains did not have a phenotype when topically applied with Gö6976 at dosages of 0.1mM, 0.01mM and 0.001mM PKCα (Figure 5.4A). As seen previously, in wildtypes a psoriasis-like phenotype was exhibited after 6 days of TPA treatment, with a less severe phenotype when applied alongside the PKCα/β inhibitor. However, we observed a slight macroscopic improvement in psoriasis-like phenotype in both strains with lower dosages of Gö6976 at 0.01mM and 0.001mM (Figure 5.4B). Surprisingly, a trend was observed with the lower dosages of Gö6976 at 0.01mM and 0.001mM which improved the psoriasis-like phenotype in comparison to the higher Gö6976 dosages 0.1mM. (Figure 5.5A-C). In PKCα knockout mice, following TPA/Gö6976 treatment, the psoriasis-like phenotype was significantly improved compared to wildtype mice with the higher dosage of Gö6976. In wildtype mice there was a trend towards an improved phenotype with lower doses of Gö6976 (Figure 5.5D), however overall, there was no significant dose dependent effect of the PKCα/β inhibitor with TPA.
Figure 5.4: Dose dependent PKCa/β inhibition during TPA treatment had macroscopic effect. (A-B) Macroscopic presentation of C57BL/6 mouse dorsal skin after 6 days of treatment (n=4). (A) Control treatments without TPA. (B) Co-treatments with TPA. Data representative of 3 independent experiments for Gö6976 at 0.1mM and 1 independent study for Gö6976 at 0.01mM and 0.001mM.
Figure 5.5: Dose dependent PKCα/β inhibition during TPA treatment has no significant effect on PASI score in wildtype or PKCα knockout mice. (A) Redness, (B) thickness, and (C) scaling of the back skin was scored daily on a scale from 0 to 4 in wildtypes and PKCα knockout mice (data not shown). (D) The cumulative PASI score (red, scaling and thickness) is illustrated (n=4). Data representative of 3 independent experiments for Gö6976 at 0.1mM and 1 independent study for Gö6976 at 0.01mM and 0.001mM. Data represented as standard error of mean (unpaired t-tests: p-value: * p≤0.05, ** p≤ 0.01, *** p≤ 0.001, **** p≤ 0.0001). Scale bar: 50μm.
5.7. High dosage of PKCα/β inhibitor is effective in reducing keratinocyte proliferation.

We stained skin sections for the proliferation-associated protein Ki67 (Figure 5.6A) to study dose dependent effects of Gö6976 on proliferation. Immunohistochemical staining showed low levels of Ki67 expressing cells in all the control groups (acetone and Gö6976 at 0.1mM, 0.01mM and 0.001mM, (Figure 5.6B), with no significant difference in comparison to untreated mice. All doses of Gö6976 reduced Ki67 expression in wildtype mice in the basal and suprabasal layer in comparison to TPA treated wildtype mice (0.1mM p ≤ 0.0001, 0.01mM p=0.0196, 0.001mM p ≤ 0.0001). 0.1mM was most effective (p<0.0001), (Figure 5.6C) however lower doses were comparable to TPA treatment of PKCα knockout mice without inhibitor.

We found that PKCα/β inhibition had no additional inhibitory effect on Ki67 expression in PKCα knockout mice.
Figure 5.6: PKCα plays a key role in proliferation. (A) Light microscope images of paraffin-embedded skin sections (5μm) stained for Ki67 at day 6 after concurrent TPA and Gö6976 treatment in C57BL/6 wildtype and PKCα knockout mice. Quantification of Ki67 expression in (B) control and (C) treatment groups after 6 days of daily TPA and Gö6976 treatment. Data representative of 1 study (n=6). Significance calculated using 2 way ANOVA with follow up T test (p-value: * p≤0.05, ** p≤ 0.01, *** p≤ 0.001, **** p≤ 0.0001). Scale bar: 50μm
5.8. K10 expression inversely correlates with PKCα/β inhibition in wildtype mice

K14 was expressed across the epidermis in control groups as well as treated groups regardless of strain or treatment or dosage (Figure 5.7A). As seen previously, quantification of histology show no significant differences following treatment with TPA or TPA co-treated with Gö6976 at all three different dosages (Figure 5.7B-C).

We had shown previously that K10 expression is significantly reduced in TPA treated PKCα knockout mice (Chapter 4.8). PKCα/β inhibition had no effect on K10 expression in normal skin (Figure 5.8A/B) in both wildtype and PKCα knockout mice.

However, in wildtype mice, K10 expression after co-treatment of TPA with Gö6976 showed an inverse relationship with PKCα/β inhibition (Figure 5.8B). High dose Gö6976 treatment (0.1mM) did not significantly affect K10 expression, whilst low dose Gö6976 (0.001mM) significantly reduced K10 compared to both wildtype and PKCα knockout untreated mice (p≤ 0.0001). Similarly, in PKCα knockout mice, high dose treatment had no effect on K10 expression (Figure 5.8B). However, TPA co-treatment with Gö6976 at low dosage in PKCα knockout mice significantly reduced K10 expression compared to medium and high dose treatment (Figure 5.8C). It is interesting that in both wildtype and PKCα knockout mice, K10 expression has an inverse correlation with Gö6976 treatment. These data are difficult to interpret, but do suggest that a factor other than PKCα is involved.
Figure 5.7: K14 expression does not change with different dosages of PKCa/β inhibition. (A) Light microscope images of paraffin-embedded skin sections (5μm) stained for K14 at day 6 TPA and Gö6976 treatment in C57BL/6 wildtype and PKCa knockout mice. Histogram showing K14 expression in (B) control group and (C) 6 days of daily TPA and Gö6976 treatment. Data representative of 3 independent experiments for Gö6976 at 0.1mM and 1 independent study for Gö6976 at 0.01mM and 0.001mM. Significance calculated using 2 way ANOVA (p-value: * p≤0.05, ** p≤ 0.01, *** p≤ 0.001, **** p≤ 0.0001). Scale bar: 50μm
Figure 5.8: PKCa/β inhibition inversely correlates with K10 expression. (A) Light microscope images of paraffin-embedded skin sections (5μm) stained for K10 at day 6 TPA and Gö6976 treatment in C57BL/6 wildtype and PKCa knockout mice. Quantification of K10 expression in (B) control and (C) treatment groups after 6 days of daily TPA and Gö6976 treatment. Data representative of 1 independent study. Significance calculated using 2 way ANOVA with follow up unpaired T test (p-value: * p≤0.05, ** p≤ 0.01, *** p≤ 0.001, **** p≤ 0.0001). Scale bar: 50μm
5.9. No dose dependent change in K6 expression during TPA treatment of either wildtype or PKCα knockout mice.

Skin sections stained for the K6 marker (Figure 5.9A) found low levels of K6 expressing cells in all the control groups (acetone and Gö6976 at 0.1mM, 0.01mM and 0.001mM) in both wildtype and PKCα knockout mice (Figure 5.9B). However high dose Gö6976 did induce significantly more hyperproliferative K6 positive keratinocyte cells when compared with untreated mice (p=0.0002) (Figure 5.9C), indicating some toxicity of the chemical to the skin.

As shown previously, we saw an increase in K6 expression in all treatment groups regardless of strain (Figure 5.9C). In wildtype mice, K6 expression inversely correlated with dose; with decreasing Gö6976 concentration, we observed a dose dependent decrease in percentage of K6 expressing keratinocyte cells, although this was not significant. These data indicated that PKCα and/or PKCβ is playing a role in hyperproliferation in wildtype mice, but that there are likely to be other factors at play. As before, in PKCα knockout mice, we saw an increase in K6 expression after TPA and Gö6976 treatment across all dosages of Gö6976 (Figure 5.9C). Again, this suggests that concurrent inhibition of PKCβ has a positive effect on K6 expression. This induction inversely correlated with dose, suggesting that in the absence of PKCα, inhibition of additional factors can restore K6 expression to wildtype levels after TPA treatment.
**Figure 5.9: K6 expression inversely correlated with PKCa/β inhibition.** (A) Light microscope images of paraffin-embedded skin sections (5μm) stained for K6 at day 6 TPA and Gö6976 treatment in C57BL/6 wildtype and PKCa knockout mice. Quantification of K6 expression in (B) control and (C) treatment groups after 6 days of daily TPA and Gö6976 treatment. Data representative of 1 independent study for Gö6976 at 0.01mM and 0.001mM. Significance calculated using 2 way ANOVA with follow up unpaired T test (p-value: * p≤0.05, ** p≤ 0.01, *** p≤ 0.001, **** p≤ 0.0001). Scale bar: 50μm
5.10. Loricrin expression is reduced at low PKCα/β inhibition

Loricrin expression was normal in all the control groups (acetone and Gö6976 at 0.1mM, 0.01mM and 0.001mM) (Figure 5.10A/B).

In wildtype mice, TPA co-treatment with high and medium dosages of Go6076 at 0.1mM and 0.01mM respectively showed loricrin expression was similar to TPA treatment only (Figure 5.10C). However, low dose of Gö6976 at 0.001mM in wildtype mice presented with significantly reduced patchy loricrin expression in comparison to other treatments (p≤ 0.0001. The data suggests that PKCα plays a role keratinization and desquamation only when inhibited at a low dosage of Gö6976 in wildtype mice. In PKCα knockout mice, both high and low dose of Gö6976 maintained loricrin expression at similar levels to TPA treated PKCα knockout mice, suggesting that PKCβ is not important in loricrin expression. Interestingly, medium dosage of 0.01mM of Gö6976 decreased loricrin expression significantly (p≤ 0.0001) in comparison to all other treatments (Figure 5.10C), however, this data point may be a technical artefact. Unfortunately time constraints meant that this could not be repeated.
Figure 5.10: Loricrin expression inversely correlates with PKCa/β inhibition in wildtype mice.

(A) Light microscope images of paraffin-embedded skin sections (5μm) stained for loricrin at day 6 TPA and Gö6976 treatment in C57BL/6 wildtype and PKCa knockout mice. Quantification of loricrin expression in (B) control and (C) treatment groups after 6 days of daily TPA and Gö6976 treatment. Data representative of 1 independent study for Gö6976 at 0.01mM and 0.001mM. Significance calculated using 2 way ANOVA with follow up unpaired T test (p-value: * p≤0.05, ** p≤ 0.01, *** p≤ 0.001, **** p≤ 0.0001). Scale bar: 50μm
5.11. Summary

In the above section, we show that PKCα/β inhibition during TPA treatment in wildtype mice can phenocopy PKCα knockout mice in some respects. Ki67 data showed that PKCα/β inhibition in wildtype mice reduces keratinocyte proliferation below that seen in PKCα knockout mice, confirming a role for PKCα in this response to TPA. Similarly, K6 expression was reduced after PKCα/β inhibition of TPA treated wildtype mice, as expected from previous PKCα knockout data (Chapter 4).

These inhibition experiments also confirmed that PKCα plays no role in K14 expression, as shown previously. Interestingly the dose response data highlighted an inverse correlation of K10 and loricrin with PKCα/β inhibition which was unexpected.

Overall, these data corroborate PKCα global knockout data, demonstrating a role for PKCα in proliferation and K6 expression. It is not clear yet whether the observed effects on differentiation, represented by K10 and loricrin, are direct or indirect downstream effects of PKCα inhibition.
5.12. **Pan inhibition of PKCs improves psoriasis-like phenotype**

As discussed previously, sotrastaurin has the potential to be a therapeutic agent for the treatment of patients with psoriasis. Here, we applied sotrastaurin on dorsal mouse skin (wildtype and PKCα knockout mice) for 6 consecutive days along with TPA.

Macroscopic images showed no changes in the control groups of wildtype and PKCα knockout mice following sotrastaurin only (Figure 5.1A). However, an improvement in psoriasis-like phenotype was seen following treatment of sotrastaurin with TPA in both strains (Figure 11B). The accumulated PASI score demonstrating redness, thickness and scaling showed a significant difference in wildtype mice with the addition of sotrastaurin in comparison to TPA only treatment from day 6 (Figure 5.1C), indicating that sotrastaurin had a positive effect on the psoriasis-like skin phenotype. Furthermore, there was a significant effect of sotrastaurin treatment in PKCα knockout mice, indicating non PKCα driven effects of sotrastaurin treatment (Figure 5.1D).
Figure 5.11: Pan inhibition of PKCs improves psoriasis-like phenotype (A) Macroscopic presentation of C57BL/6 mouse dorsal skin after 6 days of treatment in (A) control group and (B) treatment group (n=6). (C) Accumulated PASI score of redness, thickness and scaling of the back skin was scored daily (D) The cumulative PASI score at day 6 is illustrated. Data representative of 2 experiments. Data represented as standard error of mean (unpaired t-tests: p-value: * p≤0.05, ** p≤ 0.01, *** p≤ 0.001, **** p≤ 0.0001). Scale bar: 50μm.
5.13. Pan inhibition of PKCs decreases keratinocyte proliferation

Ki67 expression was quantified from stained sections of dorsal skin following 6 days of treatment. We found that in wildtype mice, pan inhibition of PKCs reduced keratinocyte proliferation in comparison to TPA treated wildtype mice (Figure 5.1A), but not as significantly as with high dose Gö6976 (Figure 5.1B). Inhibition was similar to that in TPA treated PKCα knockout mice. However, sotrastaurin treatment of PKCα knockout mice significantly inhibited Ki67 expression (p<0.001) in comparison to Gö6976 treatment.
Figure 5.12: Pan inhibition of PKCs decreases keratinocyte proliferation. (A) Light microscope images of paraffin-embedded skin sections (5μm) stained for Ki67 after 6 days of TPA and sotrastaurin treatment in C57BL/6 wildtype and PKCα knockout mice. (B) Quantification of Ki67 expression in treatment groups. Data representative of 1 independent experiment. Significance calculated using 2 way ANOVA with follow up unpaired T test (p-value: * p≤0.05, ** p≤ 0.01, *** p≤ 0.001, **** p≤ 0.0001). Scale bar: 50μm
5.14. **Pan PKC inhibition reduces K10 expression**

As shown in all previous experiments, K14 did not alter in the presence of TPA or TPA with pan PKC inhibitor, and was expressed across the entire epidermis (Figure 5.13A/C). This indicates that PKCs do not play a role in induction of K14 expression in stratified squamous keratinocytes.

In wildtype mice, sotrastaurin treatment significantly reduced K10 expression (Figure 5.13B/D), similar to that seen in the PKCα knockout mice on previous occasions, again supporting a role for PKCα in K10 induction after TPA treatment. However contrary to previous results, K10 expression increased in the PKCα knockout mice (p≤0.05) with TPA/sotrastaurin treatment.
Figure 5.13: Pan PKC inhibition reduces K10 expression. (A,B) Light microscope images of paraffin-embedded skin sections (5μm) stained for (A) K14, (B) K10 after 6 days of TPA and sotrastaurin treatment in C57BL/6 wildtype and PKCα knockout mice. Quantification of (C) K14 and (D) K10 expression after 6 days of daily TPA and sotrastaurin treatment. Data representative of 1 experiment. Significance calculated using 2 way ANOVA with follow up unpaired T test (p-value: * p≤0.05, ** p≤ 0.01, *** p≤ 0.001, **** p≤ 0.0001). Scale bar: 50μm
5.15. Pan PKC inhibition decreases K6 expression, but does not affect loricrin expression

In wildtype mice, K6 expression was significantly reduced (p≤ 0.0001) with the addition of sotrastaurin to TPA (Figure 5.14A/C), indicating that PKCs are involved in hyperproliferation of keratinocytes. We also saw a small but significant reduction in K6 expression in wildtype mice treated with sotrastaurin in comparison to Gö6976 treated wildtype mice (p≤0.05)(Figure 5.14C). This suggests that apart from PKCα, additional PKC isoforms are also contributing to hyperproliferation. In PKCα knockout mice, we found no difference in K6 expression in comparison to sotrastaurin treated wildtype mice, in particular no increase as seen previously with Gö6976 treatment, demonstrating a complex relationship between the PKC isoforms in induction of K6 expression. In fact, there was no difference between sotrastaurin treated PKCα knockout mice and TPA treated PKCα knockout mice (Figure 5.14C). As previously demonstrated, PKCα plays a role in loricrin expression following TPA treatment (p≤0.05)(Figure 5.14D). However sotrastaurin treatment had no additive effect on loricrin expression in either wildtype or PKCα knockout mice, demonstrating that pan inhibition of PKCs has no impact. This indicates that overall PKC isoforms other than PKCα are unlikely to be involved in keratinization and desquamation.
Figure 5.14: Pan PKC inhibition decreases K6 expression, but does has no effect on loricrin expression. (A) Light microscope images of paraffin-embedded skin sections (5μm) stained for (A) K6 and (B) loricrin after 6 days of TPA and sotrastaurin treatment in C57BL/6 wildtype and PKCα knockout mice. Quantification of (C) K6 and (D) loricrin. Data representative of 1 independent experiment. Significance calculated using 2 way ANOVA with follow up unpaired T test (p-value: * p≤0.05, ** p≤ 0.01, *** p≤ 0.001, **** p≤ 0.0001). Scale bar: 50μm
5.16. Summary

In corroboration of our previous observations, using an alternative approach we have demonstrated again that PKCα is important in keratinocyte proliferation during TPA treatment. However, sotrastaurin treatment has highlighted a possible role for other PKC isoforms in induction of proliferation.

Pan PKC inhibition in wildtype mice showed a significant reduction in K10 and K6, to similar levels as observed in TPA treated PKCα knockout mice, suggesting a dominant role for PKCα in keratinocyte differentiation and hyperproliferation.

In summary, topical application of sotrastaurin during TPA induction of a psoriatic phenotype appears to phenocopy many aspects of TPA treatment in PKCα knockout mice, and alternative treatment with Gö6976.

Table 5.1: Comparison of results

<table>
<thead>
<tr>
<th>Treatment / strain</th>
<th>Epidermal thickness</th>
<th>K67 /BrdU</th>
<th>K14</th>
<th>K10</th>
<th>K6</th>
<th>Loricrin</th>
<th>Cell infiltrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA-PKCα</td>
<td>6.5x</td>
<td>6.5x</td>
<td>-</td>
<td>0.5x</td>
<td>+++ (80%)</td>
<td>2.3x</td>
<td>+++</td>
</tr>
<tr>
<td>Aldara</td>
<td>4.5x</td>
<td>3.5x</td>
<td>-</td>
<td>0.66x</td>
<td>+++ (50%)</td>
<td>0.3x</td>
<td>+++</td>
</tr>
<tr>
<td>TPA</td>
<td>7.5x</td>
<td>7.5x</td>
<td>-</td>
<td>-</td>
<td>+++ (85%)</td>
<td>-</td>
<td>ND</td>
</tr>
</tbody>
</table>

PKCα deficient mice:
- Aldara 0.5x 0.3x - - - - ND
- TPA NS 0.6x - 0.5x 0.7x 0.9x ND

Topical PKCα inhibition in TPA model (wildtypes only):
- Gö6976 - 0.4x - - - 0.8x - ND
- Sotrastaurin 0.8x - 0.4x 0.7x NS ND

Topical PKCα inhibition in TPA model (PKCα deficient mice only):
- Gö6976 - - - - 1.3x - ND
- Sotrastaurin 0.6x - - - NS ND

- : no difference
a: relative to control wildtype.
b: relative to untreated control wildtype.
c: relative to treated control
NS: Not significant
ND: not done
6. Discussion

6.1. Major Findings

The principle aim of this thesis was to investigate the role of PKC\(\alpha\) in psoriasis. PKC\(\alpha\) plays a key role in keratinocyte proliferation, shown by both PKC\(\alpha\) deficiency and PKC\(\alpha\) inhibition across three different mouse models: CA-PKC\(\alpha\), Aldara- and TPA-induced psoriasis. Furthermore, alterations in keratinocyte differentiation markers were also identified in PKC\(\alpha\) knockout mice, and this was borne out in inhibition experiments. Importantly, the study revealed aspects of keratinocyte differentiation which mirrored the documented pathology of psoriasis, suggesting possible translation of these findings.

6.2. Mouse models of psoriasis

In this thesis, we have discussed 3 different mouse models. Firstly, the CA-PKC\(\alpha\) mouse model, an inducible bitransgenic mouse model generated whereby mice expressing constitutively active (CA) PKC\(\alpha\) were cross-bred with K14-GLP65 mice resulting in overexpression of PKC\(\alpha\) in keratinocytes only. In this model, a psoriasis-like phenotype was found when keratinocyte specific PKC\(\alpha\) was activated via topical application of RU486 for 5 days. We showed that keratinocyte-specific activation of PKC\(\alpha\) led to a phenotype which closely resembled human psoriasis; increased epidermal thickness and immune infiltration, a strong up regulation of K6, extended K14 expression, increase in loricrin in the granular layer, and patchy K1 expression, indicating changes in epidermal differentiation. The CA-PKC\(\alpha\) transgenic mouse model provided a novel insight into the immune pathogenesis of psoriasis, suggesting a key role for PKC\(\alpha\) in disease, corroborating published clinical trial data (He et al., 2014a) which has shown that pharmacological inhibition of PKCs after sotrastaurin treatment improved psoriasis in patients.

Secondly, we investigated the Aldara mouse model, where C57BL/6 mice received daily topical application of Aldara for 6 days to induce a psoriasis-like skin phenotype. This mouse model is well-documented (Hawkes et al., 2017b; Nerurkar et al., 2017; van der Fits et al., 2009), driven by the active ingredient imiquimod (5%), which acts as a ligand
and potent immune activator for TLR7 in mouse, and TLRs7/8 in humans. In our study, we found that absence of PKCα reduced disease severity, quantified by reduced keratinocyte proliferation, reduced epidermal thickness and reduced immune cell infiltrate. In this model however, absence of PKCα did not affect keratinocyte differentiation, hyperproliferation, keratinization or desquamation.

Finally, we also used the TPA mouse model, another well-documented model of psoriasis (Hvid et al., 2008; Palazzo et al., 2017; Sun et al., 2015). TPA is a global driver of PKCs, hence a comparison of C57BL/6 wildtype and PKCα knockout mice after treatment with TPA would give us a useful insight into the specific role for PKCα when other members of the PKC family are activated. Most interestingly, we have found that although absence of PKCα reduced keratinocyte proliferation and hyperproliferation, this was not reflected by reduced epidermal thickness in this mouse model.

Comparison of the mouse models have shown that PKC activation in CA-PKCα and TPA drives significant increase in epidermal thickness, closely mirrored by keratinocyte proliferation, and reflected by increased hyperproliferation marker K6. TPA stimulates the activation of a number of intracellular pathways through the activation of PKCs, which are known to mediate a strong inflammatory response (Cataisson et al., 2005; Mueller, 2006). Passos and colleagues demonstrated that PKCα, PKCδ, and PKCε isoforms are activated by TPA (Passos et al., 2013). PKCδ is suggested to play a role in the onset of keratinocyte differentiation (Adhikary et al., 2010; Chew et al., 2011; Efimova et al., 2004; Szegedi et al., 2009), while PKCε is suggested to contribute to keratinocyte proliferation, since K14- PKCε mouse epidermis was slightly hyperplastic (Cataisson et al., 2003; Reddig et al., 2000). Studies also show TPA induced inflammation is worsened with the overexpression of PKCα in the epidermis, along with enhanced immune infiltration and expression of inflammatory molecules, such as TNFα (Cataisson et al., 2005).

Additionally, our data show that the Aldara mouse model also drives significant hyperproliferation and epidermal thickness, but to a lesser effect. A psoriasis-like phenotype is achieved through the application of TLR7 agonist imiquimod on mouse dorsal skin. Yin and colleagues found that activation of TLR7 mediated by imiquimod caused basal epidermal cell proliferation in mice (Yin et al., 2014), which corroborates
our data and may explain the increase in epidermal thickness in our study. Treatment with TLR7 specific oligonucleotide-based inhibitor IRS661 inhibits the abnormal proliferation of keratinocytes induced by imiquimod. TLR7/α6 integrin double positive keratinocytes, potential target cells for imiquimod, showed expression of K1 terminal differentiation marker suggesting these cells are programmed to undergo epidermal differentiation, similar to epidermal stem cells (Yin et al., 2014). Early changes in keratinocytes have been reported following Aldara treatment on mouse skin. For instance, K14 expression appeared weaker, but was expressed throughout the entire epidermis and was no longer restricted to the basal layer as seen in our data, along with increased and proliferation and K6 expression (Walter et al., 2013). These changes have been reported to be independent of TLR7 and may be caused by off target effects of Aldara on epidermal keratinocytes; there is limited evidence of in vitro activation of inflammasomes and apoptosis mediated by vehicle alone in keratinocytes (Walter et al., 2013). Although IMQ alone can induce inflammasomes and apoptosis in murine keratinocytes (Drobits et al., 2012; Kanneganti et al., 2006), studies have also suggested that Aldara cream independent of IMQ has the same effect. Isostearic acid that makes up 25% of Aldara vehicle cream, may be the cause. In support of these data, studies have shown that caspase-1-deficient mice (a cysteine protease that converts inactive IL-1β to active inflammatory cytokine) are particularly protected against Aldara induced inflammation (Cho et al., 2012). One study compared Aldara vehicle cream with IMQ, and soft cream with IMQ, showing that the latter failed to induce a full psoriasis phenotype (Walter et al., 2013). Another study used different brands of 5% IMQ cream (Aldara and Likejie) on BALB/C which resulted in differential reactions in the IMQ-induced mouse models (Luo et al., 2016). This suggests that perhaps the pathogenesis of psoriasis in this model is not directly linked to TLR7 unless the component in the vehicle cream is also present (Walter et al., 2013). In our study we used soft cream as a control because it failed to induce a psoriasis phenotype (Walter et al., 2013). However, since Aldara activates cells through various separate pathways that may be independent of each other or overlapping (Nestle et al., 2009a) induce a psoriasis-like inflammation, it is difficult to study the role of PKCα in the Aldara model, especially since PKCα is involved in multiple signalling pathways itself. We therefore concluded that TPA induced psoriasis-like inflammation was a better model to study the role of PKCα in psoriasis.
6.3. PKCα drives keratinocyte proliferation

In our study, we have shown that PKCα drives keratinocyte proliferation, where we see an upregulation of BrdU in CA- PKCα mice and down-regulation of Ki67 in PKCα knockout mice with Aldara and TPA treatments. Furthermore, inhibition of PKCα with a high dose of Gö6976 (0.1mM) in wildtype mice reduced proliferation 0.4 fold; we also reported a slight recovery of proliferation inhibition with a lower dose of Gö6976 (0.001mM), demonstrating a role for PKCα in basal cell proliferation across all three mouse models.

However these data were not always corroborated by K6 expression, considered a hyperproliferation marker. In particular, PKCα knockout mice treated with Gö6976 expressed increased K6, suggesting a release of control of K6 expression. Although previous reports have described Gö6976 as PKCα/β isoform specific, it is important to note that studies have described additional targets. It has been demonstrated that Gö6976 also inhibits Chk1, JAK2 and FLT3 kinases, as well as other potential targets such as C-TAK1, Chk2 and ATM which are involved in cell cycle checkpoint regulation (Bain et al., 2007; Davies et al., 2000; Gschwendt et al., 1996). Taken together these data indicate that K6 expression is independent of PKCα but is likely to be under the control of additional factors, as yet undefined. Specific inhibitors of PKCs must be used to further investigate specific PKCα activity such as riluzole (Noh et al., 2000) or aprinocarsen (Lahn et al., 2003), and PKCβ specific inhibitors such as LY333531 hydrochloride (Ruboxistaurin) (Kunt et al., 2007).

One of the manifestations of psoriasis is scaling, which can have a high impact on self-esteem and patients’ health-related quality of life. Improvement of basal keratinocyte proliferation using inhibitors may help patients in the long term by aiding in the decrease of cell thickness and less scaling since less cells will be migrating towards the stratum corneum.
6.4. PKCα drives keratinocyte differentiation

In PKCα sufficient animals, Ki67 and K10 share an inverse correlation: increased proliferation was observed alongside reduced K10 in the CA-PKCα and Aldara models. Furthermore this correlation was seen with topical PKC inhibition using Gö6976 with TPA treatment; lower concentrations of inhibitor led to increased Ki67 and reduced K10. These data suggest that increased proliferation leads to reduced terminal differentiation. A study conducted by Reichelt et al, has suggested that K10 plays an indirect role in cell cycle control of keratinocytes (Reichelt and Magin, 2002). They showed that loss of K10 resulted in epidermal hyperproliferation (Freedberg et al., 2001), increased keratohyalin in the upper epidermis and increased number of basal cells with a significant number of Ki67-positive suprabasal cells. The increase in Ki67 positive basal and suprabasal cells in K10 knockout mice was shown using BrdU labelling, demonstrating a quicker transition time of proliferating basal cells to the upper epidermis (Reichelt and Magin, 2002). However, K10 has also been shown to be dispensable for transition of keratinocytes to the stratum corneum (Wallace et al., 2012), which is corroborated by the lack of correlation in our data between K10 and loricrin.

Although our data support these published observations, the role for PKCα in this proliferation/differentiation balance is not clear. TPA treatment of PKCα knockout mice reduced K10 expression relative to wildtype, but also reduced Ki67 whilst maintaining K6 expression, disrupting any correlation observed in wildtype mice. Since all our keratinization data were recorded at a single time point, it is not known whether Ki67 proliferation peaked earlier in the PKCα knockout mice. Since Ki67 expression is consistently reduced in the absence of PKCα, it is likely that effects on K10 are indirect.

An imbalance between keratinocyte proliferation and differentiation in patients with psoriasis (Woo et al., 2017). Lesional psoriatic epidermal keratinocytes upregulate K6 . The downregulation of K1/K10, has also been noted in the suprabasal layer of epidermal skin in patients with psoriasis (Piruzian et al., 2010). The increased proliferation of basal keratinocytes and differentiation of spinous and granular keratinocytes are the key features observed in psoriasis (Bowcock and Krueger, 2005) and in the TPA model used here.
6.5. Relationship between PKCα and loricrin expression

Loricrin expression is decreased in psoriatic patients (Guttman-Yassky et al., 2009; Katou et al., 2003; Kim et al., 2011), which is reflected in our data in the TPA and Aldara mouse models, but not in the CA-PKCα mice, where we saw a 2 fold increase in loricrin alongside the psoriatic phenotype. Loricrin is one of the major proteins that play an important role in formation of the epidermal skin barrier and is expressed in the granular layer, making up 70% of the total protein mass in the cornified layer (Candi et al., 2005; Kim et al., 2011). It is well established that corneocytes in the stratum corneum contribute to skin barrier, protecting against environmental factors (Nithya et al., 2015). The localisation of loricrin in the stratum granulosum has been verified here through immunohistochemistry. Loricrin and other proteins make up cornified envelopes which surround terminally differentiated epidermal keratinocytes (Natsuga, 2014; Nithya et al., 2015; Stamatas et al., 2012). One of the characteristics of psoriasis is the increased levels of TNFα and impaired skin barrier (Hüffmeier et al., 2008; Proksch et al., 2008). A study by Kim et al showed that increased levels of TNFα inhibits expression of loricrin (Kim et al., 2011). Our data have shown an increase in loricrin expression with overexpression of PKCα supported by research showing loricrin is a downstream target of PKCα-dependent differentiation (Dlugosz and Yuspa, 1993; Palazzo et al., 2017). Loricrin and other envelope precursor proteins covalently cross-link to form cornified envelope by transglutaminase 1, which mediates cross-linking in a calcium dependent manner (Eckert et al., 1997; Lee and Lee, 2018). In addition, when PKCs are activated by a rise in a second messenger diacylglycerol (DAG), intracellular calcium induces granular keratinocyte markers such as loricrin (Deucher et al., 2002), which correlates with our data showing a decrease in loricrin expression following TPA treatment in the PKCα knockout mice. It is likely that the Aldara mouse model does not mediate loricrin expression through a PKCα specific pathway, since there was no difference in loricrin expression in the absence of PKCα.
6.6. PKCα as a potential therapeutic target for psoriasis

All psoriasis therapy aims to improve or achieve full resolution of clinical symptoms, with a long term effect on pathology. However, even though there is a range of therapies available for patients with psoriasis, not all patients respond. Existing long-term systemic therapy still cause adverse reactions in patients and therefore new therapies are urgently needed.

For example, the IL-23/Th17 axis is a well-known contributor to the pathogenesis of psoriasis; IL-23 is responsible for the production of Th17 associated proinflammatory cytokines such as IL-17 and IL-22. Inhibition of the IL-23/Th17 axis has been shown to placate the hyperactive immune response. Existing antibodies targeting the IL-23/Th17 axis are Ustekinumab and Briakinumab, which have been proven to significantly reduce hallmarks of psoriasis and improve disease burden (Griffiths et al., 2005; Leonardi et al., 2012; Papp et al., 2012). In addition, Secukinumab, Ixekizumab, and Brodulumab, anti-IL-17/IL-17R have also been developed, which target the downstream effects of IL-23 (Hueber et al., 2010; Leonardi et al., 2012; Papp et al., 2012). Most interestingly for this thesis, a small molecule inhibitor sotrastaurin (AEB071) was developed to inhibit classical and novel protein kinase C isoforms (Wagner et al., 2009) to offer inhibition of early T-cell activation (Evenou et al., 2009). Sotrastaurin has shown positive results in clinical trials for transplant rejection and psoriasis (He et al., 2014a; Skvara et al., 2008). They reported a significant reduction in Ki67 positive proliferating keratinocytes, as well as a reduction of PASI scores by 70% which reflected improved scaling in patients with psoriasis. Our data show that proliferation and differentiation induced by topical application of TPA is reduced significantly with sotrastaurin after 6 days of treatment. We also show that in the absence of PKCα, sotrastaurin further reduces proliferating keratinocytes in comparison to Gö6976, indicating that PKCα works in concert with other PKCs to induces basal proliferation in the TPA mouse model of psoriasis. However PKCα knockout mice alone showed a significant reduction in scaling after treatment. Although further work is required to quantify scaling, a potential reduction in the appearance of disease over the short term is a novel and exciting result. These observations alone suggest that PKCα inhibition may provide significant clinical improvement for patients.
There is evidence for PKC isoforms to have a critical role in a number of diseases (Mochly-Rosen et al., 2012), including diabetes (Geraldes and King, 2010), cancer (Michie and Nakagawa, 2005; Totoń et al., 2011), heart diseases (Ferreira et al., 2011; Inagaki et al., 2006; Simonis et al., 2003), and a number of dermatological diseases (Maioli and Valacchi, 2010) and neurological diseases (Bright and Mochly-Rosen, 2005; Burguillos et al., 2011; Sun and Alkon, 2010). PKCs are also known to be involved in a number of cellular signalling pathways, and although sotrastaurin appears to be well tolerated based on published clinical trial data (He et al., 2014a; Skvara et al., 2008) long-term data is required to confirm the safety and efficacy profile of this chemical compound. Hence, specific inhibitors of PKC isoforms that are known to play a role in psoriasis may be important to be investigated. Since overexpression of PKCα is shown to increase keratinocyte proliferation and induce other psoriasis-like phenotype, we think PKCα is a potential target for therapy.
6.7. Limitations and future work

It is important to critically evaluate the overall study design of this thesis and acknowledge the various weaknesses.

As the inflammation induced by the Aldara and TPA mouse models resolves within three to four days, treatment must be applied daily. Project Licence requirements restricted the experimental design and therefore did not allow investigation of the role of PKCα in more chronic time frame to more closely mirror human pathology. Longer term treatment would also allow for investigation of inhibitors and topical treatments after establishment of disease, and would allow full utilisation of the Aldara and TPA treatments as pre-clinical models.

Alternative mouse models of psoriasis have been explored in the literature, some of which exhibit a spontaneous psoriatic phenotype such as K14-VEGF mice and lend themselves better to the study of chronic skin inflammation. For instance topical application of TPA to K14-VEGF transgenic mice results in increased infiltration of inflammatory cells and cytokine production, thereby forming a phenotype similar to human psoriasis such as epidermal thickening, acanthosis, parakeratosis and erythema, as well as T cell and neutrophil infiltration (Sun et al., 2015). It would be interesting to generate K14-VEGF-PKCα knockout mice to further investigate downstream effects of PKCα particularly in the VEGF pathway. It would also be important to improve the experimental design, by potentially developing a K14-PKCα knockout to compare data from CA-PKCα mice which exhibit overexpression of PKCα following topical activation. To further investigate the mechanism of PKCα associated proliferation, a combination of K10 or PKCα deficiency with another spontaneous mouse model such as KC-Tie2-overexpressing mice would be useful. This would also address the problem of the lack of chronicity in the mouse models. Since we have also demonstrated that PKCβ may also contribute to proliferation, it would be worth investigating the role of PKCβ in a PKCβ knockout mice.

In normal skin, epidermal keratinocytes migrate through the epidermis from the basal layer to the granular layer, where upon terminal differentiation, they lose their organelles and convert into enucleated cells (Akinduro et al., 2016; Roberson and Bowcock, 2010). However, keratinocytes in psoriatic skin retain their nuclei in the
stratum corneum, which is a hallmark of psoriasis. Unfortunately, although we saw keratinocytes retaining their nucleus following the induction of psoriasis-like inflammation in all three mouse models used here, we were not able to quantify them at this point. We also saw increased cell size of keratinocytes following treatment, similar to results found in other studies (Reichelt and Magin, 2002). This will also be part of future work. Given more time, it would be beneficial to investigate chemokines and cytokines up and downstream of PKCα in these mouse models, particularly those known to be involved in human disease. The pathology of psoriasis involves various cell types, the innate and adaptive immune system as well as the epidermis (Boehncke and Schön, 2015), and the contribution of environmental factors and genetic predisposition. These factors promote the development of psoriasis through secretion of proinflammatory cytokines and antimicrobial peptides (Büchau and Gallo, 2007), which have chemoattractants that activate different subsets of dendritic cells (Nestle et al., 2009b). Activated plasmacytoid dendritic cells secrete high levels of IFNα (Büchau and Gallo, 2007). These recognise, capture and present antigens to T cells in order to induce T cell differentiation (Griffiths et al., 2005). The IL-23/Th17 axis has been identified as crucial induction in the pathology of psoriasis (Nestle et al., 2009a; Nestle et al., 2009b).

The Aldara mouse model employs imiquimod to induce a psoriasis-like phenotype with histopathological changes along with CD4+ T cells and CD11c+ dendritic cells, both of which are important in the IL-23/Th17 axis (van der Fits et al., 2009). A study by Li et al showed that imiquimod induced the expression of TLR7 in both non-differentiated keratinocytes and differentiated keratinocytes after calcium treatment (Seo et al., 2005), resulting in the induction of IL-8 and TNFα, as well as expression of other cytokines and chemokines such as CCL20, IFNα and IFNβ (Li et al., 2013). It may be that PKCα plays a role in the induction of psoriasis-associated cytokines in the Aldara model, and this was not investigated here.

Interestingly, PKCα knockout mice have been found to have decreased STAT3 DNA binding within the IL-17a promoter. STAT3 is documented to be involved in the pathogenesis of psoriasis, where epidermal keratinocytes in psoriatic lesions are characterised by increased levels of cytokines that promote STAT3 activation (Sano et al., 2005; Sano et al., 2008). For example cytokine IL-22 released by Th17 and Th22 cells can induce phosphorylation of STAT3 in keratinocytes, which in turn contributes to
crosstalk between immune cells and keratinocytes in psoriasis (Sa et al., 2007; Zheng et al., 2007). Our preliminary experiments significant reduction in IL-17a mRNA following TPA treatment in PKCα knockout mice in comparison to wildtype mice.

Stained sections were photographed on the Slide Scanner at the University of Manchester, where the epidermis was measured on each photo using Image Pro Premier software (Media Cybernetics). The percentage of positive staining was calculated by drawing around the epidermis first to establish the area of concern. A colour intensity was set as a standard for each experiment, which was used to quantify the positively stained area as a percentage. It was ensured that the immunohistochemistry experiments for controls and the treatment groups were carried out together at the same time to ensure consistency and to reduce variability in results. Although the percentage of positive staining provides us with information to understand how much more cells were producing the protein in question, it does not provide information about the size of the epidermis, or the increase in the number is positively stained cells. Given time, this can be overcome by employing immunofluorescent staining for all the keratinocyte markers in question.

We have built this thesis on the wide understanding that application of TPA induces an increased expression of PKCα (Cataisson et al., 2003; Cataisson et al., 2005; Palazzo et al., 2017; Passos et al., 2013). However, it remains important to analyse the precise location and to quantify PKCα in the epidermis with and without TPA treatment. These studies have been hampered by the complex phosphorylation events that determine the precise function of PKCα in cellular events (Freeley et al., 2011; Seki et al., 2005), but it is now possible to track PKCα expression and phosphorylation using immunohistochemistry and western blot approaches.
7. References


Benhadou, F., D. Mintoff, and V. Del Marmol, 2019, Psoriasis: Keratinocytes or Immune Cells - Which Is the Trigger?: Dermatology, v. 235, p. 91-100.

Benjegerdes, K. E., K. Hyde, D. Kivelevitch, and B. Mansouri, 2016, Pustular psoriasis: pathophysiology and current treatment perspectives: Psoriasis (Auckl), v. 6, p. 131-144.


Ferreira, J. C., P. C. Brum, and D. Mochly-Rosen, 2011, βIIIPKC and εPKC isozymes as potential pharmacological targets in cardiac hypertrophy and heart failure: J Mol Cell Cardiol, v. 51, p. 479-84.


Steinberg, S. F., 2012, Cardiac actions of protein kinase C isoforms: Physiology (Bethesda), v. 27, p. 130-9.


