The role of Hedgehog signalling in atopic dermatitis

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Declaration of originality

I, Eleftheria Papaioannou, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Abstract

Hedgehog (Hh) proteins are morphogens which regulate embryonic development and adult tissue homeostasis, with distinct outcomes dependent on the strength and duration of their signals. In skin, aberrant Hh pathway activation is linked to cancer and malformations, but the role of the pathway in skin inflammation remains largely unknown. Here I show that the Hh signalling pathway modulates the induction and pathology of mouse atopic dermatitis. Sonic hedgehog (Shh) and Hh pathway target genes were upregulated on induction of atopic dermatitis, and the Hh pathway was activated in skin T cells, showing that they respond *in vivo* to Hh signals secreted from the skin. Higher Shh upregulation reduced skin inflammation in mice, whereas pharmacological Smoothened-inhibition exacerbated skin pathology and reduced Shh upregulation. Hh-signalling to T cells prevented skin inflammation on induction of dermatitis, while inhibition of Hh-mediated transcription in T cells substantially exacerbated the disease. RNA-sequencing analysis of skin $CD4^+$ T cells from mice with chronic atopic dermatitis revealed decreased expression of immune regulatory genes in mice with conditional inhibition of Hh-mediated transcription in T cells, and increased expression of inflammatory and chemokine genes. In contrast, constitutive Hh mediated transcription in T cells led to increased expression of immune regulatory genes in skin $CD4^+$ T cells from mice with chronic atopic dermatitis and protected against inflammation. Hh-mediated transcription in T cells resulted in increased regulatory T (T_{reg}) cells in the periphery and skin of dermatitis-induced mice,

and increased TGF-β expression, supporting their immunoregulatory phenotype, whereas, inhibition of T cell specific Hh-mediated transcription, resulted in impaired T_{reg} function, which permitted progression of skin inflammation. Thus, my data demonstrated a critical role for the Hh pathway through Shh upregulation, in the prevention of skin inflammation through upregulation of Shh signalling to T cells to increase T_{reg} populations and promote their immunoregulatory function. This opens the possibility of novel therapeutic strategies for chronic inflammatory skin diseases and conversely suggests that Hh inhibitors could be used to enhance T cell immunity to Hh-secreting tumours.

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Abbreviations

-/-	deficiency
+/-	heterozygous
AD	atopic dermatitis
BBB	brain blood barrier
BCC	basal cell carcinoma
BOC	brother of CAM-related/downregulated by oncogene
CCL	C-C motif chemokine ligand
CCR	C-C motif chemokine receptor
CD	cluster of differentiation
CDO	CAM-related/downregulated by oncogene
CFSE	Carboxylfluorescein succinimidul ester
CLA	Cutaneous lymphocyte-associated antigen
cDNA	complementary DNA
ChiP	Chromatin immunoprecipitation
Ci	Cubitus interruptus
CKI	Cistein kinase I
Cos2	Costal 2
cRPMI	complete RPMI
CXCL	C-X-C motif chemokine ligand
CTLA-4	Cytotoxic T-lymphocyte associated protein 4
D	Drosophila
DAPI	4’,6-diaminidino-2-phenylindole
DC	Dendritic cells
Dhh	Desert hedgehog

DEG	Differentially expressed genes
DISP	Dispatched
DMSO	Dimethyl sulfoxide
DN	Double negative
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphates
DP	Double positive
eBayes	empirical Bayes statistics
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FCS	Foetal calf serum
FSC/SSC	Forward scatter/ Side scatter
Foxp3	Forkhead box p3
Fu	Fused
FLG	filaggrin
GAS1	growth arrest-specific 1
GATA3	GATA binding protein 3
GBS	Gli-binding site
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony-stimulator factor
Gli	Glioma-associated oncogene family
GSK3	Glycogen synthase kinase 3
GWAS	Genome wide association studies
HIV	Human immunodeficiency virus

Hh	Hedgehog
Hhip	Hedgehog interacting protein
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
HSC	Hematopoietic stem cells
H&E	Haematoxylin and eosin
Ig	Immunoglobulin
Ihh	Indian hedgehog
IL	Interleukin
ILC	Innate lymphoid cell
IFN	Interferon
i.p.	intraperitoneal
i.v.	intravenous
K	keratin
kDa	kiloDaltons
Kif7	Kinensin motor protein 7
KO	knock-out
LAP	Latency associated peptide
LC	Langerhans cells
Lck	Lymphocyte-specific protein tyrosine kinase
dLN	draining lymph nodes
M	Molar
MFI	mean fluorescent intensity
MHC	Major histocompatibility complex
mRNA	messenger RNA
NF-κB	Nuclear factor kappa-light-chain enhancer of B cells

ng	nanograms
nM	nanomolars
NKT	Natural killer T cell
OCT	optimal cutting temperature
Oxa	Oxazolone
P	p value
PBS	Phosphate buffer solution
PCA	Principle component analysis
PCR	Polymerase chain reaction
PKA	Protein kinase A
PMA	Phorbol 12-myristate 13-acetate
Ptch	Patched
q	quantitative
r	recombinant
Rag	Recombination activating gene
rcf	relative centrifugal force
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
Rorγt	RAR-related orphan receptor gamma
SCC	side scatter
SEM	Standard error of the mean
Shh	Sonic hedgehog
Smo	Smoothened
SP	Single positive
STAT	Signal transducer and activator of transcription

Su(Fu)	Suppressor of Fu
TAE	Tris base, acetic acid, EDTA
Tbet	T-box expressed in T cells
Tc	T cytotoxic
T_{cm}	T central memory
TCR	T cell receptor
TEC	Thymic epithelial cell
T_{eff}	T effector
TGF-β	Transforming growth factor β
Th	T helper
TLR	Toll like receptor
TNF-α	Tumour necrosis factor α
T_{reg}	T regulatory cell
TSLP	Thymic stromal lymphopoietin
TSLPR	Thymic stromal lymphopoietin receptor
WT	Wild type
µm	micrometre
µg	microgram
µl	microliter

Chapter 1

Chapter 1: Introduction

1.1 Skin biology and skin resident cells

1.1.1 Structure of mammalian skin

Skin is in direct contact with the environment and acts as a physical and chemical barrier, which protects from mechanical injuries and infections, restricts water loss, controls body temperature and maintains the body's homeostasis (Di Meglio et al., 2011). The ability of the skin to perform multiple roles is due to its complex structure. It is formed principally of two components: the outer layer, called the epidermis, which is in direct contact with the environment and the inner layer, which is the dermis.

The two compartments are separated from each other by the basement membrane, which is a complex network of Collagen IV, laminin, entactin and proteoglycans (Yurchenco and Schittny, 1990). The basement membrane behaves as the connective tissue allowing epidermal to dermal adherence via anchoring proteins. It is crucial for wound healing but it is also involved in cancer development (Hashmi and Marinkovich, 2011, Watt and Fujiwara, 2011). Below the dermis lies the subcutaneous region which is dominated by fat tissue. As expected the skin structure differs between humans and mice, with mouse skin being covered by a thick fur layer, having a thinner epidermis and undergoing faster wound healing compared to human skin (Gudjonsson et al., 2007). Skin proteomic studies have revealed the presence of more than 150 different proteins in the skin, which mainly consists of keratins, collagens, elastin and other extracellular matrix proteins and cellular proteins such as actin, myosin, vimentin, desmoplakin

and annexins (Mikesh et al., 2013). A diagram of the skin structure is represented in Figure **1-1** and the detailed epidermal anatomy is described below.

From the lower to the upper skin layer, the basal stratum is attached to the basement membrane, where the renewal of epidermal cells takes place. The basal keratinocytes are barrel-shaped cells resident in this layer which are not differentiated and are constantly dividing (McKelvey et al., 2014). Upon differentiation they move to the higher compartment, the stratum spinosum, where the terminal differentiation of keratinocytes starts. At this stage the keratinocytes stop dividing, their shape changes to polygonal and they start producing different keratins (Nestle et al., 2009). Keratins are intermediate filament proteins produced by keratinocytes. Different types of keratins are present in different layers. The basal layer expresses keratins (K) K5, K14 and K17 while in the spinous layer K1 and K10 are abundant (Ekanayake-Mudiyanselage et al., 1998, Roop, 1995, Santos et al., 2002, Woo et al., 2010). K17 is an inflammation-associated keratin which induces T helper type 1 chemokines in skin and is a known target gene of Hedgehog signalling, whose function and mechanism of action are described later (Callahan et al., 2004, Depianto et al., 2010, Xiao et al., 2015).

As the cells become more mature they migrate higher towards the stratum granulosum. Keratinocytes present in this layer are now distinguished by their cytoplasmic granules, which contain lipids and proteins that will form the cornified cells of the outermost layer. They produce K2 which is co-localised with K10 (Herzog et al., 1994, Smith et al., 1999). At the final stages of epithelial differentiation, the expression of the various keratins is

downregulated whereas the expression of genes associated with cornified envelope proteins (loricrin, filaggrin, involucrin) is favoured (Fuchs, 2007, Nagarajan et al., 2008, Proksch et al., 2008). Finally, the outer visible layer, called the stratum corneum, is formed. This is a cornified layer made up of dead keratinocyte-derived cells which lack organelles (Proksch et al., 2008). Terminal differentiation of keratinocytes is a process that lasts four weeks, leading to the formation of an impenetrable physical barrier constructed by tight junctions between desmosomes and lipids. This is considered as the home of most skin microbiota, such as bacteria, fungi and parasites (Blanpain and Fuchs, 2014, Grice and Segre, 2011, Proksch et al., 2008).

The dermis is the internal part of the skin, responsible for providing mechanical strength to the skin. It consists of various types of collagen including mainly Collagen type I, but also Collagen type III and IV (Epstein and Munderloh, 1978, Oguchi et al., 1985). The upper part of the dermis interacts with the epidermis, and the lower part is formed of collagen and elastin fibres creating the dermal matrix (Hellstrom et al., 2014, Naylor et al., 2011, Wong et al., 2016). While the epidermis is characterised by the presence of hair, hair follicles and sebaceous glands are located in dermis. Hair follicles are important for barrier maintenance, tissue renewal and regeneration in case of injury (Jaks et al., 2010). Sebaceous glands are exocrine glands, which release sebum lipids, acids, anti-microbial proteins, chemokines and cytokines, participating in first line of defence against bacteria and viruses trying to penetrate the skin (Ottaviani et al., 2010, Thody and Shuster, 1989, Schmid-Wendtner and Korting, 2006, Schneider

et al., 2011). A variety of stem cells are located in hair follicles and interfollicular epidermis and their proliferation and differentiation maintain epidermal structure (Fuchs, 2008, Jaks et al., 2010, Watt and Jensen, 2009). Furthermore, in dermis a rich supply of lymphatic vessels and blood capillaries are found, which enable the movement of immune cells that are abundant in the dermis (Skobe and Detmar, 2000, Olszewski, 2005). The different types of immune cells that reside in dermis and epidermis are discussed in detail in following sections.

1.1.2 Filaggrin

Filaggrin is a histidine-rich protein located in the stratum corneum, where it specifically associates with keratins and other intermediate filaments (Irvine et al., 2011, Sandilands et al., 2009). The initial form of Filaggrin is a precursor protein called profilaggrin, which is synthesized in the granular layer of epidermis and is insoluble and phosphorylated. During the epidermal terminal differentiation process, profilaggrin gets dephosphorylated and proteolysed into Filaggrin. This complex process allows Filaggrin to bind to keratins and interact with cornified envelope lipids and proteins, forming at the end the highly impermeable and insoluble stratum corneum. The Filaggrin (*FLG*) gene is located in the epidermal differentiation complex on chromosome 1q21 and consists of three exons and two introns. The third exon is responsible for encoding the profilaggrin protein (Markova et al., 1993, McAleer and Irvine, 2013, Presland et al., 1992).

Profilaggrin comprises various filaggrin repeats and N- and C-terminal domains. The N-domain which plays a role in keratinocyte differentiation

and epidermal homeostasis, is further divided into two parts, the A domain, which is highly conserved between humans, mice and rats and the B domain, which is less conserved between the species (Aho et al., 2012, Pearton et al., 2002, Presland et al., 1992, Sandilands et al., 2007). When these two domains are cleaved, they trigger N-domain translocation to the nucleus promoting the denucleation of keratinocytes during the transition from the granular layer to stratum corneum (Ishida-Yamamoto et al., 1998). Profilaggrin deficiency in the C-domain leads to almost complete lack of Filaggrin, showing that this domain is important for the profilaggrin to Filaggrin transition. However, its exact role remains elusive (Sandilands et al., 2007, McAleer and Irvine, 2013).

The process of Filaggrin maturation is a strictly controlled process that is characterised by phosphatase-driven dephosphorylation. Profilaggrin remains highly phosphorylated in an immature state in order to prevent premature interaction with the keratin filaments. This is due to lack of keratin binding properties of the immature Filaggrin, which are only acquired after its dephosphorylation (Lonsdale-Eccles et al., 1982). Since phosphorylated Filaggrin is insoluble, it enables its package into granules. Dephosphorylation allows the cleavage of N- and C-terminal domains (Sandilands et al., 2007, Sandilands et al., 2009). Following dephosphorylation, proteases degrade Filaggrin monomers to release their amino acids and derivatives, which are crucial for epidermal hydration and barrier function (Rawlings and Harding, 2004). Additionally, skin pH is maintained at acidic levels, contributing to the antimicrobial action of the skin (Kamata et al., 2009).

Loss-of-filaggrin mutations are responsible for many skin malformations such as disorganised keratin filaments, impaired lamellar body loading and altered structure of the lamellar bilayer (Gruber et al., 2011). These mutations can also cause diseases such as ichtyosis vulgaris and atopic dermatitis (eczema; AD) (Irvine et al., 2011, McAleer and Irvine, 2013, Sandilands et al., 2007). Regarding AD, genome-wide association studies (GWAS) have shown a clear relationship between *FLG* gene and the disease (Brown and McLean, 2009, Brown et al., 2009, Brown et al., 2012, Irvine, 2007, Irvine et al., 2011, Nemoto-Hasebe et al., 2009, Palmer et al., 2006). The details of atopic dermatitis are discussed in subtopic **1.2**.

1.1.3 Immunity in skin

Crosstalk between keratinocytes and resident and migratory immune cells, forms an intact compartment allowing skin to act as a primary site of immune defence against viruses and other pathogenic organisms and maintain tissue homeostasis (Di Meglio et al., 2011, Heath and Carbone, 2013, Nestle et al., 2009, Pasparakis et al., 2014). Epidermis is colonised by densely packed memory tissue resident αβ T cells (mostly $CD8^+$ T cells), γδ T cells and Langerhans cells (Bos et al., 1987, Clark et al., 2006, Gebhardt et al., 2009, Macleod and Havran, 2011, Zhu et al., 2013), while dermis contains innate immune cells such as mast cells, macrophages, distinct types of dendritic cells (DCs), tissue resident and circulating $CD4^+$ and $CD8^+$ T cells and γδ T cells (Bromley et al., 2013, Heath and Carbone, 2013, Nagao et al., 2009, Poulin et al., 2007, van Furth et al., 1985). A schematic representation of skin immune cell localisation is shown in Figure **1-2**.

- *Keratinocytes*

As previously mentioned, keratinocytes are located in the epidermis. Their position in stratum corneum favours them to be the first skin immune sentinels which send the danger signals that are received by immune cells. By expressing various pattern recognition receptors either in the cell surface or in endosomes, they detect pathogens entering the skin (Kuo et al., 2013, Lebre et al., 2007) and they initiate T helper (Th)1 immune responses and type I interferon responses (Miller and Modlin, 2007). A useful tool of keratinocytes against pathogen invasion is the activation of the inflammasome, an organ containing many proteins including procaspase 1, which releases caspase 1 and activates IL-1β and IL-18 pro-inflammatory cytokines (Feldmeyer et al., 2010, Martinon et al., 2009). In addition, keratinocytes can also produce chemokines such as CXCL9, CXCL10, CXCL11, CCL20 and CCL27, which recruit innate immune cells and T cells to the skin. They also produce cytokines such as TNF-α, IL-1α, IL-6, IL-10 and IL-33, which activate effector cells in order to achieve defence against harmful pathogens (Nagao et al., 2012, Nestle et al., 2009, Meephansan et al., 2012, Zhu et al., 2010). Thymic stromal lymphoprotein (TSLP) is another cytokine produced by keratinocytes which promotes expression of MHC I and II on dendritic cells, favouring $CD4^+$ T cell differentiation into Th2 subtype (Liu et al., 2007). Recently it was found that keratinocytes directly control regulatory T cells (T_{regs}) via TSLP and loss of TSLPR signalling on T_{regs} triggered progression to pro-inflammatory skin conditions (Kashiwagi et al., 2017).

- *Innate-, innate lymphoid- and γδ T cells in skin*

Skin DCs are grouped according to their localization in distinct skin layers: Langerhans cells (LC) are located in basal and suprabasal layers of epidermis, while in dermis three different types of DCs are detected, with different functional properties. LC in mice have multiple roles. They are found to induce Th17 responses against extracellular pathogens and cross-present exogenous antigens to skin $CD8^+$ T cells. They also have an immune regulatory role since they promote T cell anergy (Igyarto et al., 2011, Igyarto and Kaplan, 2013, Stoitzner et al., 2006). $CD103^+$ dermal DCs are involved in immunity against viral infections (Bedoui et al., 2009, Shortman and Heath, 2010). Dermal $CD11b^+$ DCs are reported to act as antigen presenting cells mainly to $CD4^+$ T cells and they induce T_{reg} populations (Bedoui et al., 2009, Guilliams et al., 2010). Monocyte-derived DCs migrate in skin under pro-inflammatory conditions and activate $CD4^+$ and $CD8^+$ T cells (Lopez-Bravo and Ardavin, 2008).

Skin resident macrophages are responsible for cutaneous surveillance and they are amongst the first cells that detect a pathogen and carry out antigen presentation. They can be divided into three groups according to their ability to induce acute and chronic skin inflammatory conditions (M1 macrophages), to regulate skin inflammation (M2 macrophages) and to promote wound healing (M3 macrophages) (Mantovani et al., 2013, Mosser and Edwards, 2008, Sugaya et al., 2012). Another type of innate immune cells found in the skin are the mast cells, whose degranulation enhances skin inflammation in various cases including atopic dermatitis (Kawakami et al., 2009). However, in an oxazolone model of atopic dermatitis these

cells were found to produce IL-2 causing T_{regs} accumulation and suppression of skin inflammation (Hershko et al., 2011).

Innate lymphoid cells (ILCs) comprise a small family of cells with lymphoid cell morphology, which however lack the classic lymphoid markers and antigen specificity (Artis and Spits, 2015). Three groups of ILCs (ILC1, ILC2 and ILC3) are found in the skin dermis and their dysregulated activation is responsible for skin disorders (Pantelyushin et al., 2012, Roediger et al., 2013, Villanova et al., 2014). ILC2s specifically are implicated in pathogenesis of atopic dermatitis since they are expanded in skin lesions and express high levels of IL-5 and IL-13 in murine skin (Imai et al., 2013, Roediger et al., 2013, Salimi et al., 2013).

Epidermal γδ T cells have a slow proliferation rate under steady state conditions. They monitor epidermal integrity as they are capable of recognising stress antigens in the epidermis (Chodaczek et al., 2012, Hayday, 2009) and they promote wound healing by sending signals to keratinocytes (Havran et al., 1991). Apart from epidermal γδ T cells, there is also a distinct γδ T cell population found in the dermis, where it facilitates $CD4^+$ T cell responses upon infections and mice deficient in these cells, show ameliorated symptoms of dermatitis (Gray et al., 2013, Hayday, 2009, Pantelyushin et al., 2012).

- *Skin αβ T cells and differentiation of $CD4^+$ T cell subsets*

Conventional αβ T cells ($CD4^+$ and $CD8^+$ T cells) in skin have three main characteristics. The vast majority of them are memory T cells. They express CCR4, CCR10 and CLA skin homing markers, which favour skin tropism and they are primarily found in dermis with $CD4^+$ T cells being the most

abundant group. Only a few $CD8^+$ T cells are present in dermis and they mostly have effector-memory phenotype (Gebhardt et al., 2011). Although a few $CD4^+$ T cells are found in epidermis, $CD8^+$ T cells with tissue-resident memory phenotype are detected in this layer, and they protect the body from infections (Clark et al., 2012, Heath and Carbone, 2013).

In skin, $CD4^+$ T cells consist mainly of Th1, Th2, Th17 and T_{reg} subtypes (Nomura et al., 2014). Th1 cells are characterised by the production of their hallmark cytokine IFN-γ. They differentiate by IL-12-driven STAT4 activation, which activates STAT1 with subsequent expression of the Tbet transcription factor, the master regulator of Th1 cells (Lazarevic and Glimcher, 2011). Th1 cells offer protection against pathogens and are elevated in skin lesions of dermatitis patients.

Th2 cells are differentiated in response to parasites and extracellular pathogens. They are characterised by expression of key cytokines IL-4, IL-5, IL-13, IL-25 and IL-31, the latter being responsible for pruritus in skin inflammatory conditions leading to barrier disruption (Kabashima, 2013). IL-4 induces Gata3 expression, a master regulator of Th2 cells. Gata3 inhibits Tbet expression and induces STAT6 upregulation, resulting in further IL-4 upregulation and production of additional Th2 cytokines (Abbas et al., 1996, Farrar et al., 2002). These cells are found in skin lesions of dermatitis patients. The disease will be discussed in detail in the next section. IL-4 and IL-13 have been shown to damage the skin barrier, by downregulating Filaggrin expression (Kabashima, 2013).

Th17 function is attributed to IL-17 expression, which offers protection against fungi and extracellular bacteria. Their differentiation requires the

Rorγt transcription factor, which in combination with TGF-β, IL-6, IL-21 and IL-23 induces STAT3 phosphorylation and Th17 differentiation (Steinman, 2007). Upon detection of stress signals, keratinocytes produce the pro-inflammatory cytokines IL-1β and IL-6, which promote Th17 differentiation and IL-17 production (Yoshiki et al., 2014). These cells further induce IL-33 and antimicrobial peptide production by keratinocytes (Nomura, 2014). Finally, Th17 cells play a role in skin inflammatory conditions (Di Meglio et al., 2011, Koga et al., 2008, Nakajima et al., 2014).

T_{regs} is another $CD4^+$ T cell subset detected in skin. 25% of $CD4^+$ T cells in murine skin express Foxp3, and this expression is strikingly increased in inflamed skin up to 60% (Sather et al., 2007). Skin T_{regs} have the capacity to migrate between skin and lymphoid organs in normal skin and during inflammatory disease (Honda et al., 2013, Tomura et al., 2010). The population mediates its immunosuppressive function either by expressing immune suppressive cytokines (IL-10 and TGF-β) or by upregulating immune suppressive molecules such as CTLA-4 (Schmidt et al., 2012).

$CD8^+$ T cells are found in skin epidermis and their cytotoxic capacity allows them to protect skin against viral infections (Gebhardt et al., 2009, Jiang et al., 2012). However, their uncontrolled activation via the pro-inflammatory cytokine IL-1β exacerbated skin inflammation in a mouse model of cutaneous leishmaniasis (Novais et al., 2017). IFN-γ-, IL-17 and IL-22-producing $CD8^+$ T cells are linked with skin disorders such as dermatitis and psoriasis (Hennino et al., 2007, Hijnen et al., 2013) and IFN-γ production by $CD8^+$ T cells is responsible for keratinocyte apoptosis,

causing further skin barrier disruption (Hijnen et al., 2013, Trautmann et al., 2000).

1.2 Atopic dermatitis

Atopic dermatitis (AD) or eczema is a chronic relapsing inflammatory disease of the skin that often coexists with other atopic diseases such as asthma and allergic rhinitis (Akdis et al., 2006, Leung et al., 2004). AD is a disease of unknown aetiology that places a heavy burden on the economy of developed countries. Although it is not lethal, it reduces the quality of life of patients (Beattie and Lewis-Jones, 2006, Boguniewicz et al., 2007, Mancini et al., 2008). It affects 15-20% of children but also appears in 1-3% of adults and half the patients suffering from AD will develop symptoms of the so-called atopic march within the first five years of their lives (Kapoor et al., 2008, Nutten, 2015). According to data from the International Study of Asthma and Allergies in Childhood (ISAAC), the incidence of the disease varies between countries. Although it has reached a plateau in some countries such as the UK, where it was previously expanding, the prevalence of the disease is massively increasing in developing countries (Mallol et al., 2013). In the following sections I will present the different hypotheses suggested about AD pathogenesis and the mechanism of the disease by focusing on the contribution of T cells in it.

1.2.1 Symptoms of atopic dermatitis

The main symptoms of AD are redness, dry and scaly skin due to transepidermal water loss and extensive pruritus leading to disruption of the epidermal barrier, which makes it permeable to the entrance of

pathogens. AD can be divided into three phases according to the time point that the symptoms emerge, their location and the reaction of immune system (Bieber, 2008). The first stage during infancy is the non-atopic stage, where sensitization has not yet occurred and there are no obvious AD symptoms. The second stage is characterised as atopic dermatitis. Patients are sensitised against food or environmental allergens causing IgE upregulation and the first eczematous lesions in cheeks and scalp arise. This is followed by extensive pruritus (third stage), which follows a few weeks later that causes damage to skin cells and plaque formation in the chronic stages of the disease. More plaques appear during childhood and adulthood in various parts of the body including flexures, head, neck and limbs.

Complex interactions of genes, defective function of skin barrier, altered immune system and variable environmental factors are responsible for AD pathogenesis (Johansson et al., 1998). There are two hypotheses proposed about the possible disease pathogenesis. The outside-inside and the inside-outside hypotheses are both discussed in the following sections.

1.2.2 Genetics in atopic dermatitis

There is a high probability that AD will be inherited to offspring, as shown by a study where children were more probable to inherit AD when one or both parents had the disease, compared to children that inherited asthma from their parents (Dold et al., 1992). However, the fact that the disease failed to appear in some identical twins, showed that environment in addition to genetics plays an important role in AD (Strachan et al., 2001).

GWAS studies have uncovered chromosomes and polymorphisms that are linked to AD. The locus which correlates most with the disease is the 1q21, where epithelium-related genes are detected, amongst them *FLG*, terminal differentiation and epidermal cornification genes including *LORICRIN*, *HORNERIN* and *S100* proteins (Esparza-Gordillo et al., 2009, Guttman-Yassky et al., 2009, Segre, 2006). *FLG*-deficiency decreases hydration of stratum corneum and reduces its water-binding capacities resulting in increased transepidermal water loss. *FLG* mutations are largely studied in AD patients and a strong association between either loss-of-function mutations or polymorphisms are identified mainly in early onset AD patients (Akiyama, 2010, Barker et al., 2007, Barnes, 2010, Gao et al., 2009b, Marenholz et al., 2006, Nomura et al., 2007, Palmer et al., 2006, Sandilands et al., 2007, Weidinger et al., 2006). Although not all AD patients have *FLG* mutations, infants with *FLG* mutations but without AD have partially compromised skin barrier functions (Henderson et al., 2008). Abnormalities in tight junctions can also lead to AD. Tight junctions consist of proteins that control water movement. Loss-of function mutations of *CLAUDIN-1* gene, a tight junction gene, are found in patients with non-lesional AD (De Benedetto et al., 2011).

Apart from barrier integrity genes, multiple other candidates have been found to be responsible for AD pathogenesis. Genes found on the 5q31-33 locus encode Th1 and Th2 cytokines such as IL-4, IL-5, IL-12, IL-13 and GM-CSF that regulate IgE synthesis (Hoffjan and Epplen, 2005, Morar et al., 2006). Mutations in RANTES, a lymphocyte-attracting chemokine, were detected in 17q11 locus and gain-of-function mutations of *IL4RA* gene in

16q22 locus, were also strongly correlated with AD, contributing to epidermal barrier disruption (Novak et al., 2005). In addition, polymorphisms in genes encoding for IL-18, which is responsible for Th2 to Th1 switch and in genes encoding for innate immune system genes and receptors such as CD14 and TLRs, are detected, and might play a role in Th1 to Th2 imbalance observed in AD (Ahmad-Nejad et al., 2004, Lange et al., 2005).

TSLP and its receptors IL7R and TSLPR have a high association with pathogenesis of AD as TSLP, produced by keratinocytes, activates DCs to polarise $CD4^+$ T cells towards the Th2 phenotype (Gao et al., 2010). Finally, mutations in wound healing genes, which delay barrier regeneration are strongly linked to AD (Grigoryev et al., 2010).

All these studies contributed to knowledge about the crosstalk between the immune system and genetic variations responsible for AD susceptibility. However, it is still not known if defects in barrier cause the increased immune responses (outside-inside) or if deficient immune responses cause a disruption of epidermal barrier (inside-outside) further resulting in AD.

1.2.3 The outside-inside hypothesis

According to this hypothesis the compromised skin barrier permits allergens to penetrate into the skin and elevated immune responses appear as an epiphenomenon of the disease (Vijayanand et al., 2012). This favours the extrinsic type of dermatitis which is strongly correlated with loss-of-function mutations of Fillagrin, and high levels of serum IgE (Tokura, 2010). Many mouse model studies have confirmed this hypothesis, where tape striping caused disruption of the skin barrier and cytokine production by

keratinocytes including TNFα, IL-1α, IL-1β and GM-CSF, Th1 and Th2 chemokines and eosinophil dermal infiltration and triggered Th2 chemokine production by activated LCs (Mori et al., 2008, Onoue et al., 2009, Strid et al., 2004, Wood et al., 1992). This model was also applied to human studies, which showed that keratinocyte activation was followed by pro-inflammatory cytokine production (Nickoloff and Naidu, 1994).

Use of haptens as an alternative model of skin disruption also caused LC activation and maturation increasing their antigen presenting capacity (Lutz et al., 1996, Nickoloff and Naidu, 1994, Wood et al., 1992). In line with this, human studies showed that upon hapten application to skin, there was an upregulation of chemokines leading to LC migration, and the degree of LC migration to the skin was highly correlated with the severity of skin integrity disturbance (Dieu-Nosjean et al., 2000, Proksch et al., 1996, Schmuth et al., 2002).

T cell populations were affected by barrier disruption models. Lymphocytes that infiltrated skin upon cutaneous allergen application, showed an activated phenotype and finally Th1, Th2 and Th17 axes were augmented (Fallon et al., 2009, Oyoshi et al., 2009). All these studies suggested that disruption of the epidermal barrier was sufficient and necessary to cause activation of immune responses, resulting in AD symptoms.

1.2.4 The inside-outside hypothesis

There is strong evidence which support the outside-inside hypothesis, however equally strong arguments support the opposite hypothesis, where AD is driven by abnormal immune responses that drive keratinocyte apoptosis and a defective skin barrier. Experiments performed using

genetically engineered mice that overexpress Th2 cytokines in skin such as IL-4 and IL-31, showed spontaneous signs of AD followed by skin barrier defects (Chan et al., 2001, Dillon et al., 2004, Ziegler and Artis, 2010). In addition, treatment of keratinocytes with Th2 cytokines caused keratinocyte hyperplasia, downregulation of epidermal integrity proteins and finally disruption of the epidermal barrier (Cornelissen et al., 2012, Gittler et al., 2012, Howell et al., 2009). Finally, the strongest evidence that supported the intrinsic atopic dermatitis hypothesis is that many AD patients do not have *FLG* mutations, and children with AD can recover from the disease despite the presence of *FLG* mutations, showing that other factors apart from skin barrier defects regulate the disease (Henderson et al., 2008, Irvine et al., 2011, McAleer and Irvine, 2013).

1.2.5 *Mechanism and kinetics of atopic dermatitis*

Initially AD was characterised as a Th2-driven disease with augmented IgE levels, eosinophils and mast cells while T cells produced IL-4 and IL-13 (Bieber, 2008). However, it was later demonstrated that AD is a biphasic disease, separated into an early-acute phase and a late-chronic phase, and each of the phases is dominated by a different cytokine profile (Grewe et al., 1995, Bieber, 2010). During the onset of the acute phase Th2 and Th22 cytokines are detected in the skin, but as the disease progresses to the chronic stage, there is a partial shift from a Th2 to Th1 cytokine milieu (Biedermann et al., 2004, Gittler et al., 2012, Hattori et al., 2010). Although Th17 conditions are related to other kinds of skin inflammation, such as psoriasis, recently the Th17 axis was shown to play a critical role in the

disease (Kabashima, 2013). A schematic representation of the disease kinetics is shown in Figure **1-3**.

When a pathogen manages to penetrate the disturbed skin, mast cells, stromal cells and epithelial cells (mainly keratinocytes), produce TSLP (Soumelis et al., 2002, Ziegler and Artis, 2010). This cytokine is crucial for the initiation of allergic responses as it induces migration of DCs into the dermis, where they start producing Th2-attracting chemokines and they prime naïve $CD4^+$ T cells to be polarised towards Th2 cells (Soumelis et al., 2002). This is the acute phase of AD and at this stage Th2 cells produce IL-4, IL-5, IL-13 and IL-31 cytokines (Dillon et al., 2004, Grewe et al., 1995). IL-4 together with IL-13 downregulates Filaggrin expression on skin, causing further barrier disruption (Howell et al., 2009). Moreover, IL-4 acts on monocytes and DCs triggering them to produce high amounts of CCR4-binding chemokines (Andrew et al., 1998, Vulcano et al., 2001). The CCR4 receptor is important in AD, as $CCR4^+CD4^+$ T cells are able to migrate into the inflamed skin (Nickel et al., 1999). IL-4 and IL-13 act on B cells, causing class switching which leads to elevated serum IgE, an additional characteristic of acute phase AD, whereas IgE responses are absent from intrinsic AD and are low in chronic AD (Kabashima, 2013, Tokura, 2010).

IL-5 production induces increased eosinophilia, a characteristic of the acute phase of AD, while IL-31 is highly expressed in AD skin lesions, causing extended pruritus that leads to further barrier disruption (Islam et al., 2011, Raap et al., 2008). Another cytokine upregulated in the acute phase of the disease is IL-33, which is produced by Th2 cells, mast cells and keratinocytes (Iikura et al., 2007). This is responsible for the maturation and

activation of mast cells and also increases IgE responses and IL-13 production (Liew et al., 2010).

After a period of time, AD skin inflammation becomes chronic, which is characterised by dominant Th1 responses, including elevated IFN-γ, IL-12 and GM-CSF production (Yamanaka and Mizutani, 2011). Furthermore, histology of skin lesions revealed dermal thickening caused by tissue remodelling and collagen deposition in chronic AD. Stimulation of fibrosis and skin hypertrophy are attributed to IFN-γ, one of the main cytokines found in chronic skin lesions (Kosaka et al., 2008, Spergel et al., 1999). What causes the switch from acute to chronic phase of the disease remains unknown but there are well established hypotheses. TLR2 activation by certain microbial products in skin has been suggested to cause the Th2 to Th1 switch (Jin et al., 2009b, Volz et al., 2010). In addition, increased IL-12 production by DCs induces maturation of IL-18, which up to this point was in the inactive pro-IL-18 state (Tsutsui et al., 2004). This leads to priming of naïve $CD4^+$ T cells to Th1 cells which inhibit the IgE class switch of B cells. IFN-γ cytokine is not only produced by $CD4^+$ T cells. $CD8^+$ T cells found in the skin lesions of AD patients are the major producers of this cytokine, that causes keratinocyte apoptosis (Trautmann et al., 2000). Furthermore, $CD8^+$ T cells produce granzyme B in chronic lesions that leads to pro-IL-18 maturation enhancing Th1 cytokine production (Omoto et al., 2010). $CD8^+$ T cells are involved in the initiation of AD and they produce a variety of cytokines including IFN-γ, IL-13, IL-17 and IL-22 in skin AD lesions (Hennino et al., 2007, Hijnen et al., 2013).

Although when AD was first characterised, attention was not paid to the role of IL-17, recently it has been shown to correlate with AD severity. IL-17 expression is elevated in acute skin lesions where it drives Th2 responses and although its expression is decreased with disease progression, it is still present in chronic lesions (Guttman-Yassky et al., 2008, Koga et al., 2008). Its role in promoting skin inflammation is demonstrated by its capacity to regulate GM-CSF, IL-18 and TNFα production in epidermis (Boniface et al., 2007). Finally, IL-22 produced by both $CD4^+$ and $CD8^+$ T cells, is highly upregulated in chronic AD, favouring epidermal thickening and downregulating Filaggrin, resulting in inhibition of terminal differentiation of keratinocytes (Boniface et al., 2005, Nograles et al., 2009).

Circulating T cells in both stages are recruited to the skin by upregulation of the CLA homing receptor. Additionally, CCR4 and CCR10 receptors responsible for migration to the skin are upregulated in both T cell subsets, allowing skin-homing chemokines CCL17, CCL22 and CCL27 to induce T cell chemotaxis in the site of inflammation (Berg et al., 1991, Biedermann et al., 2002, Campbell et al., 1999, Homey et al., 2002).

The last subset of T cells that is highly implicated in AD are the regulatory T cells, characterised as $CD4^+CD25^+Foxp3^+$. This subset represents a very high percentage of $CD4^+$ T cells in the skin, much higher compared to the circulating T_{regs} (Kabashima, 2013). Their presence in skin AD lesions is controversial. Although studies showed that there are less T_{regs} in inflamed AD skin, later studies showed that this population is highly upregulated upon AD skin inflammation (Caproni et al., 2006, Schnopp et al., 2007,

Verhagen et al., 2006). Other studies, showed no significant change between healthy and AD samples (Malhotra et al., 2018, Szegedi et al., 2009). It is well established however that when these cells are present in the skin and under skin inflammatory conditions, they are characterised by reduced immunosuppressive activity, or they switch towards Th2 or Th17-producing cells, further enhancing the disease (Kashiwagi et al., 2017, Sanchez Rodriguez et al., 2014). T_{regs} migrate to the skin via *Fut7* under homeostatic conditions and failure to express this gene, leads to reduced migration into the skin under inflammatory conditions enabling AD progression (Dudda et al., 2008). Finally, a recent study has established direct regulation of T_{regs} by keratinocytes via TSLP and absence of TSLPR on T_{regs} led to a lethal skin inflammatory condition in mice (Kashiwagi et al., 2017).

1.3 Hedgehog signalling

1.3.1 Discovery and function of the Hedgehog genes

The Hedgehog (Hh) genes were firstly described in *Drosophila melanogaster* in the early 1980s by Nüsslein-Volhard and Wieschaus. The group focused on possible embryonic lethal mutations that could be implicated in the distribution of larval cuticular denticles and in pattern formation of adult appendages such as limbs. After genetic screening experiments, they demonstrated that Hh mutant larvae had a stubby and hairy appearance, which resembled hedgehog spines (Nusslein-Volhard and Wieschaus, 1980). Later, Hh was determined to be a secreted protein responsible for pattern formation in Hh-receiving cells (Ingham and

McMahon, 2001). In mammals three Hh proteins were discovered, Sonic Hedgehog (Shh), Indian Hedgehog (Ihh) and Desert Hedgehog (Dhh), with distinct roles in pattern formation but a common signalling pathway. Hh proteins are highly conserved amongst metazoa although the Hh genes are lost in some Nematodes (Burglin, 2008, Echelard et al., 1993, Hao et al., 2006, Krauss et al., 1993, Riddle et al., 1993, Zardoya et al., 1996).

Ihh is expressed in certain tissues such as primitive endoderm, gut, thymus and chondrocytes and shares some functions with Shh but is mainly responsible for chondrocyte proliferation and bone and cartilage formation. It also plays a role in thymocyte differentiation (Dyer et al., 2001, Outram et al., 2009, St-Jacques et al., 1999, van den Brink et al., 2002, Zhang et al., 2001). Dhh is necessary for Schwann cell formation, spermatogenesis and acts as a regulator of erythropoiesis in spleen and bone marrow (Bitgood et al., 1996, Lau et al., 2012, Parmantier et al., 1999). Shh is the most expressed mammalian ligand with functions in left-right symmetry formation and development of various tissues such as teeth, heart, lung, gut, kidney, bone, limbs, thymus and skin (Adolphe et al., 2004, Ahn and Joyner, 2004, Chang et al., 1994, Cobourne et al., 2001, Goddeeris et al., 2008, Hu et al., 2006, Madison et al., 2005, Sampath et al., 1997, Shah et al., 2004, St-Jacques et al., 1999, Watanabe and Nakamura, 2000, White et al., 2007).

Dysregulation of the Hh pathway is responsible for malformations and in some cases, it is embryonic lethal. Loss of Hh signalling during embryonic development can cause holoprosencephaly, polydactyly, skeletal and craniophacial defects (Hill et al., 2003, McMahon et al., 2003, Muenke and Beachy, 2000). Since the canonical pathway requires primary cilia function,

human syndromes caused by defective cilia, might also have an altered Hh pathway (Kyttala et al., 2006, Pan et al., 2005). Mice with homozygous mutations for the Shh and Ihh die around birth, Dhh-deficient mice are viable but the males are sterile, due to lack of mature sperm and Shh-deficient mice develop cyclopia and defects in neural tube and somites (Bitgood et al., 1996, Chiang et al., 1996, St-Jacques et al., 1999). Furthermore, failure of the pathway to regulate tissue homeostasis in adults and constant pathway expression leads to many types of cancers including basal cell carcinoma (BCC) and the brain tumour medulloblastoma (Beachy et al., 2004, Gupta et al., 2010, Takebe et al., 2015).

1.3.2 *Hedgehog protein synthesis, modification and secretion*

Hh proteins act as morphogens which play a role in the development of a plethora of tissues and in embryonic and adult homeostasis. They are synthesized as 45kDa pro-proteins that are cleaved producing two fragments: The N-terminal domain, which drives the signalling activity of Hh proteins and the C-terminal domain, which is responsible for the cleavage of Hh protein in the endoplasmic reticulum, a process that recruits a cholesterol molecule to this end (Beachy et al., 1997, Lee et al., 2016). The N-terminal end gets palmitoylated and the double lipid modification of Hh proteins increases their membrane association properties and secretion (Burglin, 2008, Ingham et al., 2011, Peters et al., 2004).

The distance that Hh proteins need to cover is between 50-300μm (Zhu and Scott, 2004). Secretion and long-range trafficking of Hh proteins is dependent on different factors. The transmembrane protein Dispatched (DISP) allows the secretion of the Hh proteins by binding directly to

cholesterol and DISP-deficiency results in Hh accumulation in the producing cells (Burke et al., 1999, Caspary et al., 2002). Additionally, the cholesterol modification enhances the long-range spread of Hh proteins, as lack of cholesterol restricts the capacity of Hh proteins to form multimers which are crucial for their long-distance movement (Lewis et al., 2001, Zeng et al., 2001). Certain proteins called glypicans also regulate the movement of Hh proteins across the tissue by inducing their association with lipophorins (Eugster et al., 2007).

When the spread of Hh proteins needs to be regulated, this is achieved by proteins involved in the Hh pathway. Patched (Ptch), receptor of the Hh molecules, binds to Hh and internalises it, promoting its degradation (Chen and Struhl, 1996). Hh co-receptors such as CAM-related/downregulated by oncogenes (CDO), brother of CDO (BOC) and growth arrest-specific 1 (GAS1) also restrict Hh diffusion via a negative feedback loop (Briscoe et al., 2001, Briscoe and Therond, 2013). Furthermore, Hedgehog interacting protein (HHIP) is upregulated upon Hh signalling and restricts Hh spread but its role is restricted to Hh movement and it is not related to signal transduction (Chuang et al., 2003). Hh proteins are transported into vesicles in order to leave the plasma membrane until their reception by the Hh receptor.

1.3.3 The Hedgehog signalling pathway

The three Hh ligands share a common signalling pathway which starts with their binding to their Ptch receptor, a 12-pass transmembrane protein expressed on Hh-responsive cells, and is itself a Hh target gene (Agren et al., 2004). The common fruit fly has only one Ptch receptor, while mammals

have two primary receptors: Ptch1 and Ptch2 (Lee et al., 2016). Hh binding to Ptch is facilitated by CDO and BOC transmembrane proteins (Lum et al., 2003, Tenzen et al., 2006). These proteins together with GAS1 form the multimolecular receptor complex which enhances signal transduction and absence of all three results in loss of Hh activity (Allen et al., 2011, Izzi et al., 2011). Binding of ligands to Ptch, reverses Ptch inhibition of Smoothened (Smo), a 7-helix transmembrane protein, allowing the Glioma (Gli)-associated oncogene family of transcription factors (Gli1, Gli2 and Gli3 in mammals) which are downstream Smo, to be activated (Taipale et al., 2002, Stone et al., 1996). The transcription factors bind DNA at consensus Gli-binding sites activating the transcription of their target genes. In the absence of Hh ligands, Ptch remains attached to Smo, inhibiting the activation of the Hh signalling cascade. A Hh signalling pathway scheme is shown in Figure **1-4**.

The general model of Hh signal transduction was confirmed by studies that showed that loss of Shh or Smo had similar phenotypes while deficiency in Ptch, which acts downstream of Hh and upstream of Smo, resulted in a phenotype similar to the one caused by Hh overexpression (Alcedo et al., 1996, Ingham et al., 1991, van den Heuvel and Ingham, 1996). However, it still remains unknown how Ptch induces Smo inhibition.

One study suggests a catalytic mechanism for Ptch, which regulates the levels of a certain ligand that controls Smo (Taipale et al., 2002). Another possible mechanism is that Ptch transports activating secondary messages (like oxysterols, which bind Smo and activate Hh signalling cascade) away from Smo or drives an inhibitory ligand to attach to Smo (Corcoran and

Scott, 2006, Dwyer et al., 2007, Nachtergaele et al., 2012). Additional studies in the fruit fly have demonstrated that lipophorin transports sterol derivatives to Ptch, responsible for negative regulation of Smo and low levels of lipophorin result in augmented Smo stability, suggesting that Ptch can modify the lipid composition of Smo-trafficking endosomes (Callejo et al., 2008). Finally, Smo activation requires a change in alignment of its cytoplasmic tails from close (inactive) to open (active) conformation. This switch is achieved by phosphorylation of specific proteins that allow Smo to accumulate in the cell membrane and trigger the Hh pathway (Chen et al., 2010, Zhao et al., 2007).

At the end of the pathway, the Gli transcription factors are activated. These are zinc finger proteins and all three contain the C-terminal activation end, while only Gli2 and Gli3 contain the N-terminal domain, which acts as a repressor (Dai et al., 1999, Sasaki et al., 1999). This allows the Gli1 transcription factor to act only as an activator of transcription, while Gli2 and Gli3 have both suppressive and activation functions, with Gli2 acting primarily as an activator and Gli3 acting mainly as a suppressor in the presence or absence of the pathway respectively (Bai et al., 2002, Park et al., 2000, Sasaki et al., 1999, Wang et al., 2000). The functions of Gli2 and Gli3 are discussed in detail in Chapters **5** and **4** and the role of Gli1 in the following section.

Gli activity needs to be regulated in order to avoid aberrant Gli function which can lead to cancer (Hui and Angers, 2011). In the absence of Hh signalling, the C-terminal domains of the Gli transcription factors undergo phosphorylation by protein kinase A (PKA), that further activates

phosphorylation by other kinases such as glycogen synthase kinase 3 (GSK3β) and cysteine kinase I (CKI) family members (Briscoe and Therond, 2013, Ingham et al., 2011, Hui and Angers, 2011, Jiang and Hui, 2008). The multiple phosphorylation steps cause ubiquitylation of the specific regions that bind the Gli proteins to the proteasome, which cleaves the C-activation domain. The remaining N-terminal repressor end, and the DNA-binding sites move to the nucleus where they suppress transcription (Jia et al., 2005, Tempe et al., 2006). When Hh signals are present, Smo activation inhibits the phosphorylation processes, allowing the full-length protein which contains the C-domain to induce the transcriptional processes (Briscoe and Therond, 2013).

In mammals a cytoplasmic protein called suppressor of (Fu) [Su(fu)] acts as an additional mechanism to repress the Gli activation and maintain it in inactive state by binding to it and creating the Gli-Su(fu) complex. Studies have shown that its absence results in uncontrolled Gli activation which is lethal in embryos (Svard et al., 2006). In *Drosophila (D.) melanogaster* Su(fu)'s role is taken by the Cos2 protein (Merchant et al., 2004).

In mammals, Hh signalling is dependent on the primary cilium, an organelle found in many metazoa but is absent from *D. melanogaster* (Han et al., 2003). In the absence of Hh signalling, Ptch is gathered around the primary cilium but upon Hh pathway activation the receptor is replaced by Smo, which enters the organelle by lateral movement and gets phosphorylated (Kovacs et al., 2008, Milenkovic et al., 2009, Ye et al., 2013). Smo translocation increases the levels of Gli2, Gli3 and Su(fu) at the tip of the primary cilium and allows the Gli-Su(fu) complex dissociation, which leads

to translocation of Gli transcription into the nucleus and activation of transcription (Chen et al., 2010, Wen et al., 2010).

In response to Hh signalling a kinesin motor protein (Kif7), which acts downstream of Smo, interacts with Gli proteins and plays a role in the movement of Gli2 and Gli3 though cilia and Gli-Su(fu) dissociation (Liu et al., 2005, Maurya et al., 2013). It also reduces the length of the cilium (He et al., 2014). However, in the absence of the signal, Kif7 together with PKA are localised in the base of cilia and promote the proteolytic cleavage of the Gli proteins into their repressor forms regulating Gli overexpression this way (Ingham and McMahon, 2009, Liu et al., 2005).

There is a plethora of Gli target genes. Apart from the previously mentioned *Ptch* and *Gli1*, other components of the Hh pathway (*Kif7*) are target genes, as well as genes related to proliferation, cell growth and division (*Igbp6*, *Opn*, *CyclinD1*, *Myc*), genes related to migration (*Muc5ac*, *Foxc2*, *Jag2*, *Twist2*), transcription factor families regulating animal development (*Myf*, *Pax*, *Nkx*, *Dbx*, *Irx*) and genes related to immunity (*Il6*, *Il1b*, *Tnfa*, *Il8*, *Il13*, *Il4*), (Smelkinson, 2017, Varjosalo and Taipale, 2008).

1.3.4 Hedgehog gradient formation

Since the Hh proteins are morphogens, the outcome of their signalling depends on the duration and concentration of the signal received and the competence of the Hh-responding cells (Ahn and Joyner, 2004, Dessaud et al., 2007, Harfe et al., 2004). Examples of tissue patterning dependent on these variables are wing disc formation in D. melanogaster, limb and neural tube formation in the vertebrates (Jacob and Briscoe, 2003, Fuccillo et al., 2006, Methot and Basler, 1999). Different ligand concentrations

result in the formation of a Gli gradient, between the Gli repressor and the Gli activator forms, which provides information to the cells according to their distance from the Hh-producing cell determining cell fate. The strength and duration of Hh signals received by the cells affect this ratio. Cells closer to the Hh-producing cells receive a bigger ratio of the Gli activator form, while the more distant ones receive a bigger Gli repressor ratio (Ingham and Placzek, 2006, Crompton et al., 2007).

1.3.5 *The Gli1 transcription factor*

The Gli1 transcription factor is highly conserved in mammals and it is not required for mouse development or to initiate the signalling pathway, and its role is restricted to amplifying the responses (Park et al., 2000). However, its measurement is a useful tool indicative of the cells' response to the pathway, as Gli1 itself is a Hh target gene (Dai et al., 1999). During mouse development Gli1 activity is completely dependent on Gli2 and Gli3 levels (Bai et al., 2004). Adult Gli1-deficient mice appear to have a normal phenotype since the activator form of Gli2 transcription factor seems to replace Gli1, the functions of the latter become obvious under Gli2 deficiency (Bai and Joyner, 2001, Bai et al., 2002, Park et al., 2000). Gli1 is expressed in both adult and foetal thymocytes. Double negative (DN)2 and DN3 populations highly express Gli1, while its expression is downregulated in DN4 and double positive (DP) stages to become high again in single positive (SP) cells (Outram et al., 2009, Rowbotham et al., 2007). Its activity promotes thymocyte development up to pre-TCR signalling in embryos, while in adult thymus, it is essential for regulation of TCR repertoire selection (Drakopoulou et al., 2010).

1.4 Hedgehog signalling and immunity

The role of Hedgehog signalling as a morphogen in embryogenesis has been widely studied (Ingham et al., 2011, Lee et al., 2016, Ramsbottom and Pownall, 2016). Additionally, it has an established role in haematopoiesis and lymphopoiesis which is described in the following sections. Recently there is a focus on the role of Hedgehog signalling upon encounter with pathogens and its implication in the immune system in the context of various diseases, discussed in section 1.4.3.

1.4.1 Hedgehog signalling and haematopoiesis

Haematopoiesis is supported by stem cells which differentiate to progenitors that will give rise to the different blood cell populations. Since the role of Hh signalling in stem cells is well established, its role in haematopoiesis has also been a subject of study. Human primitive blood cells were found to express Hh signalling components like Shh, Ptch, Smo and the Gli transcription factors and Hh on these cells was able to enhance their proliferation (Bhardwaj et al., 2001).

In a study from our group Dhh was shown to affect erythrocyte development. More specifically, Dhh deficiency promoted progression of common myeloid progenitors towards erythropoietic cells. Dhh-deficient mice had a high population of erythropoietic cells, larger spleens and were more efficient in recovering from irradiation and stress-induced anaemia (Lau et al., 2012). In contrast, Ihh is reported to support the final stages of erythropoiesis (Cridland et al., 2009). In this study although hematopoietic stem cell (HSC) progenitors from $Ihh^{-/-}$ embryos were normal, there were

defects in terminally differentiated erythrocytes leading to induction of anaemia. That is in line with another study, which shows that addition of rIhh to embryonic ectoderm was sufficient to induce HSCs activation and differentiation towards erythrocytes (Dyer et al., 2001).

Another study by (Trowbridge et al., 2006), has highlighted the regulatory role of Hh signalling in balancing hematopoietic homeostasis and regeneration. Hh signalling was found to expand bone marrow HSC, however its continuous expression led to HSC exhaustion while treatment with the Hh inhibitor cyclopamine, restored HSC function. However, several studies have revealed that conditional Smo deletion had no impact on any stage of haematopoiesis. Smo deficiency did not affect the capacity of HSCs to proliferate and differentiate or their self-renewal (Gao et al., 2009a, Hofmann et al., 2009, Merchant and Matsui, 2009).

1.4.2 *Role of hedgehog signalling in T and B cell and thymic epithelial cell development*

In T cell development, T cells undergo different stages of differentiation before they acquire their mature SP phenotype. Early thymocytes which are characterised as DN for CD4 and CD8 markers, rearrange the T cell receptor T cell receptor (TCR)β chain and form the pre-TCR, then pass to the DP stage, where both CD4 and CD8 markers are expressed, TCRα chain is rearranged and TCRαβ is formed, to finally differentiate towards mature helper $CD4^+$ T cells or towards mature cytotoxic $CD8^+$ T cell phenotype. By TCR rearrangement, T cells acquire the ability to recognise foreign peptides. The DN population is further divided into four stages according to the expression of CD44 and CD25 markers DN1 ($CD44^+CD25^-$

), DN2 ($CD44^+CD25^+$), DN3 ($CD44^-CD25^+$) and DN4 ($CD44^-CD25^-$) (Takaba and Takayanagi, 2017).

The presence of Hh components in the thymus of both murine foetus and adults was revealed by (Outram et al., 2000) and thymocytes have been reported to express Ihh (also expressed by thymic stroma), Ptch, Smo and the Gli transcription factor but not Shh, which was found to be expressed by thymic epithelial cells (TEC) (Drakopoulou et al., 2010, Hager-Theodorides et al., 2005, Rowbotham et al., 2009, Sacedon et al., 2003, Saldana et al., 2016, Outram et al., 2009).

In the embryonic thymus, Gli2 expression is highest in the DN1 and DN2 stages. As thymocyte development progresses to the DN3 stage, Gli1 expression is the highest, to be substituted by Gli3 with highest expression in the DN4 populations (Barbarulo et al., 2016). At the DN1 to DN2 transition, Shh, Gli2 and Gli3 drive DN2 differentiation and expansion. Loss of Shh in embryonic thymus results in a reduced DN2 population, and loss of Gli2 and Gli3 causes impaired DN2 differentiation, while Gli1 does not affect DN1 to DN2 progression (Drakopoulou et al., 2010, Hager-Theodorides et al., 2005, Rowbotham et al., 2009, Shah et al., 2004). At this stage of T cell maturation Ihh deletion alone was not sufficient to affect DN2 differentiation (Outram et al., 2009).

Hh has a controversial role in the pre-TCR differentiation to DP cells. It has been shown to act as a negative regulator of the differentiation towards DP stage, as *in vitro* studies with recombinant (r)Shh inhibited the DN to DP transition in foetal thymus and thymocyte development was arrested in DN3 stage, while treatment with a Shh neutralising antibody had the opposite

outcome (Outram et al., 2000). Gli2 and Gli3 are also implicated at this stage of development, with Gli2 acting as negative regulator of DP progression after pre-TCR signalling, as transgenic mice overexpressing Gli2 activator form in T cells only, had a slower progression to DP stage, while mice with inhibition of physiological Gli2 function in T cells, had the opposite phenotype (Rowbotham et al., 2007, Rowbotham et al., 2008, Rowbotham et al., 2009). Gli3, which acts as a transcriptional repressor of the Hh signalling and has opposing effects to Shh, was found to be essential for DN to DP progression and Gli3 deficient embryonic thymi were partially arrested at the DN stage after pre-TCR signalling (Hager-Theodorides et al., 2005).

In contrast to these experiments, a study by (Shah et al., 2004) suggested that Shh acts as a positive regulation of DN to DP transition, as reduced production of DP thymocytes was observed in foetal $Shh^{-/-}$ thymi. Additionally, *in vitro* treatment of $Shh^{-/-}$ thymus with recombinant Shh restored the DP population. According to a different experimental approach, Shh does impact thymocyte differentiation after DN2 stage, as conditional Smo deletion on T cells showed DN and DP numbers with normal TCRβ expression (El Andaloussi et al., 2006).

Moreover, an interesting role of Ihh has been revealed at this stage of transition. Ihh acts as both positive and negative regulator of thymocyte development according to the stage of maturation. Before pre-TCR signalling, it promotes T cell development, however after pre-TCR signalling it arrests the transition to DP stage. Therefore, it has been

proposed that Ihh might have a homeostatic role in thymocyte development and numbers (Outram et al., 2009).

During the DP to SP transition Hh signalling acts a negative regulator. Studies on constitutively mutant Shh, Gli1 and Gli2 embryonic thymi and on conditional Gli2 overexpression or inhibition have shown higher SP:DP and CD4:CD8 ratios of thymocytes, suggesting increased DP to SP progression in the absence of Hh signalling, possibly via increased TCR signal strength (Drakopoulou et al., 2010, Furmanski et al., 2012, Rowbotham et al., 2007, Rowbotham et al., 2008). In addition, Gli3 expression on TECs promotes DP to $CD4^+$ T cell transition by repressing Shh, as Gli3 conditional deletion from TECs reduced $CD4^+$ T cell differentiation and inhibition of Hh proteins in the Gli3 deficient thymus restored this population (Solanki et al., 2018).

Hh is also involved in B cell development although it has not been studied as in depth as T cell development. It has been reported that Shh produced by follicular DCs, rescues B cells from apoptosis, and Hh inhibition causes increased B cell apoptosis (Sacedon et al., 2005). A recent study has shown that Gli3 acting in embryonic liver stromal cells, is indispensable for B cell development. Shh deficient liver was characterised by increased B cell development, while loss of Gli3 transcription factor had the opposite effect and reduced expression of key B cell development transcriptional regulators (Solanki et al., 2017).

The role of Hh signalling in TEC development has been investigated in embryos and adults by using a fluorescent tagged mouse that reports Gli-transcription. Hh signalling was present in embryonic thymi and its

expression was tracked in different developmental stages of TEC progenitors. Furthermore, Hh signalling was present in different subtypes of TEC in adults and Shh induced normal TEC development (Saldana et al., 2016).

1.4.3 Implication of Hedgehog signalling in peripheral T cells and immune diseases

The role of Hh signalling in T cell development was previously described. In this section I will focus on its role in peripheral T cells and in the context of certain immune diseases.

Our group has shown that conditional overexpression of Gli2 only in the T cells, reduces T cell activation as determined by CD69 and CD25 activation markers (Rowbotham et al., 2007). Also, the same study demonstrated attenuated proliferation of $CD4^+$ T cell spleenocytes overexpressing the Gli2 transcription factor. Furthermore, Gli2 overexpression on naïve $CD4^+$ T cells compromised their ability to activate, proliferate and produce IL-2 cytokine when stimulated, and suppressed key activation molecules (Furmanski et al., 2015). In contrast, conditional repression of Gli2 specifically in T cells, enhances peripheral T cell populations. T cells from these mice responded quickly to activation by upregulating the above-mentioned activation markers (Rowbotham et al., 2008). Therefore, these studies confirm that increased Gli2-driven Hh signalling attenuates T cell activation in periphery. However, one study showed that Ihh expressed on mature $CD8^+$ T cells, increases their cytotoxic activity and the formation of immunological synapse in a Smo-dependent manner (de la Roche et al., 2013).

Hh signalling primes certain immune populations to proliferate and produce cytokines, implicating the pathway in immune diseases. However, its role is controversial. In a murine asthma model, where Shh was activated in the lungs and Gli2-mediated activity only in T cells, Th2 differentiation was induced, exacerbating asthmatic allergic responses. *Il4* was confirmed to be a Hh target gene (Furmanski et al., 2013). Similarly, by using a fluorescent tagged mouse to detect Gli *in vivo* activity, it was shown that in the same asthmatic model, Gli transcription was activated in T cells and Gli repression compromised Th2 cell recruitment to the lungs (Standing et al., 2017). These studies highlight the detrimental role of Gli2-driven transcription in asthma.

Upon influenza infection, IL-6 pro-inflammatory cytokine is expressed and this is partly attributed to direct Hh signalling interactions with influenza genes. *Il6* gene, which is a target gene of Hh signalling, was detected at higher levels in infections with more pathogenic viral mutations (Smelkinson et al., 2017).

In a liver fibrosis model, natural killer T cells expressed Shh ligand. Additionally, Hh signalling was responsible for the expansion, activation and inhibition of apoptosis of IL-13-producing NKT cells upon liver fibrosis damage (Syn et al., 2009).

However, other studies have revealed the anti-inflammatory role of Shh in different diseases. Ihh expression in epithelial intestinal cells, is important for the integrity of the intestine, as its loss led to increased numbers of fibroblasts and macrophages in the gut and persistent Ihh loss, was detrimental for intestinal mucosa, causing leukocyte infiltration and fibrosis

(van Dop et al., 2010). In another study, Shh was also implicated in intestinal inflammation and persistent loss of Hh signalling reduced the villus and resulted in death of mice, while restoration of Shh and Ihh in cultured mesenchymal cells from the intestine reduced pro-inflammatory cytokine expression of *Il1b* and *Il6* (Zacharias et al., 2010).

The protective role of Shh is additionally described in brain, where Shh is responsible for the integrity of brain-blood barrier (BBB). Loss of Shh resulted in compromised BBB function upon HIV infection, whereas Shh restoration decreased the viral burden at the BBB (Singh et al., 2016, Singh et al., 2017). The results were confirmed in human studies where Shh reduces $CD4^+$ T cell infiltration in primary human BBB endothelial cell cultures, their Th1 and Th17 differentiation and production of pro-inflammatory cytokines (Alvarez et al., 2011).

Finally, Shh is upregulated in acute pancreatitis acting as an anti-inflammatory mediator by inducing the production of IL-10 anti-inflammatory cytokine by pancreatic cells in a Gli1-dependent mechanism (Zhou et al., 2012). In ischemic skeletal muscles, Shh was upregulated and attenuated macrophage recruitment and their action to induce cell migration and angiogenesis in the ischaemic muscle, limiting this way the inflammation (Caradu et al., 2018).

1.5 Objectives of the thesis

Hedgehog proteins are important morphogens that regulate cell fate during embryogenesis. In addition to their role in T development, recently there is a focus on Hedgehog pathway involvement in the context of immune

diseases. Our group has shown that Gli2-driven Shh signalling exacerbates asthma by promoting Th2 responses. Given the fact that atopic dermatitis and asthma belong to the atopic march and they are ruled by Th2 responses, I aim to study the role of Hh signalling in atopic dermatitis. I will test the hypothesis that Hh morphogens are expressed in the skin and I will examine if they are increased in the inflamed tissue. By modulating Hh-mediated transcription in T cells, I will explore if it affects the disease progression and pathology and I will try to uncover a possible mechanism via which it acts on AD. This study is of importance as it can give further insight about the role of developmental molecules in AD and pave the way for potential new therapeutic strategies for the disease.

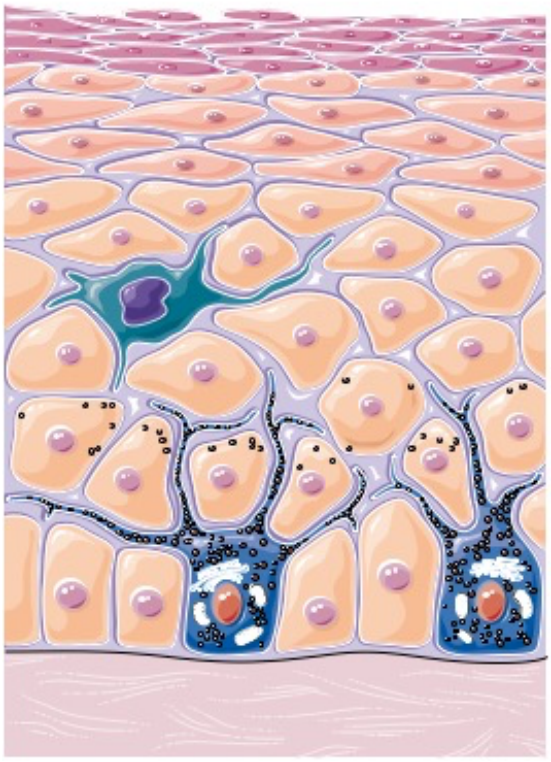


Figure 1-1: Skin anatomy

Starting from the outermost part of the skin, the four layers of epidermis, stratum corneum, stratum, granulosum, stratum spinosum and stratum basal, are represented. Basement membrane links the epidermis with the dermis, which is the inner part of the skin containing lymphatic cells and different types of cells.

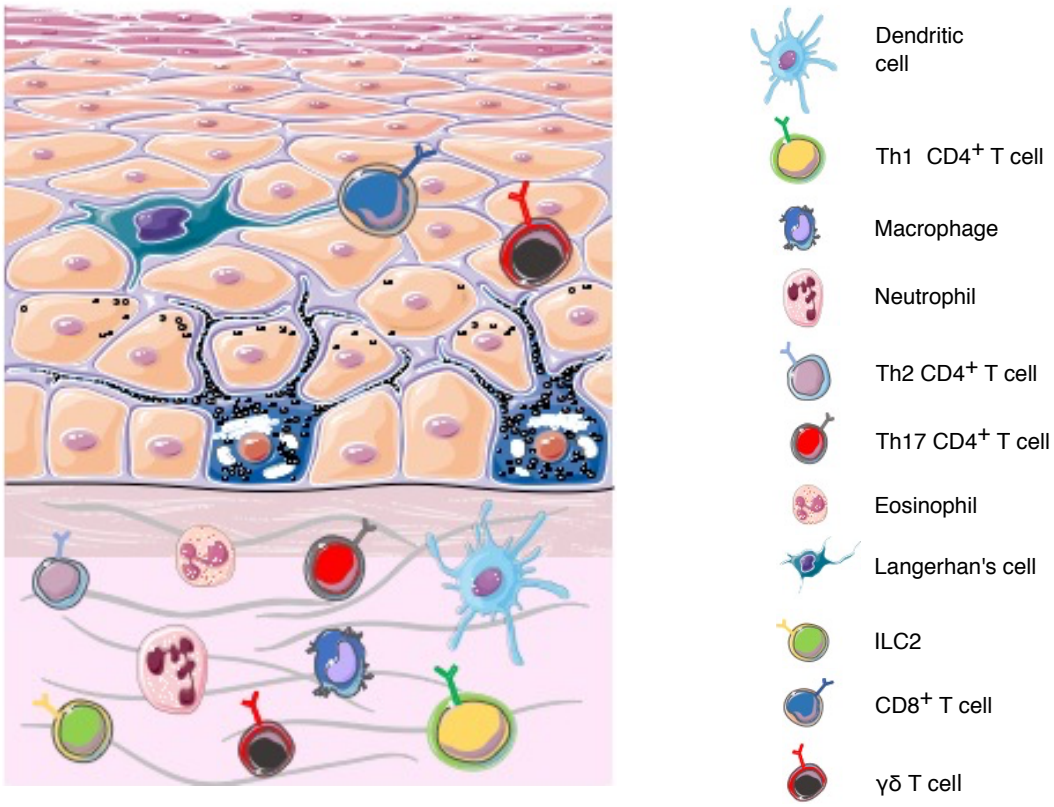


Figure 1-2: Skin resident immune cells

The epidermis (represented by the four layers described in Figure **1-1**), contains epidermal $CD8^+$ T cells, Langerhans cells and epidermal dendritic cells. The dermis contains the majority of immune cells: $CD4^+$ T cells, γδ T cells, ILCs, neutrophils, eosinophils and macrophages.

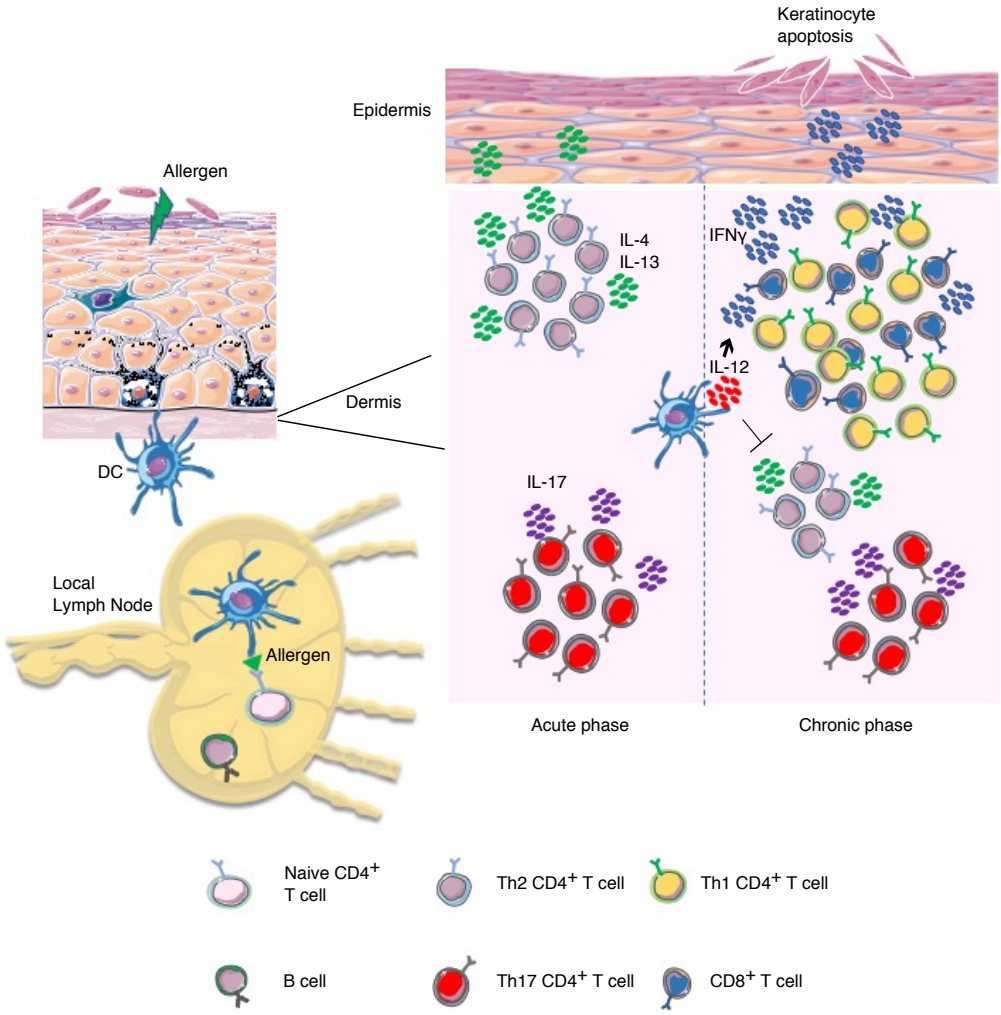


Figure 1-3: Mechanism of atopic dermatitis

The figure represents the acute and chronic phase of atopic dermatitis. Dendritic cells encounter naïve $CD4^+$ T cells in the local draining lymph nodes and drive their Th2 differentiation, leading to B cell IgE class switch and Th2 survival. These cells migrate to dermis and by producing IL-4 and IL-13 they inhibit keratinocyte terminal differentiation. Th17 in the dermis promote Th2 survival and triggers the initiation of the inflammatory responses. As the disease progresses DCs start producing IL-12, which causes a partial shift towards Th1 $CD4^+$ T cells. These together with $CD8^+$ T cells start producing IFNγ, which causes keratinocyte apoptosis. Th2 and Th17 are still present, although in lower levels, in the chronic phase of the

disease and they produce IL-4, IL-13 and IL-17. Th17 levels in the chronic stage of dermatitis are correlated with the severity of the disease.

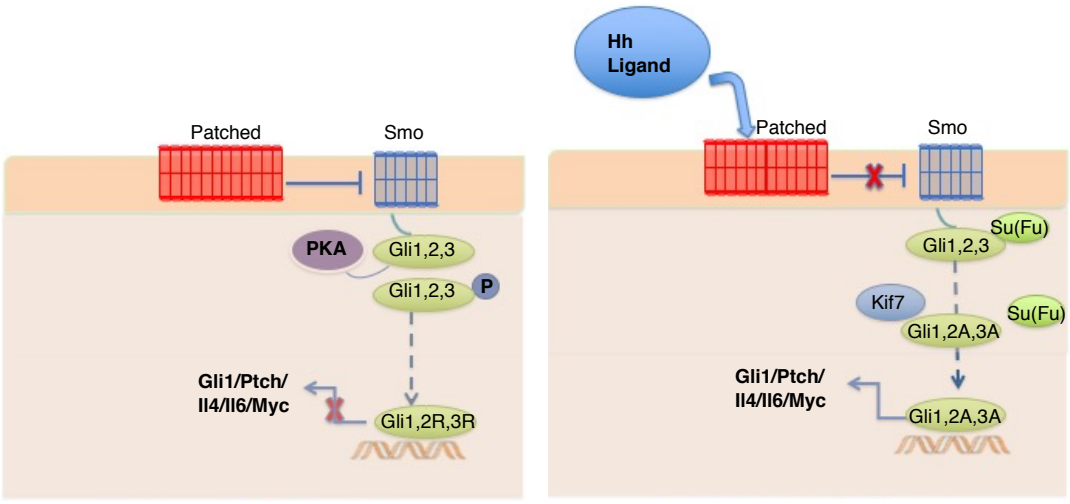


Figure 1-4: Summary of Hedgehog signalling pathway

The figure shows the mammalian Hedgehog signalling components. In the absence of the Hh signal, Ptch inhibits Smo, allowing the PKA kinase to phosphorylate Gli2 and Gli3 transcription factors. This generates their repressor forms, which inhibit their target genes when translocated to the nucleus. In the presence of Hh ligands and upon its binding to Ptch, Smo inhibition is relieved. That causes the Kif7 phosphorylation which permits Gli proteins to be dissociated from the Su(Fu) complex. Gli2 and Gli3 are not phosphorylated, therefore they acquire their activated forms and when they translocate to the nucleus they activate their target genes.

Chapter 2

Chapter 2: Materials and Methods

2.1 Mice

Mice were housed and maintained in specific pathogen-free environment at University College London under UK Home Office regulations and the experimental procedures were in accordance with protocols approved by the University College London ethical approval committee. All mice used were backcrossed for at least ten generations on C57BL/6 background. Mouse strains used are represented in Table **2-1**. All mice used for experiments were age- (between 5 and 8 weeks old) but not sex-matched. Gli3 knockout mice are not viable, for this reason Gli3 heterozygotes ($Gli3^{+/-}$) were used for the experiments.

Table 2-1: Strains and origins of mice used

Mouse strain	Source
C57BL/6	Envigo
GFP-GBS	Gift from James Briscoe (Balaskas et al., 2012)
$Gli3^{+/-}$	Purchased from Jackson Laboratories (USA)
Lck- Gli2ΔN2	Described in (Rowbotham et al., 2007)
Lck- Gli2ΔC2	Described in (Rowbotham et al., 2008)

2.2 Mouse genotyping

In order to genotype the mouse strains, DNA was extracted from 2mm ear clips followed by PCR with the corresponding primers and the PCR products were electrophoresed.

2.2.1 DNA extraction

Ear biopsies were placed in 100μl of digestion buffer [50mM KCL, 1.5mM MgCl2, 10mM Tris HCL (pH=8.5), 0.01% gelatin, 0.45% Nonidet P-40, 0.045% Tween 20 in ultra-pure water (Life Technologies)]. 2μl of 0.5μg/ml Proteinase K (Sigma-Aldrich) were added in the cocktail and the samples were left shaking overnight at 56°C and 750rpm in order to release DNA by tissue enzymatic disruption. After digestion the samples were centrifuged at 13000rpm for 5min in order to separate DNA from impurities.

2.2.2 Polymerase Chain Reaction (PCR)

PCR was carried out in 20μl total volume. 1μl of DNA extract (containing 1μg of DNA) was used from each sample, 50% Golden Taq Polymerase (Sigma) and 1μM of the relevant primers (shown in Table **2-2**) in nuclease free water (Life Technologies). PCR was carried out on an Alpha Cycler 1 (PCRMax, UK) as described below:

- $Gli3^{+/-}$ mice
  - ✓ Denaturation: 5min at 94°C
  - ✓ Annealing: 60sec at 94°C, 80sec at 58°C (34 cycles), 60sec at 72°C
  - ✓ Elongation: 10min at 72°C
- Lck-Gli2ΔN2 and Lck-Gli2ΔC2 mice: The same program was used to genotype both strains.

- ✓ Denaturation: 5min at 94°C
- ✓ Annealing: 60sec at 94°C, 60sec at 58°C (30 cycles), 60sec at 72°C
- ✓ Elongation: 10min at 72°C

Table 2-2: Oligonucleotide sequence of forward and reverse primers used for mouse genotyping and product sizes

Transcript	Primer Direction	Oligonucleotide Sequence	Product Size
Gli2ΔN2/ Gli2ΔC2	Fw Rev	CGA ACC ACT CAG GGT CCT GTG GGA TTT CTG TTG TGT TTC CTC	850
Gli3 Mutant	Fw Rev	GGC CCA AAC ATC TAC CAA CAC AT GTT GGC TGC TGC ATG AAG ACT GAC	580

2.2.3 Gel electrophoresis

The PCR products were electrophoresed on gel containing 1% agarose (Sigma), 1xTAE (Life Technologies) and 0.001mg/ml gel red (Biotium), which allows DNA to be visualised under ultraviolet light. A 50-2000bp Hyperladder (Bioline) was used as a control in order to estimate the band sizes. Gel was visualised under ultraviolet light (Herolab, Germany) and images of the products were acquired (Sony).

2.2.4 GBS-GFP mice

Genotyping of the green fluorescent mice was achieved by measuring the presence of GFP construct in the blood. Mice were tail-bled and a drop of blood was added in 1x PBS (Life Technologies). Samples were acquired

on Accuri C6 cytometer (BD) in order to confirm the presence of GFP on the green channel.

2.3 Oxazolone model of dermatitis-like skin inflammation

2.3.1 *Protocol of skin inflammation*

Age-matched mice (6 to 8 weeks old) were treated with 4-ethoxymethylene-2-phenyloxazolin-5-one (Oxazolone; Sigma). For the sensitization phase, 40μl of 3% Oxazolone were applied on shaved mouse abdomen. After five days, mice were challenged topically on the ears. Mice were anesthetized and 40μl of 0.6% Oxazolone was applied on the ears. Control mice were treated only with vehicle (ethanol). For chronic induction of dermatitis, a total of five applications of 0.6% Oxazolone were performed, starting from day 5 until day 13. Doses were applied every second day. The next day mice were sacrificed. A schematic representation of the protocol used is shown in Figure **3-1 A** in the following chapter. Ear thickness was measured using a micrometer (Mitutoyo, Kanagawa, Japan) at days 5, 7, 9, 11, 13 before applying the next Oxazolone or vehicle dose on the ears and at the end of each experiment.

2.3.2 *Protocol of skin inflammation together with Smoothened-inhibitor injections*

Mice were treated with Oxazolone as described previously. In order to perform Hedgehog inhibition experiments, mice were given daily intra-peritoneal (i.p.) injections of PF-04449913 (Pfizer). 40μg per mouse were being injected during 14 days. Control mice were injected with vehicle

(Dimethyl sulfoxide; DMSO; Sigma) only, as above explained. Ears thickness was measured at the days indicated above.

2.4 Cell isolation and counting

2.4.1 Spleen and lymph nodes

Once dissected, spleen and draining lymph nodes were mashed through a 70μm cell strainer (VWR) in order to obtain single cell suspensions. Cells were processed with FACS buffer [5% Fetal Calf Serum (FCS; Life Technologies) and 0.01% sodium azide (Severn Biotech Ltd) in 1x PBS], before being counted and stained with antibodies. Samples from each suspension were diluted 50x in FACS buffer and counted using Accuri C6 (BD). Forward Scatter (FSC) and Side Scatter (SSC), which determine cell size and granularity were used to draw live gates. Cell numbers were calculated based on the percentage of cells falling within these gates and the volume that the organs were initially resuspended in. Cells were washed at 300rcf for 10min and were prepared for staining (described in 2.5.1).

2.4.2 Skin

Ear tissues were detached from the mouse, split into dorsal and ventral halves and cut into small pieces using scissors in order to facilitate skin enzymatic digestion. In order to achieve leukocyte release from the whole tissue, skin pieces were digested for 30min in 37°C water bath in a cocktail containing 1mg/ml Collagenase D (Roche) or 0.25mg/ml Liberase TM Research Grade (Roche) and 0.5mg/ml DNaseI (Roche) in RPMI (Life Technologies) with 2% FCS and 2% Penicillin/Steptomycin (Pen/Strep; Life

Technologies). Next, tissues were filtered through a 70μm cell strainer to obtain single cell suspensions. Cells were washed once with RPMI containing 10% FCS and 5% Pen/Strep 300rcf for 10min and resuspended in RPMI. Cell samples were [1:1] diluted in 0.1% Trypan blue (Sigma) in order to exclude dead cells. Dead cells were distinguished as they acquire a blue colour. Finally, cells were counted under Alphashot-2 YS2 (Nikon) microscope with a 20x objective, using a haemocytometer (Hawksley). Cell numbers were calculated based on the cells measured in the grid lines of the haemocytometer, the dilution and the final RPMI volume that they were resuspended in.

2.5 Antibodies and flow cytometry

Cell suspensions were stained using combinations of different directly conjugated antibodies (as listed on Table **2-3**; supplied by Biolegend, eBioscience or BD Pharmigen). Samples were acquired on the LSR II (BD) flow cytometer.

2.5.1 Surface staining

$2X10^6$ cells were used for surface staining. For exclusion of dead cells, where necessary, Zombie Aqua Fixable Viability kit (Biolegend) was used. Cells were first washed in 1x PBS at 300rcf for 5min and were stained with the viability dye (L/D). The dye was used in 1:100 dilution in 1x PBS and cells were stained for 15min on ice in dark. After that cells were washed in FACS buffer in order to stop the dye action and an antibody master mix was prepared in FACS buffer. Antibodies were used in 1:100 dilution. 100μl of master mix were added to the cell pellet. Samples were incubated for

30min on ice in dark and they were washed once at 300rcf for 5min in order to wash away the excess amount of antibodies. Cell pellets were resuspended in 250µl of FACS buffer. Samples were acquired on LSR II. A minimum of 10^5 cells was acquired within the gate determined by FSC/SSC and double cells were excluded by gating them on FSC-H/FSC-A. Compensation for each experiment was based on the unstained and single stained controls. Finally, results were analysed by FlowJo 10.4.1 version (Tree Star, US).

2.5.2 *Intranuclear staining for transcription factors*

$5X10^6$ cells were used for transcription factor staining. Staining for Foxp3, Ki67, Tbet and Gata3 was performed using the eBioscience Fixation/Permeabilization (Fix/Perm) kit according to manufacturer's instructions. 100µl of Fix/Perm reagent were added on the surface stained samples and samples were incubated for 15min on ice in dark. Next, cells were washed once using 1x Permeabilization buffer (eBioscience) at 300rcf for 5min and the master mix containing the antibodies corresponding to transcription factors was added to the cells. Cells were incubated for 50min on ice in dark. Samples were washed once in 1xPermeabilization buffer and were ready for acquisition.

2.5.3 *Intracellular staining for cytokines*

In order to detect cytokines secreted from the cells, $5X10^6$ cells from each sample were stimulated in complete RPMI (cRPMI) [containing 10%FSC, 1% PenStep and mercaptoethanol (Sigma)] with cell activation cocktail (1:2 dilution; Biolegend) for 4 hours in a 37°C and 5% CO_2 incubator. Cell activation cocktail is a mix which contains optimised concentrations of

Phorbol 12-mysistate-13-acetate (PMA), ionomycin and protein transport inhibitor, Brefeldin-A. The latter captures cytokines in rough endoplasmic reticulum or Golgi apparatus, which are produced under PMA/ Ionomycin activation making them detectable by intracellular flow cytometry. After four hours, cells were stained first with Viability dye and surface stained (as described in 2.5.1). Then they were fixed using the Fix/Perm solution (as described in 2.5.2). After being washed once with 1X Permeabilization solution at 300rcf for 5min, 100µl of the cytokine master mix was added to the cells. They were left for 30min on ice in the dark and after a final wash with 1x Permeabilization solution, cells were ready for acquisition.

2.5.4 Staining for phosphorylated Smad2 and Smad3

I order to detect phosphorylated (p)Smad2 and pSmad3 in skin T cells, I first stained at least $5x10^6$ cells with LD dye as described previously. After washing the cells with FACS buffer, I stimulated them with rTGF-β (5ng/ml) in RPMI, for 45min at 37°C and 5% $C0_2$. Cells were fixed with 1x pre-heated BD PhosphoFlow Perm Buffer II (BD) for 10min at 37°C and 5% $C0_2$. Cells were washed with PBS and permeabilised with ice-cold BD PhosphoFlow Perm Buffer III (BD) from 30min. To block unspecific binding, cells were treated with 1.5% donkey serum (Sigma) in PBS for 30min. After washing the cells with PBS, they were stained with primary anti-Smad2+Smad3 (Phospho T8) antibody [1:50; Abcam (ab63399)] for 1h. Finally, cells were stained with donket ant-rabbit PE secondary antibody (1:200; Biolegend) and with the rest of the antibodies of interest for 45min as described above.

Table 2-3: Clones and origin of conjugated antibodies used

Antibody	Clone	Company
CD3	17A2	Biolegend
CD4	RM4-5	Biolegend
CD8	53-6.7	Biolegend
CD11b	M1/70	Biolegend
CD11c	N418	Biolegend
CD25	PC61	Biolegend
CD44	IM7	eBioscience
CD45	30-F11	Biolegend
CD62L	MEL-14	eBioscience
CD69	H1-2F3	Biolegend
F4/80	BM8	Biolegend
Klrg1	2FI/KLRG1	Biolegend
MHCII	M5/114.15.2	Biolegend
SiglecF	E50-2440	BD Pharmigen
LAP	TW7-16B4	Biolegend
γδ-TCR	GL3	Biolegend
Foxp3	MF14 or 150D	Biolegend

CTLA-4	UC10-4B9	Biolegend
Ki67	16A8	Biolegend
Tbet	Ebio4B10	eBioscience
Gata3	TWAJ	Biolegend
IFNγ	XMG1.2	Biolegend
IL4	Ebio11b11	eBioscience
IL5	TRFK5	Biolegend
IL13	Ebio13A	eBioscience
IL17	TC11-18H10.1	Biolegend

2.6 Cell sorting

2.6.1 Naïve CD4⁺ T cells

CD4⁺ T cell populations were purified from spleens of WT mice. Spleens were mashed and cell suspensions were obtained as described in 2.4.1 and were treated with 3ml red blood cell (RBC) lysis buffer for 3min, in order to clean the samples from red blood cells. Cells were washed twice with PBS at 300rcf for 7min. CD4⁺ T cells were magnetically purified using EasySep mouse CD4⁺ T cell negative selection kit (StemCell Technologies) according to manufacturer's instructions. Samples were stained for L/D, CD3, CD4, CD62L and CD44 as described above. Samples were sorted at the ICH/GOSH Flow Cytometry Core Facility using FACS Aria III cell sorter (BD). Cells stained as L/D⁻CD3⁺CD4⁺CD62L⁺CD44⁻ were collected in

cRPMI. Purified cells were pelleted by washing once in cRPMI at 300rcf for 5min. Cells were resuspended in 10^5/ml concentration and were cultured as described below (2.7.4).

2.6.2 Skin

For $CD4^+$ skin T cell sorting, ears from 3 mice per strain were pooled together. Skin was processed as described above (2.4.2) and cells were stained with Z/A (L/D marker), CD45, CD3, γδTCR, CD4 and CD8 (2.5.1). Sorting was performed using FACS Aria III cell sorter. Cells stained either as $L/D^-CD45^+CD3^+\gamma\delta TCR^-CD8^-CD4^+$ or $L/D^-CD45^+CD3^+\gamma\delta TCR^-CD4^-CD8^+$ were collected. Populations were sorted directly into Trizol (ThermoFisher Scientific). Sorted populations were vortexed for 2min and were placed at -80°C for future RNA extraction.

2.7 Cell cultures

2.7.1 T cell differentiation assay

Sorted naïve $CD4^+$ T cells were cultured under Th0, Th1, Th2 and Th17 conditions. Round-bottom 96-well plates (Costar) were coated with 5μg/ml anti-CD3 (100μl/well; Tonbo Biosciences) for 5h in a 37°C and 5% $C0_2$ incubator. Plates were then washed with PBS twice in order to wash away unbound anti-CD3 and 100μl of cRPMi was added to each well. For Th0 conditions, I prepared master mix containing 1μg/ml anti-CD28 (Tonbo Biosciences) and recombinant (r)IL-2 (20ng/ml; Peprotech) in cRPMI. For Th1 conditions the master mix contained 1μg/ml anti-CD28, 20ng/ml rIL-2, 20ng/ml rIL-12 (eBioscience) and purified anti-IL-4 (10μg/ml; eBioscience) in cRPMI. For Th2 conditions the master mix contained 1μg/ml anti-CD28,

20ng/ml rIL-2, 20ng/ml rIL-4 (eBioscience), purified anti-IL-12 (10μg/ml; eBioscience) and purified anti-IFN-γ (10μg/ml; eBioscience) in cRPMI. For Th17 conditions the master mix contained 1μg/ml anti-CD28, 20ng/ml rIL-2, rIL-6 (50ng/ml; Biolegend), rTGF-β (5ng/ml; Biolegend), 10μg/ml purified anti-IL-4 and 10μg/ml purified anti-IFN-γ in cRPMI. Each master mix was added to the appropriate wells and 100μl of cells were added to each well. Cells were cultured for 10 days in a 37°C and 5% $C0_2$ incubator. Medium was replaced on day 4 and the appropriate recombinant cytokines were added to each well. On day 7, cells were restimulated with anti-CD3 as previously described and were cultured again under Th0, Th1, Th2 and Th17 conditions. Throughout the differentiation protocol, either 1μl of PF-04449913 Smo-inhibitor (1:10000 dilution) or 1μl dimethylsulfoxide (DMSO) (1:10000 dilution) were added to the appropriate wells. On day 10, cells were stimulated with cell activation cocktail, stained for cytokines (as described in 2.5.3.) and analysed in LSR II flow cytometer.

2.7.2 T cell activation assay

Spleenocytes were isolated and plated in a 12-well plate (Costar) at a concentration of $5x10^6$ cells per well with 0.01μg/ml anti-CD3 and 0.01μg/ml anti-CD28 in cRPMI for 24 hours in a 37°C and 5% $C0_2$ incubator. Cells were harvested and stained for surface markers as described in 2.5.1. Finally, they were analysed in LSR II flow cytometer.

2.7.3 Carboxyfluorescein succinimidyl ester (CFSE) proliferation assay

Spleenocytes were isolated, washed in ice-cold PBS and were counted as described before. Cell concentration was adjusted at $10x10^6$ cells/ml in PBS. 1μM CFSE (eBioscience) was added and cells were incubated for

10min in a 37°C and 5% $C0_2$ incubator. Cells were washed once with PBS containing 10% FCS in order to stop CFSE reaction. Then cells were washed twice with ice-cold PBS at 300rcf for 7min. Cells were cultured with 0.01µg/ml anti-CD3 and 0.01µg/ml anti-CD28 in cRPMI at 37°C and 5% $C0_2$. Cells were harvested on days 4 and 6, were surface stained as described before and were acquired on LSR II flow cytometer.

2.7.4 *Suppression assay*

$CD4^+CD25^-$ T cells and $CD4^+CD25^+$ T_{reg} cells from WT and Gli2ΔC2 spleens of Oxa-treated mice were FACS-sorted as described in 2.6. $CD4^+$ T cells were first stained with CFSE as mentioned above, and were used as responder cells. T_{regs} were used as suppressor cells. Responder cells were used at a fixed concentration of 50000 cells per well and were co-cultured with suppressors at 1:1 and 1:4 ratios in plates which were pre-coated with anti-CD3 as above mentioned. Anti-CD28 (1µg/ml) and rIL-2 (20ng/ml) were added to the cultures. Cells were cultured for 4 days at 37°C, 5%CO_2.

2.8 Skin histology

2.8.1 *Immunofluorescence*

Ear tissue was embedded in OCT (ThermoFisher Scientific) and was cut into 7.5µm thick sections on cryostat. Samples were left at room temperature for 2 hours to dry. Non-specific binding was blocked by incubation in blocking buffer [containing 0.2% fish gelatin (Sigma), 0.1% Tween (BioRad) in PBS] for 20min. Cells were stained overnight at 4°C with

primary unconjugated anti-Shh (1:50 dilution; 171018; Santa Cruz Biotechnology), followed by incubation with donkey anti-goat IgG Alexa Fluor 594 (1:500 dilution; Invitrogen) secondary antibody in blocking buffer for 1 hour. Cells were washed twice in blocking solution. Slides were mounted with Gold anti-fade reagent with DAPI (Invitrogen) and were visualised using 10x magnification in Leica upright 3 color microscope. Image analysis was performed with Fiji software.

2.8.2 *Hematoxylin and Eosin (H&E) staining*

Ear tissue was fixed in 2ml Bouin's solution (Sigma), paraffin-embedded and sectioned for H&E staining. Staining was performed by Histopathology, Great Ormond Street Hospital. Images were taken by Zeiss Axioplan 2 microscope and quantification of dermal and epidermal thickness on H&E sections was carried out using Fiji software.

2.9 RNA extraction and cDNA synthesis

2.9.1 *RNA extraction from the whole ear tissue*

RNA was extracted using the Arcturus PicoPure RNA isolation kit (Applied Biosystems) following the manufacturer's instructions. RNA quality and concentration were evaluated using Nanodrop (ND-1000). RNA was used for quantitative real time PCR.

2.9.2 *RNA extraction from sorted skin $CD4^+$ and $CD8^+$ T cells*

Sorted skin $CD4^+$ and $CD8^+$ T cells were kept in Trizol in -80°C. After thawing, samples were vortexed for 2min. 140µl chloroform (Sigma) was added to each sample and they were vortexed for 2min. Samples were left

to rest for 3min and they were centrifuged at 12000g for 15min. The top aqueous phase which contained RNA was transferred to a new collection tube and RNA was extracted as described in 2.9.1. The extracted RNA was used for RNA-sequencing as described in section 2.12.

2.9.3 cDNA synthesis

RNA was converted to cDNA using the High capacity cDNA reverse transcription kit (Applied Biosystems) according to the manufacturer's instructions. DNA quality and concentration were evaluated using Nanodrop (ND-1000).

2.10 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

qRT-PCR was carried out using QuantiTect primers (Qiagen) and iQSYBR Green Supermix (BioRad) following the manufacturer's instructions. Samples were run in triplicates on the CFX Connect (BioRad). 10ng of cDNA were added in each reaction combined with 0.3M primers, 10μl iQSYBR Green 2x Supermix [containing 100mM KCL, 40mM Tris-HCL; pH 8.4, 0.4mM of each dNTP (dATP, dCTP, dGTP, dTTP) iTaq^{TM} DNA polymerase (50units/ml, 6mg $MgCl_2$, SYBR Green 1, 20nM Fluorescein and stabilizers)] in ultra-pure water. Normalization was calculated by using Hypoxanthine Guanine Phosphoribosyl Transferase (HPRT) gene, which is a housekeeping gene. Amplification of HPRT was quantified with 1:10 series dilutions of cDNA made from embryonic head. Each cDNA sample was compared to the dilution series in order to be precisely quantified. Primers were used for *Shh*, *Dhh*, *Ihh*, *Ptch1*, *Gli1*, *Il4*, *Il13*, *Ifng* and *Flg* gene expression and were purchased from Qiagen.

2.11 Cytokine secretion measurements

2.11.1 Enzyme-linked Immunosorbent Assay (ELISA)

Blood was collected directly from the heart after mice were terminally anaesthetized. It was centrifuged at 1000rcf for 20min and supernatant was collected and kept in -20°C for future use. 2µl were used to perform IgE ELISA (eBioscience) according to the manufacturer's protocol. For active TGF-β ELISA (Invitrogen), supernatants were collected from co-cultured $CD4^+$ and T_{reg} spleenocytes after suppression assay which is described in section 2.7.4.

Ear pinnae of mice were snap-frozen in liquid nitrogen and were manually mashed and homogenised in PBS. Samples were centrifuged at 1400rcf for 20min and skin supernatants were collected. IFN-γ (eBioscience) levels were measured according to the manufacturer's instructions.

2.11.2 Mouse multiplex immunoassay (Firefly)

IL-1β, IL-4, IL-5, IL-6, IL-13 and TNF-α levels were measured by Firefly© (Abcam) according to manufacturer's instructions in skin supernatants. This assay uses particles which enables the quantitative measurement of many mouse cytokines at the same time. Particles have three functional regions, with central region capturing target antibodies, while the two ends serve to distinguish different particles. When particles capture the target antibodies, biotin conjugated detector antibodies bind on them followed by streptavidin-PE-Cy5 addition. Presence of particles is detected in green channel (all three regions are detected), the central region of particles is detected by red channel and end regions with different intensities are detected in yellow channel. Samples were acquired on Accuri C6.

2.12 RNA sequencing (RNA-seq) and analysis

2.12.1 RNA-seq

RNA-seq is a technique that reveals the highly dynamic nature of a cell transcriptome. Apart from being a quantification method, it further allows the detection of different genes, alternatively spliced genes and different populations of RNA such as total RNA, coding- and non-coding- RNA (Kukurba and Montgomery, 2015). The process comprises different steps. First RNA is extracted from the tissue. Then RNA molecules are enriched using a specific protocol, like poly-A selection protocol, which enriches the polyadenylated transcripts. Subsequently, RNA is converted to cDNA and the library is constructed, which is finally ready for sequencing.

RNA extracted from sorted skin $CD4^+$ and $CD8^+$ T cells were sequenced by UCL Genomics using Illumina Next Seq 500. The created datasets were processed and normalised using the Bioconductor package *DESeq2*.

2.12.2 Differentially Expressed Gene (DEG) Analysis

DEG genes were selected using the Bioconductor Package Limma (Ritchie et al., 2015). Significantly different genes ($p<0.05$) between WT and transgenic samples were evaluated using Ebayes statistic test. Gene expression heat maps were based on DEG genes, where red colour represents high gene expression and green colour represents low gene expression.

2.12.3 Principal Component Analysis (PCA)

PCA was performed using the CRAN package *ade4*. PCA is a statistical multivariable method used to analyse large datasets with thousands of genes. Highly correlated cells are clustered together, making new variables

relatively to the old ones, which are described as principal component axes (PC1, PC2, PC3, etc). Each axis shows the percentage of variation or biological variable that is different in the set of genes. Differences along the first principal component axis (PC1) are more important compared to differences along the rest of principal component axes (PC2, PC3, PC4). The scores for genes on each axis can reveal which genes are in the compared groups, which genes affect the most or the least the genotypes or populations and their relationship under a specific experimental condition or genotype. DEG and PCA analysis were performed by Anisha Solanki.

2.13 Statistical data analysis

An unpaired Student's t test was performed for comparison of the experimental groups. For comparison between treated and untreated samples of different mouse strains, datasets were analysed using two-way ANOVA statistical test. For DEG gene analysis, significantly different genes between the datasets were evaluated using Ebayes statistical test. For all the data analysed, a p-value lower than 0.05 was considered statistically significant. Results were analysed using Prism7 (GraphPad).

Chapter 3

Chapter 3: Oxazolone model of dermatitis-like skin inflammation

3.1 Introduction

Animal models are of high importance to help scientists better understand the development and different stages of atopic dermatitis. A variety of mouse models, which mimic certain characteristics of human acute or chronic dermatitis are used extensively and include the following categories:

- Mice spontaneously developing AD-like skin lesions
- Genetically deficient mice or mice overexpressing certain molecules
- Models induced by epicutaneous application of substances (Jin et al., 2009a).

The low cost and high reproducibility of the results, along with the availability of a wide range of genetically modified mice, offer a big advantage to these models, which are widely used in skin inflammation research. However, no mouse model can fully recapitulate the characteristics of human atopic dermatitis (Ewald et al., 2017).

The first mouse model used to investigate atopic dermatitis was the Nc/Nga mouse strain by Matsuda and his team. These mice, which had mutations in chromosome 9, spontaneously developed atopic dermatitis characterised by increased levels of Th2 responses and IgE levels (Matsuda et al., 1997), accumulation of eosinophils and mast cells in dermis (Vestergaard et al., 1999) and skin barrier abnormalities (Aioi et al., 2001).

A variety of mouse models have also been used to test the intrinsic hypothesis of dermatitis such as the IL-18 transgenic mouse model (Konishi et al., 2002), the role of IgE by using TSLP transgenic mice (Yoo et al., 2005) and T cell implication in the disease, with transgenic models such as RelB knockout mice (Barton et al., 2000) or models induced by allergens like ovalbumin (He et al., 2007, Laouini et al., 2005, Spergel et al., 1999, Woodward et al., 2001) and house dust mite (Huang et al., 2003). In the last category belongs Oxazolone, which I used for the induction of dermatitis-like skin inflammation in my experiments. Oxazolone is a strong hapten, which can easily penetrate the skin barrier due to its small size (Kabashima, 2013). It is frequently used to induce contact dermatitis which resembles the characteristics of chronic human dermatitis and many of its aspects indicate extrinsic driven dermatitis. Clinically the model is characterised by edema, epidermal hyperplasia, destroyed skin barrier and T cell dermal infiltration (Jin et al., 2009a). Single Oxazolone application has been shown to induce the acute phase of dermatitis (Fowler et al., 2003, Sheu et al., 2002). However, repeated applications of the allergen have been reported to induce chronic inflammation and skin barrier alterations (Aebischer et al., 2014, Man et al., 2008, Nakajima et al., 2014). Hairless mice treated with 9 to 10 Oxazolone doses developed Th2 reactions characterised by dermal cell infiltrates and elevated IgE serum levels (Man et al., 2008). Using a different model, two repeated applications of Oxazolone on BALB/c mice were enough to cause a mixed lymphocyte Th1, Th2 and Th17 response (Aebischer et al., 2014), while an elevated Th17 response in skin was reported after five Oxazolone applications in

flaky tail mice (Nakajima et al., 2014), a mouse strain widely used in skin research.

3.2 Aim

Various Oxazolone-induced dermatitis protocols have been described. These include protocols with different duration, Oxazolone concentrations, and mouse backgrounds. Therefore, I wanted to test if repeated applications of a certain Oxazolone concentration on our mice which have C57BL/6 background, would develop signs of chronic phase of skin inflammation together with impaired skin barrier and epidermal hyperplasia in a relatively short period of time. In this chapter I present data which were generated by using a specific two-week-dermatitis protocol.

3.3 Results

3.3.1 Skin inflammation induced by Oxazolone shows signs of extrinsic dermatitis followed by skin barrier disruption

In order to induce dermatitis-like skin inflammation I used a 14-day long Oxazolone protocol (Nakajima et al., 2014) on C57BL/6 mice. The schematic representation of the protocol is shown in Figure **3-1 A** and it has two phases. The first phase, is called the sensitization phase where the mice were firstly introduced to the allergen. I shaved their abdomens and I applied 3% Oxazolone resuspended in 100% ethanol (Day 0). After that, mice were not treated for five days. The second phase -the challenge phase- lasts from day 5 until the end of the experiment and mice are treated with Oxazolone every second day. Since I wanted to examine the potency

of the protocol, I treated only one ear with Oxazolone, while the other ear was used as a control and was treated with the vehicle (100% ethanol). On days 5, 7, 9, 11 and 13, 40μl of 0.6% Oxazolone were applied to one ear and 40μl of ethanol to the control ear. Mice were monitored daily in order to examine the dermatitis development and the time points when ears developed apparent signs of inflammation. On day 14 the mice were culled and images of the ears were taken (Figure **3-1 B**). The treated ears (**left image**) had developed signs of hyperplasia, redness and dryness. Also, some mice had developed flares due to the extended pruritus caused by the hapten application. On the other hand, ears treated only with vehicle (**right image**) looked completely healthy with no signs of swelling or flare induction.

Kinetics of ear swelling was created by monitoring ear thickness at the beginning of the challenge phase and every second day until the end of the protocol (Figure **3-1 C**). Ear thickness of both treated and control mice was measured and measurements were taken right before the application of the next Oxazolone dose. A final measurement was obtained when the experiment was terminated. Five days after the mice were sensitised, ear thickness of both treated (**red line**) and control mice (**black line**) was the same. However, as the protocol was developing and after each Oxazolone treatment, there was a significant increase ($p<0.001$ after the first dose application and $p<0.0001$ after all the other applications) in ear swelling of treated compared to the untreated ears. Control mice had the same ear thickness throughout the whole protocol, showing that ethanol did not cause any irritation to the skin, and confirming that any alterations provoked

were due to Oxazolone. At the end of the experiment ear thickness of treated mice was almost four times higher compared to controls.

Histological examination with H&E staining of both treated and untreated skin was conducted in order to characterise skin lesions and confirmed the previous results (Figure **3-1 D**). Analysis of treated ears (**right image**) showed signs of inflammation as there was observed increased parakeratosis and epidermal hyperplasia. Skin thickening was associated with dermal cell infiltration, while control ears **(left image**) had normal skin characteristics. Dermal and epidermal thickness were quantified and the results showed a significant increase in both dermal (377.8μm vs 26.93μm, $p<0.0001$) and epidermal (269.8μm vs 102.1μm, $p<0.01$) swelling in ears treated with Oxazolone compared to ears treated only with vehicle.

In order to evaluate if the signs of skin inflammation represent the acute or chronic phase of the disease, cytokine transcripts were examined in both skin and lymph nodes (Figure **3-2**; Left column represents cytokine transcript expression in skin and right column shows transcript expression in lymph nodes). I measured Th1 and Th2 cytokine expression and I showed that *Il4* transcript was five times higher in treated skin compared to the control (0.1589 vs 0.03677, $p<0.05$), however in the lymph nodes although there was an upregulation in treated mice compared to the controls, this was not significant (Figure **3-2 A**). *Il13* transcript showed the same trend in both skin and lymph nodes, with higher expression in treated compared to control, although the differences were not significant (Figure **3-2 B**). Finally, *Ifng* levels were tested and I showed that this transcript was significantly upregulated in skin but not in lymph nodes of treated mice

compared to controls (Figure **3-2 C**). Taken together the results showed that after two weeks of dermatitis induction, skin showed characteristics of chronic inflammation.

Apart from the cytokines, terminal differentiation marker Filaggrin was examined in the skin of treated and control mice (Figure **3-3 A**). Disruption of Filaggrin is an indication of skin barrier impairment (McAleer and Irvine, 2013) and as expected, treated skin showed significantly lower transcript expression compared to the healthy mice (0.3275 vs 3.041, $p<0.05$). In order to evaluate if Oxazolone induces an intrinsic or extrinsic model of dermatitis-like skin inflammation, IgE levels in blood serum of treated and control mice were examined (Figure **3-3 B**). I observed significantly higher IgE secretion in blood of treated mice compared to the controls, showing that my protocol induces extrinsic dermatitis.

3.3.2 T cell extraction from skin tissue

Leukocyte suspension from non-lymphoid tissues is a challenging process in order to obtain a cell yield with a high percentage of viability and maintain the cell surface receptors intact. Cell surface CD4 is very sensitive to enzymatic processes and it can get easily cleaved. I tried enzymatic digestion protocols using either Liberase or Collagenase D enzymes combined with DNAse I. Liberase is a blend of different types of collagenases which break down collagen, dissociating the skin (Yesil et al., 2009), while DNase I prevents the cells from developing clumps (Buitrago et al., 1977) and supports further skin dissociation. I used cRPMI as working medium without mercaptoethanol, which is considered to stop the action of the digestive enzymes. In order to evaluate the best protocol, I

tried different durations of skin digestion. Although a 2-hour digestion gave a cell yield with high viability, it did not allow me to detect either $CD4^+$ or $CD8^+$ T cells (Figure **3-4 A**). Trying the same protocol but followed by 30-45 minutes skin digestion, I obtained cell populations with equally high viability. Regarding T cells populations, both $CD4^+$ and $CD8^+$ T cells were detected and represented by high percentages (Figure **3-4 B**). Therefore, I used in all my experiments the 30-minute skin digestion protocol.

3.3.3 T cell infiltration in skin of Oxazolone-induced dermatitis and cytokine expression

Having shown that Oxazolone disrupts the skin barrier and causes skin lesions characterised by dermal cell infiltration, I next examined the type of cells which infiltrate skin. Atopic dermatitis is characterised by increased numbers of T cells in skin lesions (Pasparakis et al., 2014). First, I developed a skin gating strategy which allowed me to detect $CD4^+$ and $CD8^+$ skin T cell population with accuracy (Figure **3-5**). From the lymphocyte population I gated on single cells, eliminating in this way the potential false positive signals. As the skin is hard to digest, it needs to undergo both enzymatic and mechanical digestion. For this reason, the amount of cell death is much higher compared to digestion of lymphoid tissues and it needs to be determined with a viability dye. I gated single cells on the live gate, which are cells negative for the viability dye and then on $CD45^+CD3^+$ cells, which represent the leukocyte population, containing all types of T cells which are found in skin ($CD4^+$, $CD8^+$ and γδ T cells). γδ T cells are found to be double positive for CD3 and γδ TCR markers (Cron et al., 1989). A majority of them also express CD8 (Lake et al., 1991) and

some of them CD4 markers (Wen et al., 1998). Therefore, from the $CD45^+CD3^+$ population I gated out $CD3^+$ γδTCR^+ cells (which is the γδ T cell subset), ensuring this way that a pure TCRαβ $CD4^+$ and $CD8^+$ T cell population would be examined. The gating strategy described is used for the skin FACS analysis that I performed in all my experiments.

In order to test T cell infiltration, I treated both ears of WT mice with Oxazolone, while WT mice treated only with ethanol were used as a control. As expected, the percentage of lymphocytes in treated skin was higher compared to WT control skin (Figure **3-6 A**). Calculating the numbers, I saw that WT treated skin was infiltrated by triple the number of lymphocytes that normally is found in the skin (3.000.000 vs 990.140 lymphocytes, $p<0.0001$) as shown in Figure **3-6 B.** I next asked whether there is a difference in percentages and numbers of skin T cells, and I confirmed that there was a significant increase in T cell percentages (Figure **3-6 C**). Further analysing the different T cell types, I also showed that $CD4^+$ and $CD8^+$ T cell numbers were significantly upregulated in inflamed skin compared to control (Figure **3-6 D**). Overall the data suggested that the hapten-induced protocol of dermatitis which I used is characterised by T cell infiltration.

I further sought to examine the profile of T cells and their capacity of expressing inflammatory cytokines under steady state and inflammation conditions. Therefore, I stimulated skin cells from mice treated with dermatitis and control mice and I checked T cells for cytokine expression. Higher numbers of skin $CD4^+$ T cells from mice treated with Oxazolone produced Th1 (IFN-γ), Th2 (IL-13) and Th17 (IL-17) cytokines (Figure **3-7 A**) compared to control mice. As expected, more $CD8^+$ T cells from inflamed

skin produced IFN-γ and IL-17 cytokines in comparison with untreated control skin (Figure **3-7 B**). All of the differences observed were statistically significant.

3.3.4 *Leukocyte cell infiltration in skin of Oxazolone-induced dermatitis*

It is known that dermatitis is a skin condition which is also characterised by infiltration of innate immune cells (Kiehl et al., 2001, Oyoshi et al., 2012, Shi and Pamer, 2011). Apart from T cells, I examined skin for eosinophil and macrophage infiltration. In order to analyse these cells, I gated on $CD45^+$ skin populations and then I identified each population according to its markers: $CD11b^+SiglecF^+$ for eosinophils and $CD11b^+F4/80^+$ for macrophages (Figure **3-8 A**). Control skin was characterised by significantly lower percentages of both populations compared to dermatitis skin as shown on the representative FACS plots in Figure **3-8 A**.

Finally, quantification of FACS analysis showed that inflamed skin was characterised by five times higher numbers of eosinophils and tenfold increase in macrophages (Figure **3-8 B**).

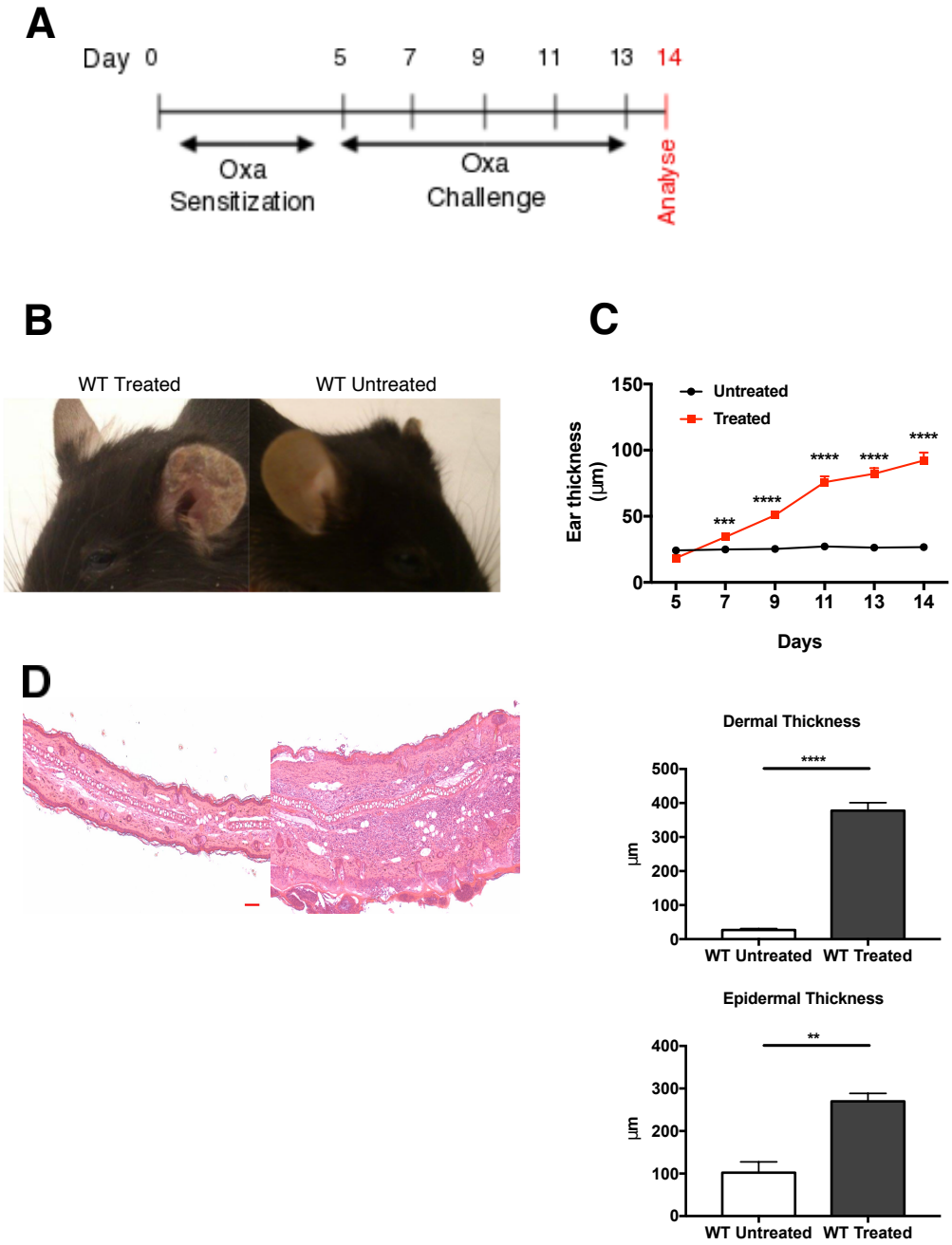


Figure 3-1: Histological features of Oxazolone-induced skin inflammation on C57BL/6 mice

(A) Sensitization and challenge schematic representation of Oxazolone (Oxa) administration.

(B) Representative images of ear pinnae from WT Oxa-treated mice (Left) and WT control mice (Right). Images were obtained when the experiment was terminated.

(C) Time course of ear thickness measurement of WT untreated (Black line) and WT treated (Red line) mice. Data were generated from one experiment with 6 mice per group.

(D) Representative H&E images of skin sections from WT control (Left) and Oxazolone-treated mice (Right). Scale bar represents 100μm. Quantification of dermal and epidermal thickness (White bars show untreated mice; Grey bars show Oxazolone-treated mice). Data were generated from one experiment with 5 mice per group.

Plots show mean ±SEM. **p<0.01, ***p<0.001 and ****p<0.0001.

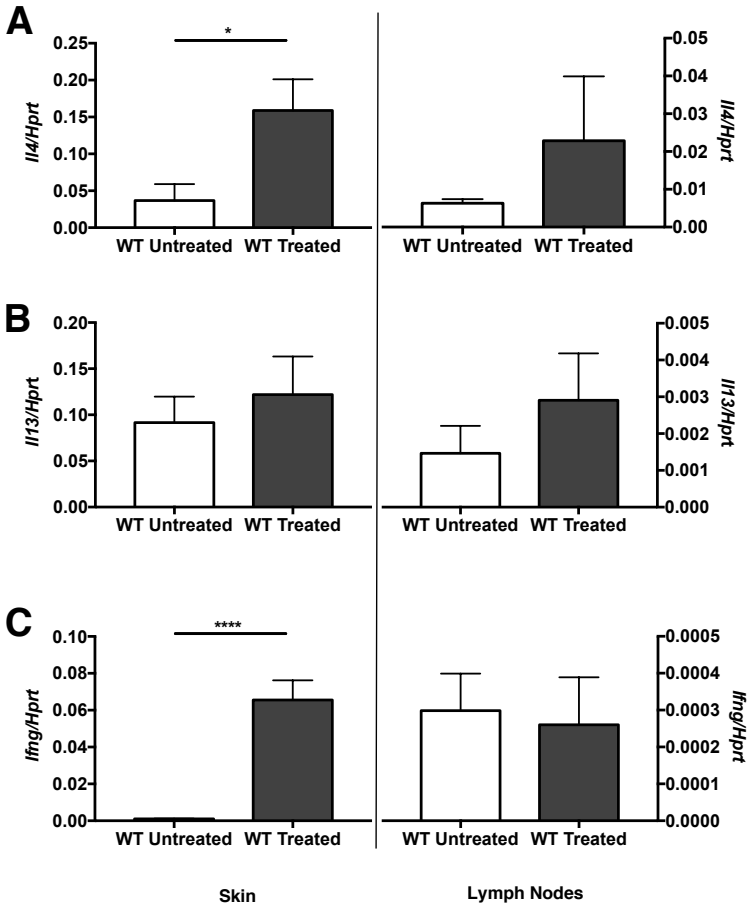


Figure 3-2: Features of chronic phase of dermatitis in skin but not in lymph nodes

Quantitative PCR analysis of **(A)** *Il4*, **(B)** *Il13* and **(C)** *Ifng* transcripts of WT control (White bars) and WT treated mice (Grey bars) on skin (Left column) and lymph nodes (Right column). Data were generated from two independent experiments with 2-4 mice per group. Plots show mean ±SEM. *p<0.05 and ****p<0.0001.

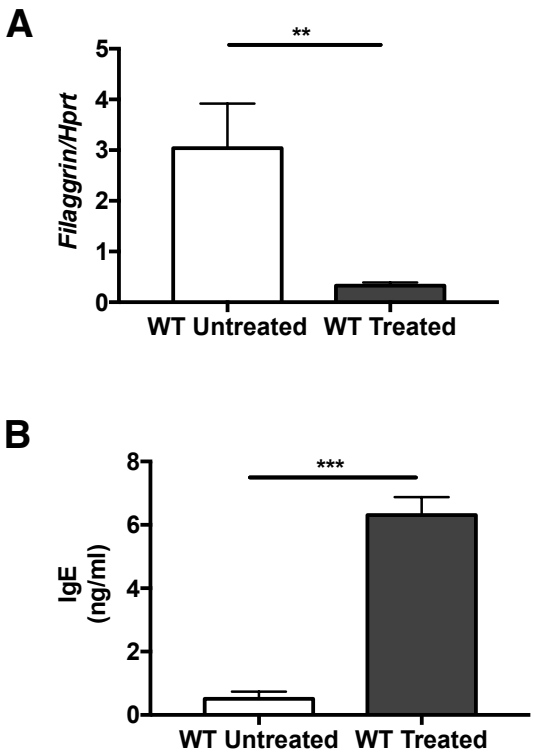


Figure 3-3: Impaired skin barrier and elevated IgE serum levels in mice with Oxazolone-induced skin inflammation

(A) Quantitative PCR analysis of *Filaggrin* transcript of WT control and Oxa-treated mice. Data were generated from one experiment with 4-6 mice per group.

(B) ELISA analysis on blood serum to measure IgE levels of WT control and Oxa-treated mice. Data were generated from two independent experiments with 2-4 mice per group.

White bars represent WT control mice, while grey bars represent mice with dermatitis-like inflammation. Plots show mean ±SEM. **p<0.01 and ***p<0.001.

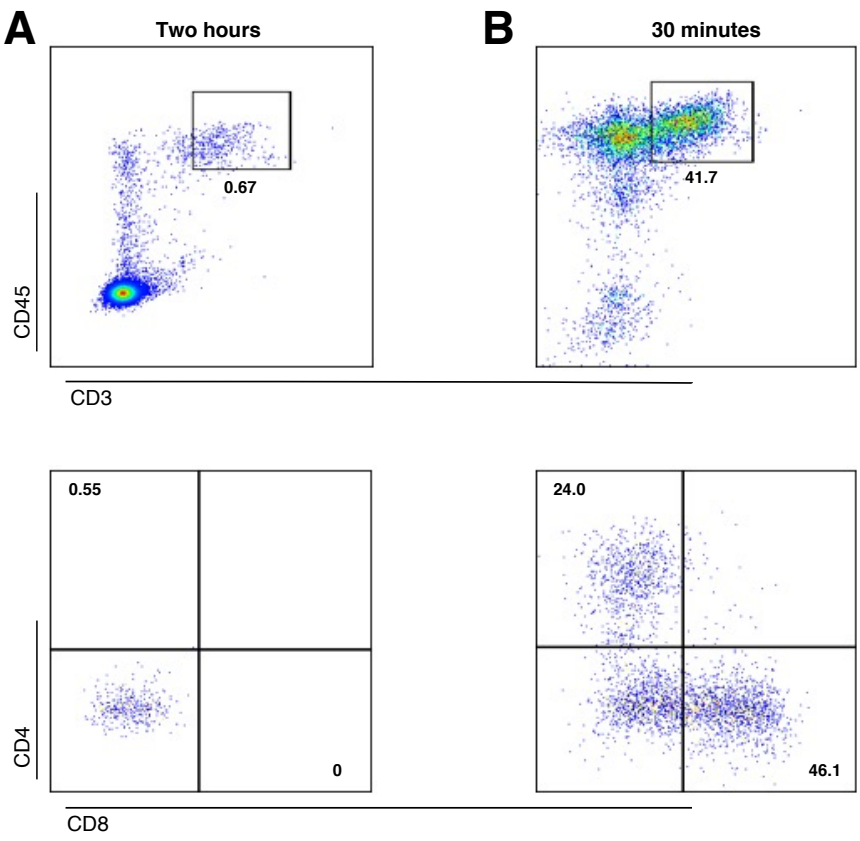


Figure 3-4: Different protocols of enzymatic digestion for skin T cell isolation from whole skin cell suspensions

Representative flow cytometric analysis of $CD4^+$ and $CD8^+$ T cells gated on $CD45^+CD3^+$ populations after skin was digested during **(A)** two hours or 30 **(B)** minutes.

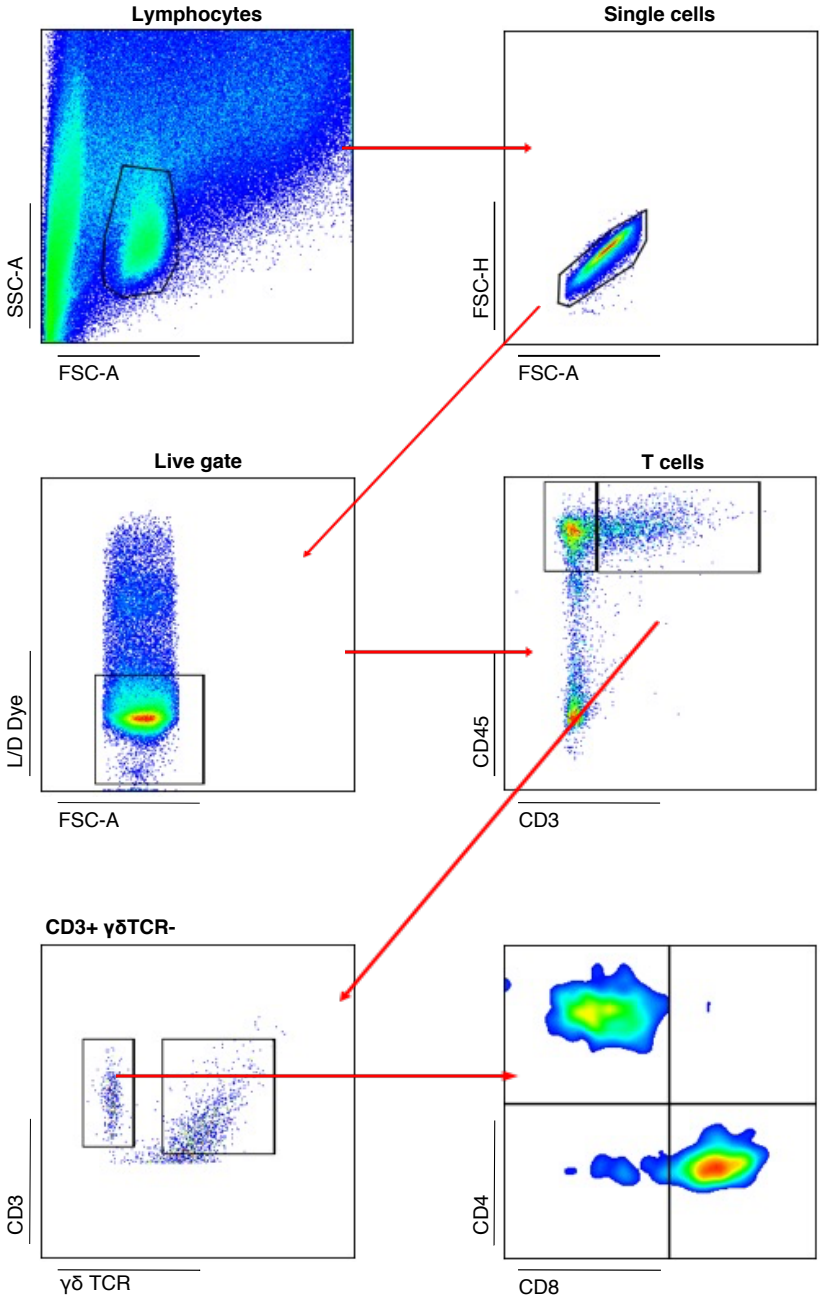


Figure 3-5: Identification of skin T cells from the whole skin

Representative FACS plots of flow cytometry gating strategy of skin $CD4^+$ and $CD8^+$ T cells. These are determined as $L/D^-CD45^+CD3^+\gamma\delta TCR^-$.

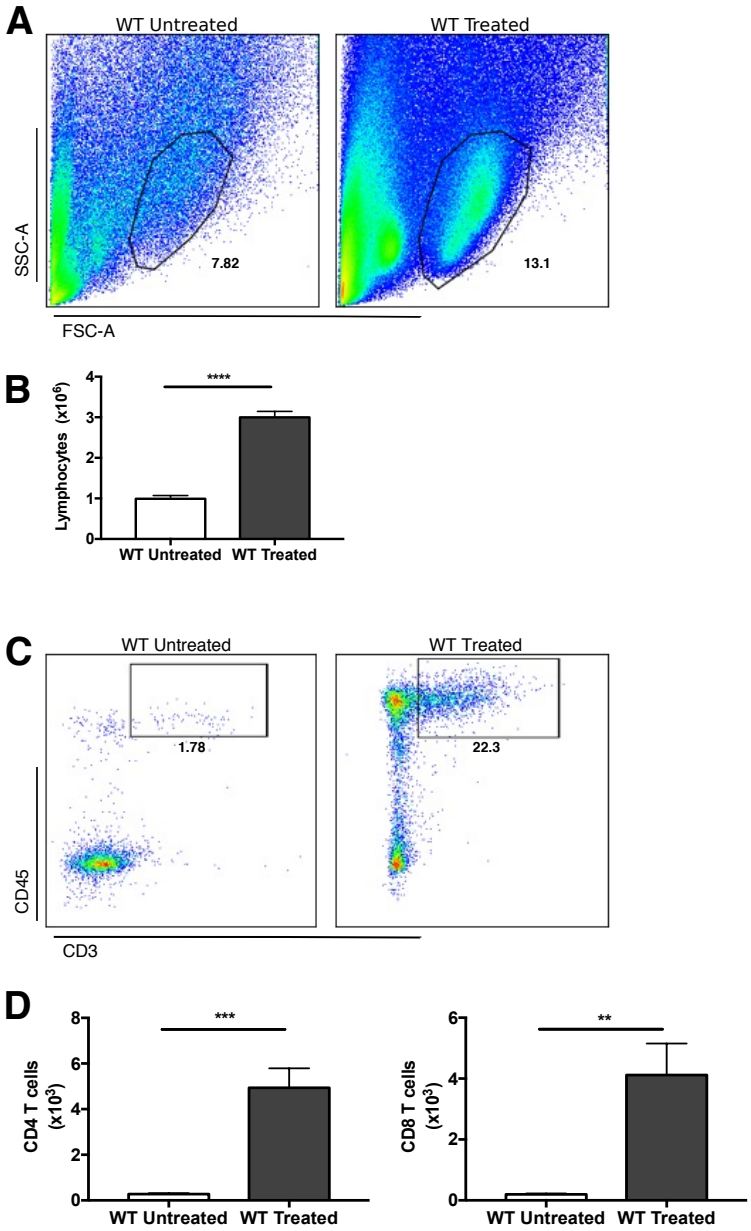


Figure 3-6: Skin cellular infiltration under steady state and upon dermatitis-like inflammation

Representative FACS plots showing **(A)** lymphocytes and **(C)** $CD4^+$ and $CD8^+$ T cells of whole untreated (Left) and treated (Right) skin. Bar charts show **(B)** numbers of lymphocytes and **(D)** $CD4^+$ and $CD8^+$ T cells of untreated (White bars) and treated (Grey bars) skin. Data were generated from two independent experiments with 3-5 mice per group. Plots show mean ±SEM. **p<0.01, ***p<0.001 and ****p<0.0001.

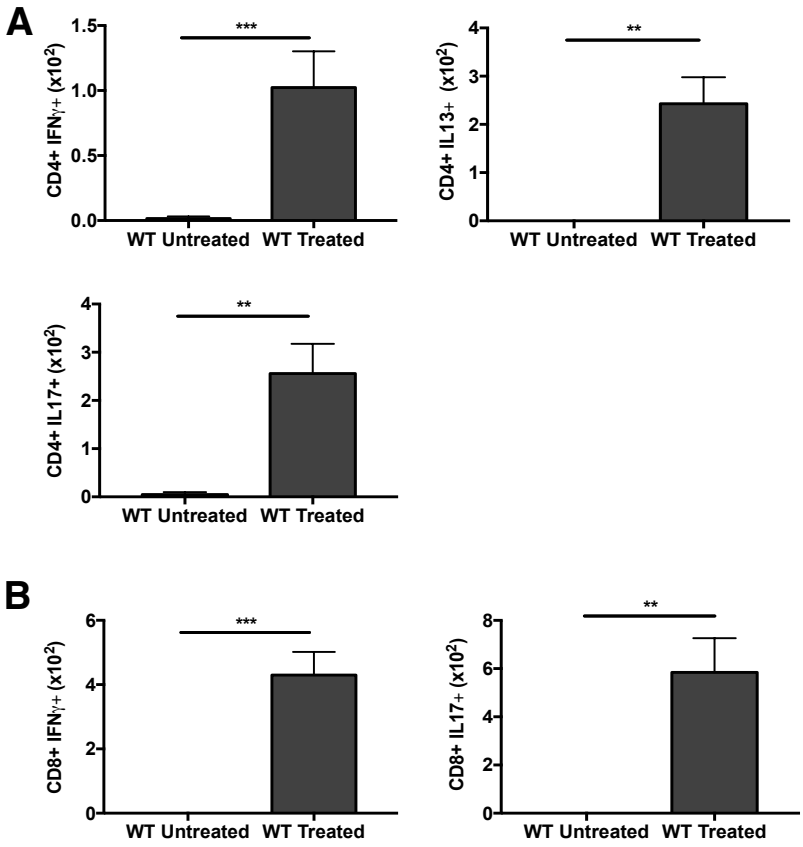


Figure 3-7: Cytokine production by skin T cells after Oxazolone treatment Bar charts show **(A)** IFNγ, IL-13 and IL-17 production in skin $CD4^+$ T cells and **(B)** IFNγ and IL-17 production in skin $CD8^+$ T cells from untreated (White bars) and treated (Grey bars) mice. Data were generated from one experiment with 4 to 6 mice per group. Plots show mean ±SEM. **p<0.01 and ***p<0.001.

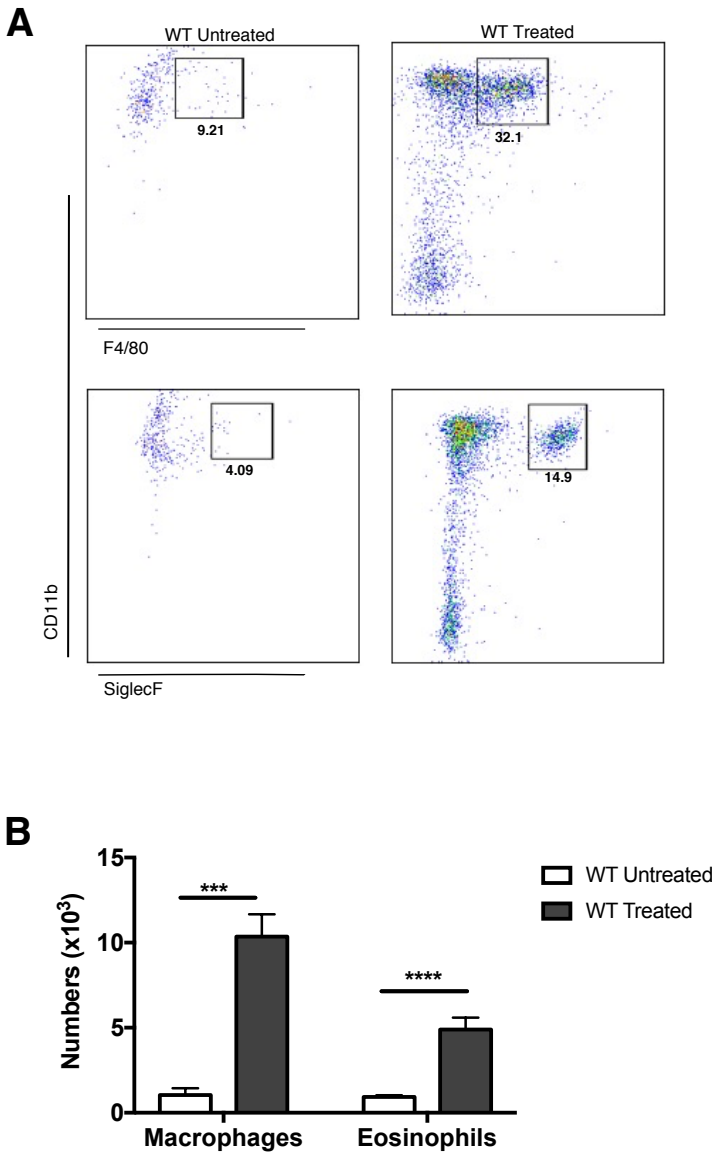


Figure 3-8: Monocyte infiltration in inflamed skin

(A) Representative FACS plots of macrophages (top plots) and eosinophils (bottom plots) of WT untreated (Left) and WT treated (Right) skin.

(B) Bar charts show numbers of macrophages and eosinophils. Untreated skin is represented with white bars and treated skin with grey bars. Data were generated from two independent experiments with 4 to 7 mice per group. Plots show mean ±SEM. ***p<0.001 and ****p<0.0001.

3.4 Discussion

In this chapter I have described a two-week Oxazolone protocol for induction of dermatitis which is capable of provoking skin inflammation that resembles the chronic phase of dermatitis in WT mice of C57BL/6 background. The mice showed impaired skin barrier as described by hyperkeratosis and parakeratosis and loss of the terminal differentiation marker Filaggrin, all histological features of chronic dermatitis. Visible signs of skin inflammation were not detected immediately after Oxazolone application, but after the third administration on ears (around day 10 of the protocol), suggesting that this was the time point where the disease switched from acute to chronic phase.

T cell percentages and numbers were precisely calculated (Figure **3-5**) after testing two different enzymatic protocols with different durations (Figure **3-4**). Skin T cell suspension processing is very challenging as skin tissue needs to be dissociated and produce viable cells and at the same time and maintain the integrity of common surface markers such as CD4 and CD8 which are very sensitive to enzymatic digestion. I confirmed that the best combination of enzymes was Collagenase D with DNase, as Dispase causes damage to immune cell receptors (Autengruber et al., 2012), while Trypsin affects the integrity of $CD4^+$ T cells (Botting et al., 2017) and mammalian cells in general (Benck et al., 2016). Also, the time that I enzymatically digested the skin was of high importance. 30-45 minutes were enough to dissociate skin, acquire a good cell yield of viable cells with normal cell surface protein expression, while after two hours digestion, although the skin was dissociated, without affecting CD45, CD3,

CD11b, F4/80 and γδ TCR markers, it failed to maintain CD4 and CD8 surface receptors, and thus T cell populations could not be detected by flow cytometry.

Macrophages and eosinophils along with T cells infiltrated skin, indicating that within two weeks of allergen-induced dermatitis there has been crosstalk between innate and adaptive immune cells, and monocytes have sent signals to activate adaptive immune cells. This was concluded as T cells were found to produce mixed Th1, Th2 and Th17 pro-inflammatory cytokines, which is an additional indication of widespread skin inflammation.

Additionally, lymph nodes of treated skin, showed no significant upregulation in cytokine transcript expression (Figure **3-2 B**), which can be explained by the kinetics of the disease. As mentioned in the introduction, atopic dermatitis is a disease with a complex cytokine profile which is affected by the time point at which mice are analysed. It can be concluded that after 14 days, lymph nodes have already been differentiated towards Th1, Th2 and Th17 effector cells, and have induced a response in the skin. If the mice were analysed at an earlier time point, it would have been possible to observe responses in the lymph nodes but probably not in the skin.

Finally, I have confirmed that the protocol that I will be using causes an extrinsic form of dermatitis as evaluated by elevated levels of IgE blood serum, however that was expected as the allergen compromises skin barrier allowing it to be penetrated.

Overall, using the protocol described in this chapter I will be investigating the role of **Hedgehog signalling** in the context of the **outside-inside hypothesis** of dermatitis. Additionally, the results presented in the following chapters describe the role of Hedgehog signalling in skin showing features of **chronic dermatitis.**

Chapter 4

Chapter 4: The role of Hedgehog signalling in skin inflammation

4.1 Introduction

4.1.1 Shh is implicated in regulation of skin homeostasis in developing and adult tissue

In addition to its roles in regulating lymphopoiesis and hematopoiesis, which are described in Chapter **1**, Hh signalling pathway is fundamental for embryonic development as it is involved in tissue patterning such as limb bud and ventral neural tube formation (Dessaud et al., 2008, te Welscher et al., 2002, Wang et al., 2000). Shh expression on notochord has been reported to promote floor-plate gene expression in invertebrates and vertebrates (Echelard et al., 1993, Krauss et al., 1993, Roelink et al., 1994). Hh signalling has also been associated with many processes in skin such as cell proliferation, survival and migration, stem cell homeostasis, tissue regeneration and repair (Beachy et al., 2004, Ingham and McMahon, 2001, Teglund and Toftgard, 2010). During skin development Shh regulates stem cell homeostasis, epidermal morphogenesis, sebaceous gland- and hair follicle development (Adolphe et al., 2004, Chiang et al., 1999, Karlsson et al., 1999). Mouse studies revealed that Shh induced differentiation of embryonic follicles into developing dermis and an increase in mass of follicle epithelium (Chiang et al., 1999). Furthermore, although Shh is not required for initiation of hair follicle development, it regulates growth and progression of hair follicles to hair germ (Chiang et al., 1999, St-Jacques et al., 1999).

Regarding stem cell regulation, Hh signalling regulates stem cell proliferation of various tissues such as brain, muscles, blood, prostate, gut and bladder (Beachy et al., 2004, Petrova and Joyner, 2014) but I will focus on its role in skin stem cells. In normal epidermis, Shh is the main Hh ligand found in postnatal skin and its presence is restricted to hair follicles undergoing the anagen (growing) phase (Oro and Higgins, 2003). There, it regulates cell cycle cyclins, such as Cyclin D and Cyclin E giving rise to hair follicles in adult epidermis (Duman-Scheel et al., 2002). This effect has also been shown in human primary keratinocytes where Shh favours their proliferation and inhibits their apoptosis via cyclin D1 (Liu et al., 2014). Moreover, Shh activation induces the interfollicular cells of adult epidermis to acquire characteristics of bulge stem cells (Silva-Vargas et al., 2005). Recent evidence also reported that Shh-expressing progenitor cells trigger stem cell activation and proliferation via signalling to their parental quiescent stem cells in the bulge region (Hsu et al., 2014).

4.1.2 Hedgehog signalling in skin cancers

Although in adult tissues Hh pathway function is minimal, its aberrant activation is usually oncogenic (Briscoe and Therond, 2013, Epstein, 2008, Pietsch et al., 1997, Tostar et al., 2006). BCC, Squamous cell carcinoma (SCC) and melanoma are types of skin cancer which are related to non-regulated Hh signalling activation (Li et al., 2011, Teglund and Toftgard, 2010).

SCC is initiated in squamous epithelium located in many tissues including skin where the majority of cases are detected in neck and head (Li et al., 2011). According to (Schneider et al., 2011), patients with skin SCC show

upregulated levels of Gli1, Ptch and Smo and lower levels of Gli2 compared to healthy patients. Also, Shh upregulation was linked with poor survival prognosis for these patients.

Melanoma is a cancer type arising in skin melanocytes (Grichnik, 2008). Mouse evidence showed that Gli1 is upregulated upon induction of melanoma in a transgenic mouse overexpressing Ras, which was suppressed after cyclopamine treatment and that high Gli2 expression is linked with high tumour metastasis (Alexaki et al., 2010, Stecca et al., 2007). However no genetically modified mouse models for Hh signalling pathway have been established for studying SCC and melanoma.

BCC originates from body areas which are exposed to the sun (Rubin et al., 2005) and analysis of BCC patients confirmed that loss of function Ptch1 mutations, which led to uncontrolled Smo activation, were responsible for the development of cancer (Gailani et al., 1996, Reifenberger et al., 2005). Also, mouse models with conditional deletion of Ptch1 show features of human BCC after exposure to UV light (Adolphe et al., 2006, Ellis et al., 2003, Siggins et al., 2009). Mutations of other Hh signalling components are also related to BCC, such as Smo overexpression (Reifenberger et al., 1998, Xie et al., 1998), aberrant Shh activation and Gli1 and Gli2 mutations which lead to a consecutive activation of the pathway (Grachtchouk et al., 2000, Ikram et al., 2004, Nilsson et al., 2000, Oro et al., 1997).

4.1.3 Sonic hedgehog signalling in tissue repair

Hh pathway is also implicated in airway, gastrointestinal and epidermal tissue repair. Lung fibrosis is characterised by elevated Gli1-expressing fibroblasts in lung airways (Liu et al., 2013). During lung fibrosis Shh upregulation results in collagen accumulation in airways and induces cell proliferation and tissue alterations resembling characteristics observed after injury (Krause et al., 2010, Liu et al., 2013).

Liver damage activates Hh pathway in humans and mice, where it has been shown that it enhances tissue reconstruction after injury (Jung et al., 2007, Omenetti et al., 2007). Studies have also reported that the Hh pathway is indispensable for hepatocyte proliferation after liver ablation (Ochoa et al., 2010). In human and murine stomach, Hh signalling promotes gastric epithelium differentiation and is downregulated during restoration of gastric tissue after ulcer injury (Kang et al., 2009, van den Brink et al., 2002).

Finally, Shh plays an important role in skin wound repair. Mice treated with cyclopamine, which is a Shh inhibitor, showed a significantly increased time for wound healing, indicating that Shh disruption attenuates wound healing (Le et al., 2008, Luo et al., 2009). Additionally, Shh is reported to upregulate angiogenic factors which facilitate blood flow to wounds (Pola et al., 2001) and to induce hair follicle development in wounded skin (Ito et al., 2007).

4.2 Aim

Although Hh signalling pathway is known to be active in skin and other tissues during embryonic development, there is growing evidence of its presence in adult skin under homeostatic conditions and activation or loss-

of-function mutations of Hh signalling components result in various types of skin cancer. However, the role of Hh signalling in skin inflammation still remains elusive. In this chapter, I aim to test the hypothesis that the Hh signalling pathway is involved in Oxazolone-induced skin inflammation. By using a fluorescent reporter mouse model, I will assess the Hh signalling activation on different skin cell types under steady state and inflammation.

I will first examine and compare the expression of Hh signalling components in WT control and WT mice on induction of dermatitis. Then I will investigate how Hh ligand expression develops throughout the resolution of dermatitis-like skin inflammation. Finally, I will evaluate if T cells show active Gli-mediated transcription.

4.3 Results

4.3.1 *Hedgehog pathway components are upregulated upon dermatitis- like skin inflammation*

In order to assess the role of the pathway in dermatitis, I applied the dermatitis protocol described in Chapter **3** to WT mice. Ear tissues from WT treated and control mice were digested and non-quantitative PCR was performed in order to examine whether Hh family member proteins are upregulated on induction of dermatitis. As shown in Figure **4-1 A**, *Shh*, *Dhh* and *Ihh* could be detected in whole skin tissue homogenates from WT-treated skin, whereas their levels were lower in control skin. *Shh* transcript was very low in control skin but it was more readily detectable in skin tissue with dermatitis. *Dhh* and *Ihh* were both detectable under both homeostatic

and skin inflammation conditions. In order to quantify the data, I performed quantitative PCR for all three ligands (Figure **4-1 B**). I observed that only *Shh* transcript was significantly upregulated in skin with dermatitis-like skin inflammation and was characterised by a threefold increase. *Dhh* and *Ihh* transcripts were altered but the differences were not significant. More precisely, *Dhh* expression was slightly increased, while *Ihh* transcript was slightly lower after dermatitis-like inflammation.

I further evaluated the levels of known Hh pathway target genes. Hh receptor Ptch1, Hh responsive transcription factor, Gli1 and IL-4 are target genes of the pathway (Ingham et al., 2011, Furmanski et al., 2013, Park et al., 2000) . Transcript levels of *Patch1*, *Gli1* and *Il4* were assessed by q-PCR and I observed a twofold increase in *Ptch1* expression (Figure **4-2 A**), a threefold *Gli1* upregulation (Figure **4-2 B**), while *Il4* transcript levels were four times higher (Figure **4-2 C**) in injured skin compared to WT controls. All differences were statistically significant.

4.3.2 *Shh expression in skin is increased as the dermatitis-like skin inflammation is induced*

Since only *Shh* ligand showed significant changes after induction of dermatitis-like skin inflammation, I wanted to understand when and where it is expressed. Therefore, Shh protein expression was evaluated during a time course experiment of Oxazolone administration. I followed the Oxazolone protocol mentioned in the previous chapter, but mice were culled on days 6, 8, 10, 12 and 14. The time points on which the experiments were ended correspond to the days that mice were given 1st, 2nd, 3rd, 4th and 5th doses respectively. Schematic representation of the

different time points when the experiment was terminated is indicated in Figure **4-3 A**.

After each time point ear tissues were cryopreserved in OCT and immunofluorescence staining for Shh was performed. As expected, untreated skin (**Day 0**) showed no obvious Shh expression. (Figure **4-3 B**). After one Oxazolone dose application on the ears (**Day 6**), Shh expression was still not detected and skin had no signs of inflammation (Figure **4-3 C**). When two Oxazolone doses were applied to the mice (**Day 8**), I could detect signs of skin inflammation as there was an apparent swelling of the dermis. Skin inflammation was followed by Shh expression mainly in the epidermis (Figure **4-3 D;** shown in red colour). After three (**Day 10**) and four (**Day 12**) Oxazolone administrations, immunofluorescence staining revealed upregulation of Shh expression, which was now apparent not only in epidermis but also in dermis (Figures **4-3 E** and **4-3 F**). Finally, after two weeks of treatment (**Day 14**), skin injury was followed by a strong Shh expression in dermis and epidermis of ear tissue (Figure **4-3 G**).

4.3.3 Skin inflammation upregulates active Hh signalling pathway in immune cells

Given that Shh expression was upregulated on dermis and epidermis of injured skin, I decided to examine if T cells are undergoing active Hh signalling in control and dermatitis skin. To perform these experiments, I used the Gli-binding site (GFP) reporter transgenic mice, which have eight binding sites for Gli transcription factors, which regulate GFP expression. GFP will be expressed when Gli activator forms bind to the GFP in the transgene. In this way we can measure the active Hh signalling pathway in a series of tissues including skin (Balaskas et al., 2012).

In order to compare GFP expression in WT-treated and WT-untreated ears, I applied Oxazolone only to one ear of the mouse using the other ear as control. In this way I evaluated GFP upregulation in the same mouse, because GFP expression is variable in every animal, even though the mice are littermates. I first tested Hh signalling activity in skin $CD3^+$ T cells, and the results showed that a lower percentage of $CD3^+$ T cells expressed GFP on control ear tissue, which was increased in treated ears (Figure **4-4 A**, 13.8% vs 19.3% in a representative contour plot; skin cells are gated on $CD3^+$ T cells), suggesting that there is more active Hh signalling in immune cells upon dermatitis-like skin inflammation. However, this difference was not significant. Therefore, I decided to test the activity of the Hh signalling pathway on T cell components. Although both percentages and numbers of skin γδ T cells with active Hh pathway were greater in treated WT skin compared to the control, the differences were not significant (Figure **4-4 B**). Next, I examined GFP expression in skin $CD4^+$ and $CD8^+$ T cells. A higher percentage of $CD4^+$ T cells (15.13% vs 2.23%) and $CD8^+$ T cells (10.2% vs 4.963%) expressed active Hh signalling in treated mice compared to controls, although the differences were not statistically significant (Figure **4-5 A**). The increased $CD4^+GFP^+$ and $CD8^+GFP^+$ T cell percentages were consistent with significantly higher numbers of both populations expressing GFP (Figure **4-5 B**) in WT treated compared to control ears. Finally, I examined mean fluorescent intensity (MFI) of $CD4^+GFP^+$ and $CD8^+GFP^+$ T cells. $CD4^+GFP^+$ T cells from treated ears had a significantly higher MFI (994.3 vs 214.7, $p<0.05$) compared to WT control mice. However, $CD8^+GFP^+$ T cells from both treated and control experimental groups had

similar MFIs (Figure **4-5 C**). These results imply that not only more skin $CD4^+$ T cells had active Hh pathway, but also the level of the *in vivo* pathway activation was higher compared to the WT untreated mice. Thus, T cells have Gli-mediated transcriptional activity in dermatitis skin, and $CD4^+$ and $CD8^+$ T cell subpopulations show significant differences in active Hh signalling expression, suggesting that Hh signalling might be implicated in skin inflammation via those cells.

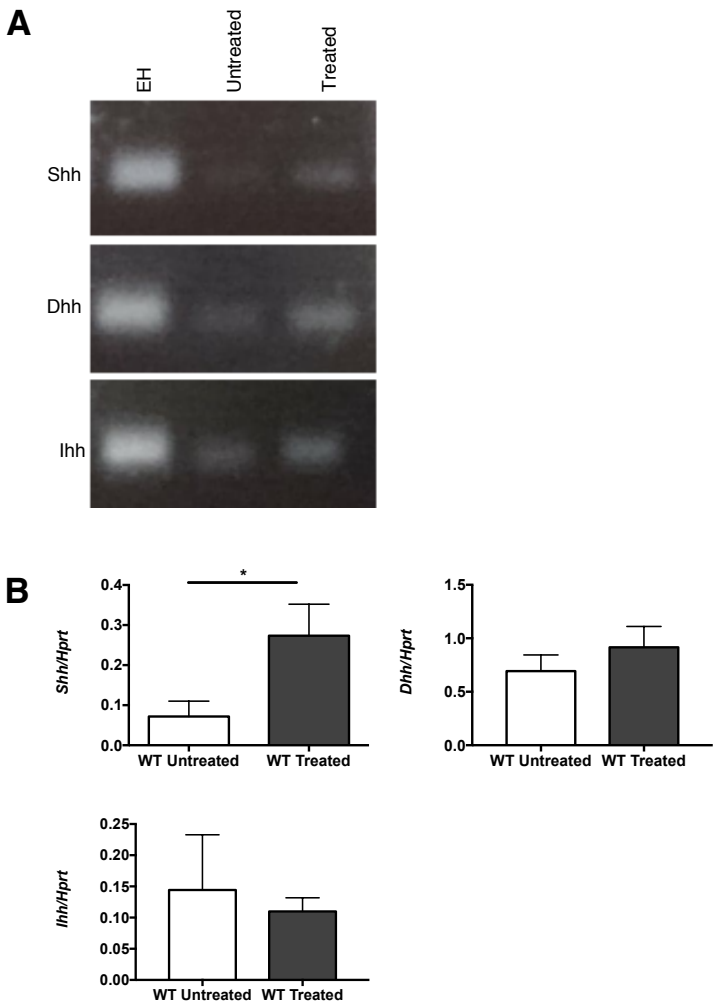


Figure 4-1: Hh signalling components are upregulated upon dermatitis-like skin injury

(A) Representative image showing the expression of Shh, Dhh and Ihh Hh ligands as determined by non-quantitative RT-PCR in homogenised skin tissue of WT untreated and WT treated mice. EH; embryonic head, positive control.

(B) mRNA expression of *Shh*, *Dhh* and *Ihh* Hh ligands as determined by quantitative PCR from WT untreated (White bars) and WT treated (Grey bars) homogenised skin.

Data were generated from two independent experiments with 3 mice per group. Plots show mean ± SEM. *p<0.05.

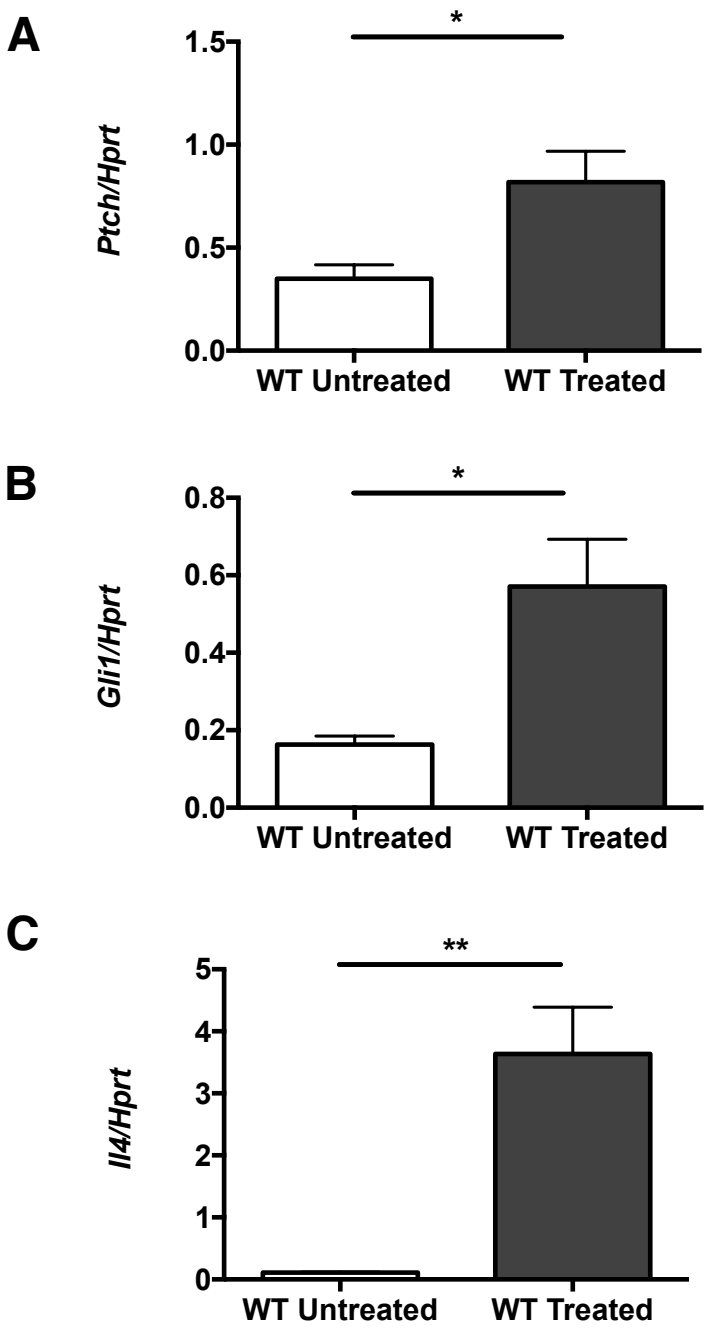


Figure 4-2: Upregulation of Hh signalling pathway target genes upon dermatitis-like skin inflammation

mRNA expression of **(A)** *Patched1* (*Ptch*), **(B)** *Gli1* and **(C)** *Il4* transcripts determined by q-PCR from the whole homogenised skin of WT untreated (White bars) and treated skin (Grey bars). Data were generated from two independent experiments with at least 5 mice per group. Plots show mean ±SEM *p<0.05 and **p<0.01.

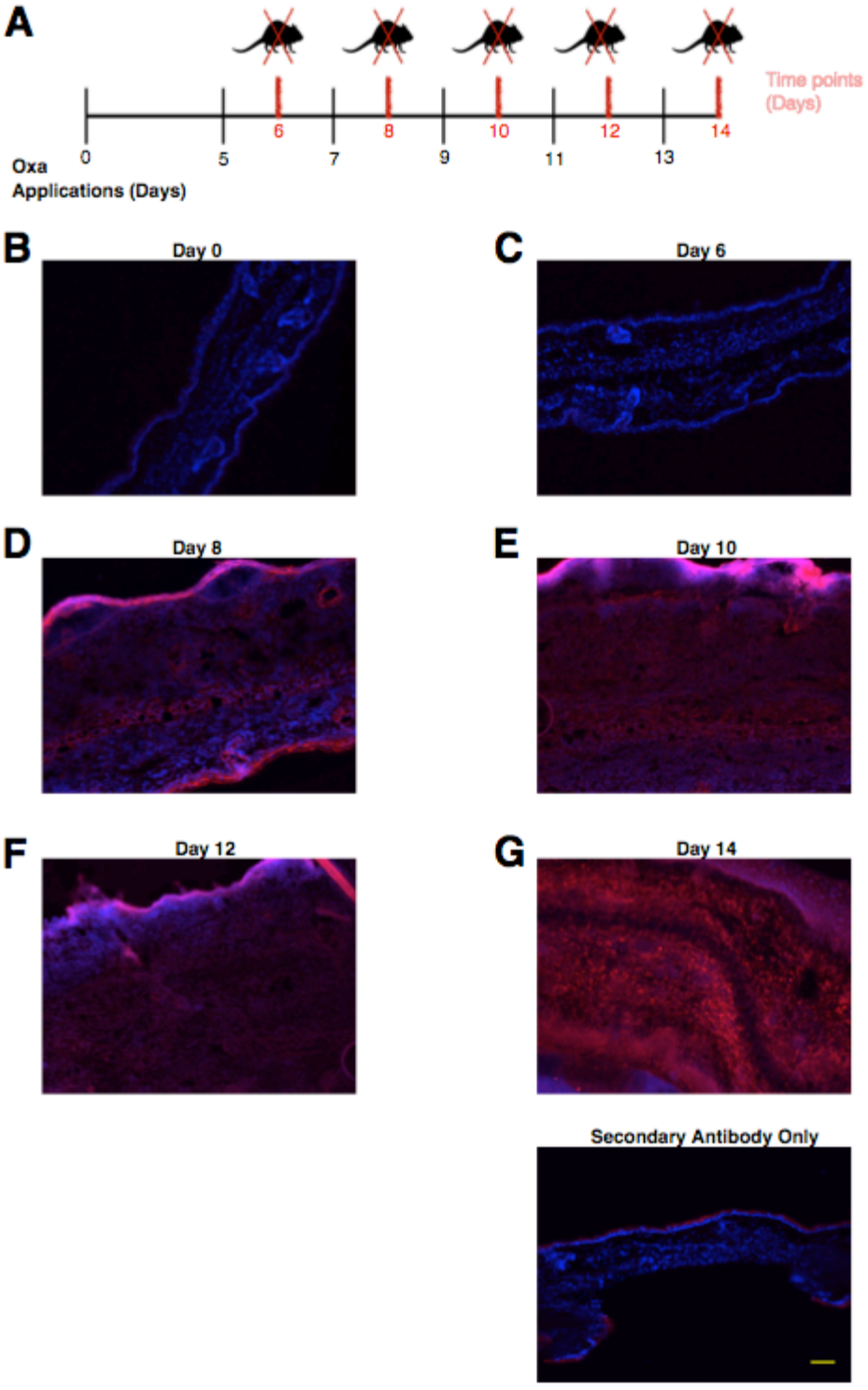


Figure 4-3: Shh is expressed by epidermis and dermis during Oxazolone-induced dermatitis-like skin inflammation

(A) Schematic representation of the time course experiment. Black numbers show the days of Oxazolone administration while red

numbers show the time points when each experiment was terminated.

Representative immunofluorescence staining images of Shh (red) expression in frozen skin sections from **(B)** WT untreated mice, and mice treated with **(C)** one, **(D)** two, **(E)** three, **(F)** four and **(G)** five Oxazolone applications. DAPI-stained nuclei are shown in blue. The last image shows staining of skin only with isotype control. Scale bar represents 100μm. Data were generated by one experiment with three mice per time point.

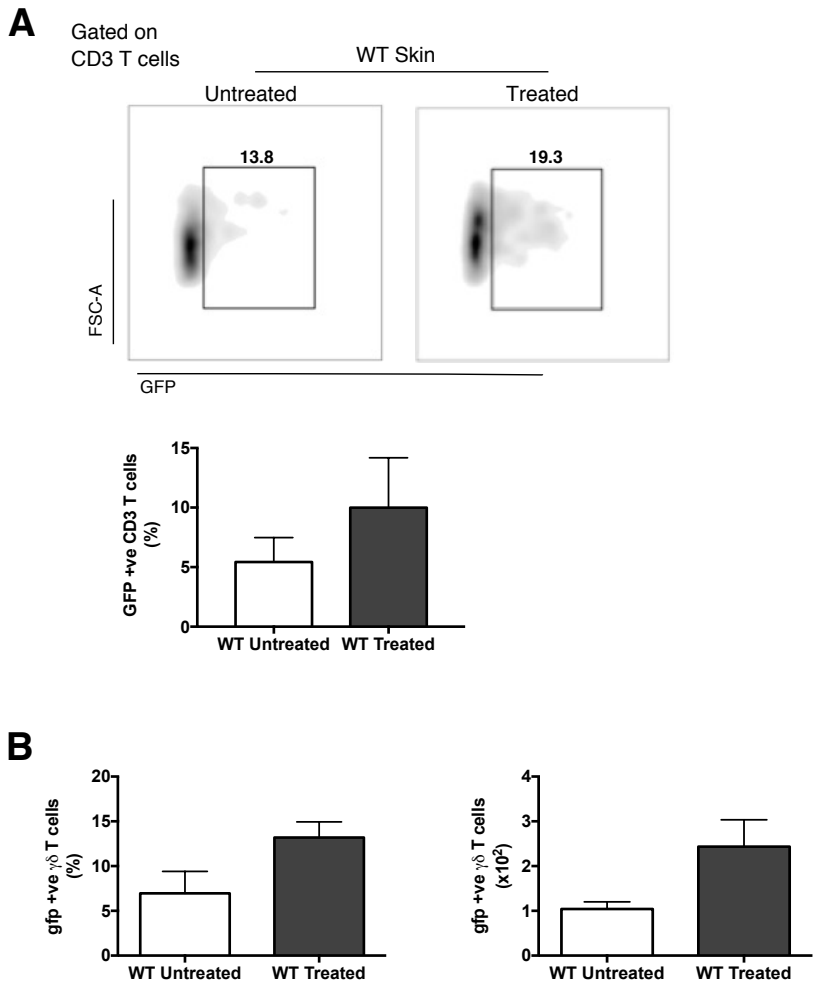


Figure 4-4: Lymphocytes show active Hh signalling pathway *in vivo* upon skin inflammation

(A) Representative contour plots of skin $CD3^{+}GFP^{+}$ T cells in WT untreated (Left) and WT treated (Right) mice. Bar charts show percentages of skin $CD3^{+}GFP^{+}$ mice in WT untreated and WT treated mice.

(B) Bar charts show percentages (Left) and numbers (Right) of skin γδ T cells from WT untreated and WT treated mice.

Data were generated from two independent experiments with 3 mice per group. WT untreated mice are represented with white bars and WT treated mice are represented with grey bars.

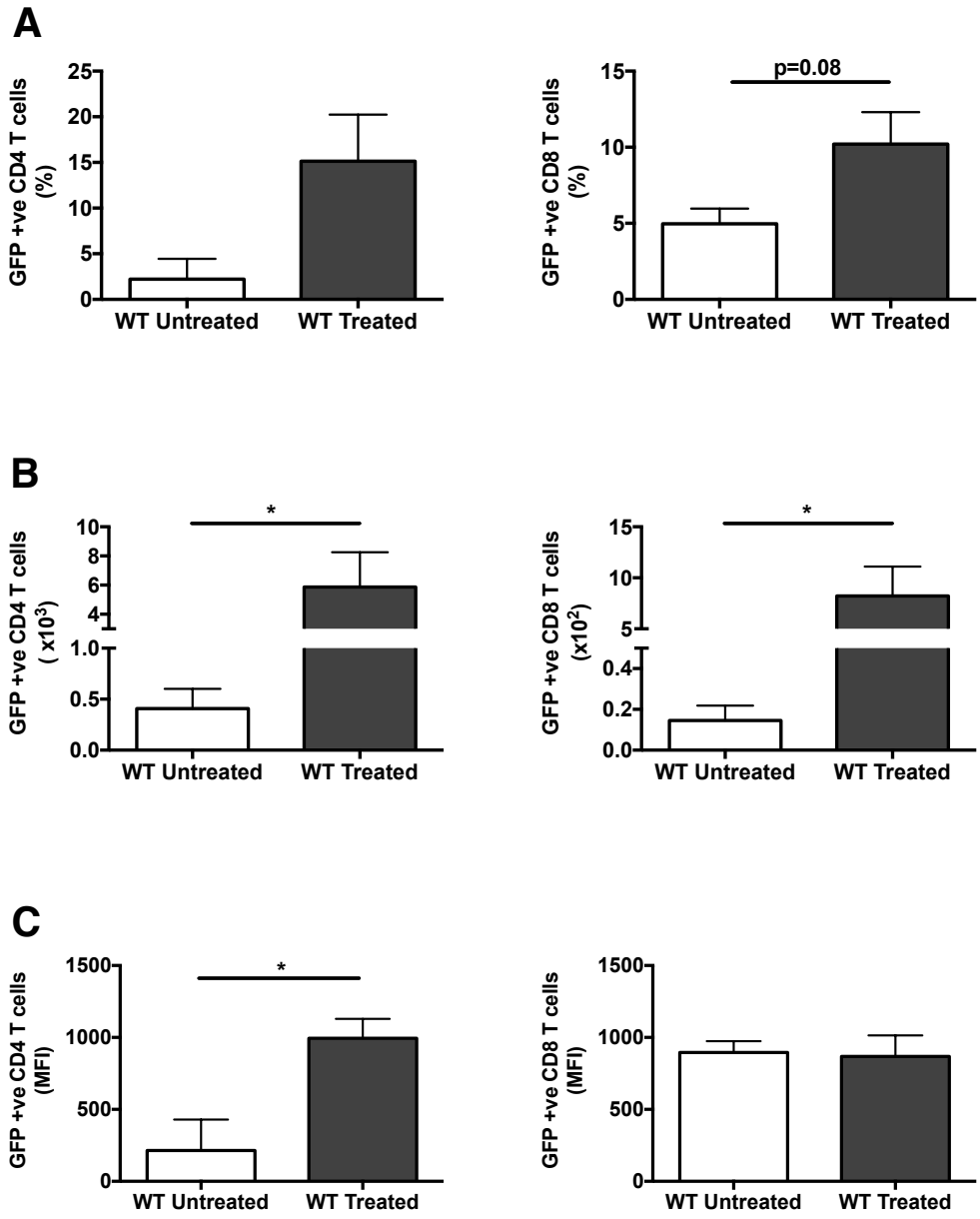


Figure 4-5: *In vivo* active Hh signalling on skin CD4$^+$ and CD8$^+$ T cells upon skin inflammation

Bar charts show **(A)** percentages, **(B)** numbers and **(C)** mean fluorescent intensity (MFI) of skin CD4$^+$ and CD8$^+$ T cells. Data were generated from two independent experiments with 3 mice per group. WT untreated mice are represented with white bars and WT treated mice are represented with grey bars. Plots show mean ±SEM *p<0.05.

4.4 Discussion

Here I have shown that the Hh signalling pathway is upregulated after induction of chronic dermatitis-like skin inflammation. After examination of the Hh ligands which are responsible for initiating the Hh pathway, I found that *Shh* is the only one significantly upregulated, while *Dhh* and *Ihh* did not show any significant alteration in their expression. That is consistent with literature previously published which suggests that Shh is upregulated and plays a role in repair in various tissues such as lung, skin and pancreas (Asai et al., 2006, Granato et al., 2016, Pereira Tde et al., 2010, Pogach et al., 2007, Watkins et al., 2003). In agreement with this finding, Hh signalling target genes are also upregulated, as shown in Figure **4-2**, suggesting that the whole pathway is activated. This upregulation could be Smo-dependent or Smo-independent (Teperino et al., 2014). For this reason, further experiments need to be done to investigate Smo function.

The time course experiment revealed a progressive induction of Shh expression in mouse skin. Shh expression was initially upregulated in epithelia, as indicated by its expression only in epidermis. In later stages of the inflammation other cells also expressed Shh, as the signal appeared in dermis (Figure **4-3 E**), which is characterised by accumulation of immune cells as mentioned in the introduction. The time point of Shh expression in epidermis (Figure **4-3 D**) coincides with the first visible symptoms of skin disruption which is discussed in the previous chapter. This experiment is supported by similar findings of Shh expression in asthmatic lungs (Standing et al., 2017), which could indicate that Shh expression has similar

behaviour in dermatitis and asthma, diseases that both belong to atopic march as mentioned in the introduction.

Moreover, in this chapter by making use of the GBS-GFP reporter mouse model, I confirmed that Hh pathway activation *in vivo* was elevated in inflamed skin, as shown by GFP measurements. Skin T cells were assessed for GFP expression. GFP upregulation however was not significant in the entire skin T cell population as indicated in Figure **4-4 A**. When T cell subpopulations were analysed individually, the results revealed that only skin $CD4^+$ and $CD8^+$ T cells were significantly upregulated *in vivo* Hh signalling. Hh proteins are reported to be expressed by epithelial and not T cells (Outram et al., 2000, El Andaloussi et al., 2006, Petrova and Joyner, 2014, Saldana et al., 2016, Standing et al., 2017). Thus, I can conclude that the Hh proteins expressed by epithelia of the damaged skin, can activate the local immune network, in this case skin $CD4^+$ and $CD8^+$ T cells. This suggests that $CD4^+$ and $CD8^+$ T cells are the major players to link Hh pathway activation with dermatitis. Therefore, in the following experiments I will focus on these two populations.

It would be interesting to investigate if Hh pathway upregulation after skin inflammation with dermatitis features is protective or contributes to further skin disruption. According to recent publications, in patients with chronic Hepatitis B and C infections, hepatocytes produced more Hh ligands and an increase in Hh pathway activity was linked to severe outcome of the disease (Pereira Tde et al., 2010). In addition, increased Hh ligand expression and Hh target genes are reported in Epstein-Barr virus and HIV infections and result in decreased host defences (Deb Pal and Banerjee,

2015, Lan et al., 2017). I will present my findings in the following chapters, where by using transgenic mouse models I will investigate the role of Hh signalling in more depth.

Finally, since there is a clear link between the Hh pathway and skin injury, in the future it would be important to examine its role in the context of other skin inflammatory diseases such as psoriasis. My experiments are conducted in animals; however, literature is poor about the role of Hh signalling in human skin inflammation. Therefore, it would be of importance to do experiments on skin biopsies of patients with acute or chronic atopic dermatitis or non-lesional skin sections of patients with eczema. These experiments would give us an insight about the role of Shh upregulation in humans and if it follows the same pattern as in mice (upregulation in chronic stages of inflammation) or if its expression in humans is elevated earlier (in the acute phase) or even in non-lesional skin, indicating that Shh could be used as a prognosis marker for the disease.

Chapter 5

Chapter 5: The impact of Shh upregulation and inhibition on dermatitis-like skin inflammation

5.1 Introduction

Gli3 is one of the three Hh-signalling responsive transcription factors found at the end of the signalling pathway. It can be processed to act as both an activator or a repressor of transcription in presence or absence of Hh pathway activation respectively (Sasaki et al., 1999). However *in vivo* it acts mostly as a transcriptional repressor and it functions to limit Hh signalling pathway in many tissues. Furthermore, $Shh^{-/-}$ and $Gli3^{-/-}$ have opposite phenotypes in many tissues. In this chapter I will first review the role of Gli3 in tissue patterning and in the development of immune cells as well as the function of Smo-inhibitors *in vivo* before presenting the experimental data.

5.1.1 *The transcription factor Gli3 and its role in regulation of tissue patterning*

Gli3 is largely known to act as a repressor of the Hh signalling pathway. However, it has a bifunctional role as it can either act as an activator (Gli3A) in the presence of Hh signalling or as a repressor (Gli3R) in the absence of the Hh pathway. The outcome of its function depends on the balance between the two forms (Jacob and Briscoe, 2003, Wang et al., 2000).

As described in the previous chapter, Hh signalling plays a fundamental role in embryonic patterning and Shh is particularly important for the development of notochord and floor plate. A similarly important role has been established for the Gli3 transcription factor in the formation of limb.

Limb formation requires processing of full length Gli zinc finger proteins, a mechanism which depends on Shh. Gli3 contributes more to limb formation

compared to Gli1 and Gli2 and when it is inactivated, Shh is expressed in the anterior region triggering limb formation (Mo et al., 1997, Park et al., 2000). However posteriorly, Gli3 is processed to a transcriptional repressor and restricts Shh expression via limiting dHAND expression, suppressing in this way the formation of posterior digits (te Welscher et al., 2002, Wang et al., 2000).

Recent findings have tried to uncover the gene regulatory pathway behind Shh and Gli3 interaction. Analysis of limb extracts by ChiP studies using a transgenic mouse model expressing a tagged Gli3 repressor form, revealed a series of direct target genes of Gli3 repression involved in Shh-mediated limb patterning (Vokes et al., 2008). RNA-sequencing analyses also showed that the expression of Gli target genes found at the posterior developing limb depended on Gli3 repressor rather than Gli3 activator activity (Lewandowski et al., 2015).

Disruption of the Gli3 gene leads to an anterior Shh gradient in mouse limbs and the formation of polydactylous phenotype (Hui and Joyner, 1993, Masuya et al., 1995, Schimmang et al., 1992). In addition, human mutations in Gli3 locus cause syndromes characterised by polydactyly and syndactyly of hands and feet such as Pallister-Hall and Greig Cephalopolysyndactyly Syndromes (Kang et al., 1997, Wild et al., 1997).

5.1.2 *The role of Gli3 in T and B cell development*

Gli3 is also involved in T cell differentiation in the thymus. It is expressed in adult and foetal thymic epithelial cells (Outram et al., 2000, Hager-Theodorides et al., 2005, Hager-Theodorides et al., 2009, Saldana et al., 2016) and it is differentially expressed during T cell development, with

highest expression observed in foetal DN4 populations, but has not been detected in adult thymocytes (Hager-Theodorides et al., 2005). Gli3 is involved in differentiation of DN2 thymocyte populations from DN1 stage (Hager-Theodorides et al., 2005). Also, FTOC studies of CD3-treated $Rag^{-/-}Gli3^{-/-}$ thymus showed that T cell development was partially arrested at the DN to DP stage after pre-TCR signalling, suggesting that Gli3 activity is essential for DN to DP T cell differentiation (Hager-Theodorides et al., 2005).

Furthermore, Gli3 plays an important role in negative selection via Nos2 upregulation, which is induced during negative selection and favours apoptosis (Hager-Theodorides et al., 2009). FTOC cultures showed that $Gli3^{-/-}$ DP thymocytes underwent decreased apoptosis, indicating that negative selection is restrained in $Gli3^{-/-}$ thymus. Finally, it is required for thymocyte positive selection, as Gli3 in foetal thymic epithelial cells promotes DP to SP4 differentiation (Solanki et al., 2018).

Apart from its role in T cell development, Gli3 is shown to be involved in B cell development in foetal liver, where it promotes B cell development via upregulation of Ebf1 and Pax5 master regulators of B cell development (Solanki et al., 2017). Overall Gli3 and Shh have opposing functions in T- and B-cell differentiation as Gli3 represses Shh signalling (Hager-Theodorides et al., 2005, Shah et al., 2004, Solanki et al., 2017, Solanki et al., 2018).

5.1.3 *Smoothened antagonists target Hh signalling in tumours*

As described in Chapter 3, aberrant Hh signalling is linked to solid and hematologic malignancies. When Smo receives Hh signals, it leads to

activation and nuclear translocation of Gli transcription factors and this signalling cascade is widely targeted in cancer therapy (Amakye et al., 2013, Takebe et al., 2015).

The first Smoothened antagonist to be discovered was cyclopamine which directly interacted with Smo (Chen et al., 2002a, Taipale et al., 2000) and helped scientists understand how Hh pathway targeting would offer therapeutic advantages, but its limited potency and oral solubility made it unsuitable for clinical trials (Ng and Curran, 2011). Therefore, new chemical compounds were discovered, with improved properties, which allowed for better *in vivo* metabolic stability, oral solubility, specific toxicity and increased potency, the majority of which targeted Smo (Chen et al., 2002b, Frank-Kamenetsky et al., 2002, Peukert and Miller-Moslin, 2010).

One of the most developed Smo-antagonists which targets ligand-dependent tumours is PF-04449913 compound from Pfizer. PF-04449913 in combination with other anticancer compounds reduced dormant leukaemia stem cells in *in vitro* co-cultures by repressing Gli2 transcription factor (Sadarangani et al., 2015). This selective Smo-inhibitor has also been tested i.v. in rat models, at an optimum dose of 2mg/kg and its half-life was 30 hours (Munchhof et al., 2012).

In clinical trials it is orally distributed to patients on a daily basis (Wagner et al., 2015) and Phase I clinical trials in patients with hematologic cancers have shown early signs of tumour remission (Jamieson et al., 2011). The compound was used in Phase I clinical trials in patients with skin cancers such as BCC, melanoma, small-cell or non-small-cell carcinoma and pancreatic carcinoma, where it has achieved a more than 80% Gli1

suppression in skin of patients (Wagner et al., 2015). Finally, in another Phase I clinical trial, PF-04449913 also inhibited Gli1 expression in skin of Japanese patients with blood cancers (Minami et al., 2017).

In total, PF-04449913 Smo-Inhibitor is largely used in clinical trials and it inhibits the Hh signalling pathway downstream of Smo.

5.2 Aim

In the previous chapter I showed that Shh is upregulated in dermatitis-like skin inflammation and that its expression in skin coincides with the chronic phase of inflammation. However, it is not known if its role is protective or if it contributes to the development of the disease. Gli3 mutation is shown to upregulate Shh expression in various tissues as mentioned previously, yet there is no reported role of Gli3 loss in the context of inflammation. Furthermore, the PF-0444913 compound has been widely used in cancer research and shown to inhibit Hh pathway components. Therefore, it can be used to specifically inhibit Smo and its downstream Hh signalling components in Oxa-induced skin inflammation. Here I present results coming from Oxa-treated $Gli3^{+/-}$ and WT littermates as well as WT mice injected with the PF-0444913 selective Smoothened antagonist.

I will examine the effect of **1)** Shh upregulation via Gli3 mutation and **2)** Shh inhibition via Smo pharmacological blockade on mice with dermatitis-like skin inflammation. I will carry out skin histology and phenotypic analysis of skin and peripheral immune cell population profile in both mouse models. I will assess if these changes are Hh-dependent and more specifically if Hh pathway acts via Smoothened. Finally, I aim to evaluate whether Hh

pathway alteration during skin inflammation has a direct or indirect effect on T cells by performing *in vitro* experiments.

5.3 Results

5.3.1 *Shh upregulation through Gli3-mutation protects skin from dermatitis-like inflammation*

Given the fact that Gli3 deletion is embryonic lethal (Hui and Joyner, 1993, Schimmang et al., 1992), I used $Gli3^{+/-}$ and WT counterparts. First, I applied the Oxazolone protocol to these mice and at the end of the experiment I performed H&E histological examination of treated and control ears. Baseline controls of both WT and $Gli3^{+/-}$ mice did not show any signs of inflammation and were characterised by anucleated epidermis and normal cell infiltration in dermis (Figure **5-1 A**). As expected, Oxa administration caused dermal and epidermal thickening with aberrant dermal cell infiltration in both experimental groups. However, Oxa-treated $Gli3^{+/-}$ mice had decreased thickening of the stratum corneum (hyperkeratosis), indicated by green arrows and decreased retention of nuclei in epidermis (parakeratosis), indicated by white arrows, compared to Oxa-treated WT mice which developed severe hyperkeratosis and epidermal hyperplasia followed by the presence of nucleated cells.

Furthermore, quantification of skin thickness in treated mice showed significantly attenuated epidermal (89.97 ± 7.695 vs 145.4 ± 25.13 $p<0.05$) and dermal thickness (339.8 ± 30.21 vs 535.6 ± 51.64 $p<0.01$) in treated $Gli3^{+/-}$ mice compared to WT littermates (Figure **5-1 B**).

Since Gli3 mutation showed reduced skin pathology, I then wanted to examine if Shh is upregulated in untreated and treated skin of Gli3-mutant mice compared to WT. Q-PCR from the whole untreated skin showed significantly elevated *Shh* transcript levels in $Gli3^{+/-}$ mice compared to WT, confirming the hypothesis that Gli3 mutation induces *Shh* expression in skin (Figure **5-2 A; Left plot**). In agreement with this, *Shh* levels showed a threefold increase in Oxa-treated skin of $Gli3^{+/-}$ compared to the WT littermates (Figure **5-2 A; Right plot**), indicating that upregulation of *Shh* expression in skin might be linked to a protective role against dermatitis-like inflammation.

In order to further test this hypothesis, I tested the blood serum IgE levels from Oxa-treated mice. $Gli3^{+/-}$ mice showed significantly diminished IgE levels compared to WT mice (Figure **5-2 B**), supporting the hypothesis that $Gli3^{+/-}$ mice exhibit attenuated response upon induction of skin inflammation. Molecular analysis of Th2 cytokines from whole skin homogenates showed significantly decreased *Il4* (Figure **5-3 A**) and *Il13* (Figure **5-3 B**) expression in $Gli3^{+/-}$ mice compared to their WT littermates. However, *Ifng* expression analysis revealed no differences between the two experimental groups (Figure **5-3 C**), suggesting that Shh inhibited the acute phase of the disease.

5.3.2 *Gli3 mutation leads to reduced pro-inflammatory cytokine production by skin T cell populations*

Next in order to examine the impact of Gli3-mutation on T cells, I analysed skin T cell infiltration. FACS analysis showed similar skin $CD4^+$ T cell infiltration from Oxa-treated $Gli3^{+/-}$ and WT mice, in terms of percentages

and numbers (Figure **5-4 A**) and the same pattern was revealed for $CD8^+$ T cells (Figure **5-4 B**). By comparing the two skin cell populations, I observed higher numbers of $CD8^+$ T cells compared to $CD4^+$ T cells. This observation could suggest that skin inflammation is at an early stage as it has previously been reported that $CD8^+$ T cell skin infiltration is important for initiation of atopic dermatitis (Hennino et al., 2007, Hijnen et al., 2013). Then I analysed cytokine expression in skin T cells. Although the two groups showed almost identical numbers of T cell subsets, skin T cells of Gli3 mutant mice were characterised by lower production of IL-4 (1.41±0.2713 vs 2.088±0.2135, p=0.054) (Figure **5-5 A**) and significantly reduced IL-13 production (2.784±0.5809 vs 3.71±0.4483, $p<0.05$) (Figure **5-5 B**). On the other hand, the proportion of skin T cells producing IFN-γ (Figure **5-5 C**) and IL-17 (Figure **5-5 D**) was not significantly different between $Gli3^{+/-}$ and WT mice.

Overall the experiments showed that *Shh* upregulation via Gli3 mutation in skin rescues skin inflammation by affecting the ability of skin T cells to produce Th2 cytokines. Therefore, I decided to examine if Hh inhibition, by pharmacological Smo inhibition would have the opposite effect in my experimental model.

5.3.3 *Smoothened inhibitor administration alone is not sufficient to provoke signs of dermatitis-like skin inflammation in skin*

In order to test this hypothesis, I first injected WT mice with PF-0444913 Smoothened inhibitor (Smo-inh) without inducing dermatitis. Smo-inh was daily administered i.p. to the mice for 14 consecutive days, at a

concentration of 40µg/ml per mouse. DMSO-only injected mice were used as a control, and the same amount of vehicle was injected.

At the end of the experiment, I performed H&E staining. Histological analysis of the samples showed a normal skin with no difference in dermal or epidermal thickness and skin cell infiltration in mice injected with Smo-inh compared to the DMSO-only control (Figure **5-6 A**).

I then carried out expression analysis of Hh pathway components and target genes, which showed that *Shh*, *Patch1* and *Gli1* expression was not altered in either group (Figure **5-6 B**). Finally, in agreement with my previous findings, *Filaggrin* gene expression was similar in both groups (Figure **5-6 C**).

T cell presence was also examined in WT untreated mice injected with DMSO or Smo-inh. As shown in representative FACS plots in Figure **5-7 A**, the percentages of $CD4^+$ and $CD8^+$ T cells did not change upon Smo-inh injection compared to mice administered only with the vehicle. Furthermore, I observed that untreated skin was characterised by a high proportion of $CD4^+$ T cells while $CD8^+$ T cells accounted for a small percentage of total T cell populations. Numbers of both $CD4^+$ and $CD8^+$ T cells were also indistinguishable between DMSO-only and Smo-inh injected untreated mice (Figure **5-7 B**). Finally, and in line with higher $CD4^+$ T cell percentages in both experimental groups, $CD4^+$ T cells were 50 to 60 times more abundant than $CD8^+$ T cells.

Taken together these results showed that injection of mice with Smoothened inhibitor alone was not sufficient to induce signs of skin

inflammation. Therefore, I decided to examine its role upon induction of the Oxa-inflammation protocol described in Chapter **3**.

5.3.4 *Selective Smoothened inhibition upon Oxa-induced skin inflammation causes exacerbation of dermatitis-like inflammation and downregulation of skin Shh expression*

In order to evaluate the role of the Smoothened inhibitor in the context of skin inflammation, I followed the protocol shown in Figure **5-8 A**. Briefly, I sensitised the mouse abdomen with 3% of Oxa and after five days I started applying 0.6% Oxa on the ears challenging them every second day in a total of five doses. A few hours before Oxazolone sensitization, I injected the mice either with Smo-inh or with DMSO. Smo-inh or DMSO were injected i.p. to the mice daily throughout the dermatitis protocol and the injections were carried out 4-5 hours prior to Oxa epicutaneous application. First, I examined the kinetics of ear thickness. I picked specific time points which correspond to the initiation of the ear challenge (Day 5), the time point when I have observed signs of skin inflammation in the ears (Day 9) and the end of the experiment (Day 14). Measurements revealed that although skin thickness was similar before Oxa-application, after two Oxa doses there was a significant increase in ear thickness of mice injected with Smo-inh compared to DMSO injected mice. Smo-inh-injected mice maintained the significantly elevated thickness compared to the vehicle-injected mice until the end of the experiment (Figure **5-8 B**). This observation was confirmed by histological examination of skin sections from DMSO-only (**left image**) and Smo-inh-injected (**right image**) treated ears (Figure **5-8 C**). As shown in the representative images, skin of Smo-

inh-injected mice was characterised by intense dermal cell infiltration and more severe hyperkeratosis and parakeratosis signs compared to the treated mice injected only with vehicle control.

To further examine the impact of Smoothened inhibition on skin inflammation, I performed q-PCR of the whole skin homogenates in order to examine cytokine gene expression. I observed that *Il13* and *Il4* expression were doubled in Smo-inh injected mice, while *Ifng* expression was not altered (Figure **5-9 A**). After checking serum IgE levels, I observed significantly augmented IgE secretion in Smo-inh compared to DMSO-injected treated mice (5.019 ±1.173 vs 2.005 ±0.443, $p<0.05$). Since IgE is predominantly produced by B cells, I also examined this population in the draining lymph nodes (dLNs) and as expected B cell proportions were significantly higher in Smo-inh treated mice (Figure **5-9 B**). Finally, *Shh* levels in Oxa-treated mice were twofold decreased upon Smo-inh injection compared to DMSO administration (Figure **5-9 C**). These results could be indicative of a heightened Th2 response in Oxa-treated mice after Smo-inh injection.

5.3.5 *Increased skin cellular infiltration after Smo-inh administration on treated mice*

I then examined the impact of Smo-inhibition on skin cell infiltration of innate immune cells, which are implicated in AD (Leung et al., 2004). Representative FACS plots of innate immune cells including neutrophils (Nφ), eosinophils (Eos) and macrophages (Mφ) are shown in Figure **5-10 A**. Skin of Smo-inh injected treated mice was characterised by significantly increased leukocyte infiltration. Further analysis of the leukocytes revealed

that there were significantly higher proportions of eosinophils and macrophages in Smo-inh injected compared to vehicle-injected treated mice. Surprisingly neutrophils in Smo-inh injected mice were significantly lower (Figure **5-10 B**). Examination of T cell populations showed that $CD4^+$ and $CD8^+$ skin T cells were similar in terms of proportions in both groups. In addition, numbers of $CD4^+$ and $CD8^+$ T cells were decreased in Smo-inh injected compared to DMSO treated mice but this difference was not significant (Figure **5-10 C**). In agreement with the untreated skin T cell populations, $CD4^+$ T cells were more abundant in treated skin compared to $CD8^+$ T cells.

5.3.6 *Skin T cells of Oxa-treated Smo-inh mice show a more activated phenotype with signs of chronic dermatitis-like inflammation*

Although the differences in percentages and numbers of skin T cells were not significant, I decided to further investigate their phenotype. I examined T cell proliferation by measuring Ki67 expression in skin T cells. $CD4^+$ T cells from Oxa-treated Smo-inh injected mice were characterised by higher Ki67 expression (**blue line**) compared to Oxa-treated DMSO injected mice (**red line**) and Ki67 expression was significantly higher in terms of percentages (80.04 ± 4.151 vs 62.52 ± 4.586, $p<0.05$) (Figure **5-11 A**). $CD8^+$ T cells were also examined for Ki67 expression. There was a high percentage of proliferating $CD8^+$ T cells (approximately 80%) however no difference was detected between Smo-inh-injected and DMSO-injected treated mice (Figure **5-11 B**).

I decided to further examine activation of peripheral $CD4^+$ and $CD8^+$ T cells. Therefore, T cell spleenocytes were stained for early (CD69) and late

(CD25) activation markers. The $CD4^+$ T population from Oxa-treated Smo-inh-injected mice contained higher percentages of $CD69^+$ cells compared to their control Oxa-treated littermates, but there was no difference in $CD8^+$ T cells between the groups (Figure **5-12 A**). Spleen $CD4^+$ T cells from Smo-inh-injected mice were also characterised by an elevated CD25 expression although the difference was not significant. On the other hand, $CD8^+$ T cells upon Smo-inh administration showed a significant increase in CD25 expression compared to DMSO-injected littermates (Figure **5-12 B**).

Having shown that both T cell populations are displaying a more activated phenotype upon Smo-inh injection I decided to examine skin T cells for key transcription factors. Tbet transcription factor, which controls the expression of its hallmark cytokine IFN-γ was significantly elevated in both $CD4^+$ T cells and $CD8^+$ T cells in treated mice with Smo-inh administration compared to DMSO-only injection (Figures **5-13 A** and **5-13 B**). The difference in expression was particularly striking in $CD4^+$ T cells (top histogram, blue line: Smo-inh injected mice, red line: DMSO-injected mice). In agreement with elevated Tbet expression, treated Smo-inh-injected skin $CD4^+$ T cells had a twofold significant increase in IFN-γ expression compared to DMSO-only-injected mice (Figure **5-14 A**). Skin $CD8^+$ T cells of Smo-inh-injected treated mice, were also characterised by a significantly higher IFN-γ expression compared to their DMSO-injected treated counterparts (Figure **5-14 B**).

Since chronic dermatitis involves a mixed lymphocyte response (Gittler et al., 2012), I tested whether Smo-inh administration affected Th2 and Th17 features of inflammation. Therefore, I performed intracellular staining for IL-

13 on skin leukocytes and I showed that treated skin expressed higher levels of the cytokine upon Smo-inh injection (Figure **5-15 A**). For this reason, I decided to check Gata3 expression, a master regulator of Th2 cytokines, in skin $CD4^+$ T cells. Indeed, Gata3 expression was markedly elevated after Smo-inh injection (**blue line**) on treated mice compared to DMSO-injected controls (**red line**) and the difference was significant (65.1 ± 2.824 vs 44.29 ± 6.424, $p<0.05$) (Figure **5-15 B**). Surprisingly skin $CD4^+$ T cells from Smo-inh-injected group did not show elevated expression of either IL-13 or IL-4 despite the increased Gata3 expression (Figure **5-15 C**), suggesting that elevated IL-13 expression as indicated in Figure **5-15 A** might be due to other immune cells such as basophils or B cells.

Finally, I examined skin T cells for IL-17 production. Skin $CD4^+$ T cells of treated Smo-inh mice showed a twofold upregulation of IL-17 compared to DMSO-only treated mice (15.06 ± 2.256 vs 8.764 ± 1.383, $p<0.05$) (Figure **5-16 A**). The same trend was observed in $CD8^+$ T cells which had increased IL-17 expression compared to the vehicle-injected group, although this difference was not significant (Figure **5-16 B**).

5.3.7 *Smoothened inhibitor does not act directly on $CD4^+$ T cells*

So far, I have shown that Smo-inh administration in Oxa-treated skin resulted in increased skin inflammatory T cell response followed by reduced *Shh* expression. The findings suggest that the Smo-inh could increase skin inflammation either by reducing Shh signalling to immune cells or by directly inhibiting Smo in T cells.

To test if Smo-inh has a direct impact on T cell differentiation, I FACS-sorted naïve $CD4^+$ T cells from spleens of WT mice and cultured them *in*

vitro under Th0, Th1, Th2 and Th17 skewing conditions in the presence of Smo-inh or DMSO only (vehicle control). After 10 days of polarization, I performed intracellular staining in order to evaluate key cytokine expression: IFN-γ for Th1, IL-4, IL-5 and IL-13 for Th2 and IL-17 for Th17 conditions. Under Th0 conditions, I detected higher expression of IFN-γ and IL-13 compared to the rest of the cytokines. IFN-γ and IL-13 were both upregulated when naïve cells were cultured under Smo-inh compared to DMSO only, however the differences were not significant. Regarding IL-4, IL-5 and IL-17 expression, their levels were relatively low and no difference was detected between Smo-inh and vehicle culture (Figure **5-17 A**).

Under Th1 conditions IFN-γ expression was markedly upregulated compared to the other cytokines, but its expression was equally high in both Smo-inh and DMSO cultured $CD4^+$ T cells (Figure **5-17 B**). Both Smo-inh and DMSO culture caused similarly elevated Th2 cytokines especially IL-13 and IL-5 under Th2 polarization (Figure **5-17 C**) as well as upregulation of IL-17 under Th17 conditions (Figure **5-17 D**). Thus, Smoothened inhibition failed to act directly *in vitro* on $CD4^+$ T cell cytokine profile.

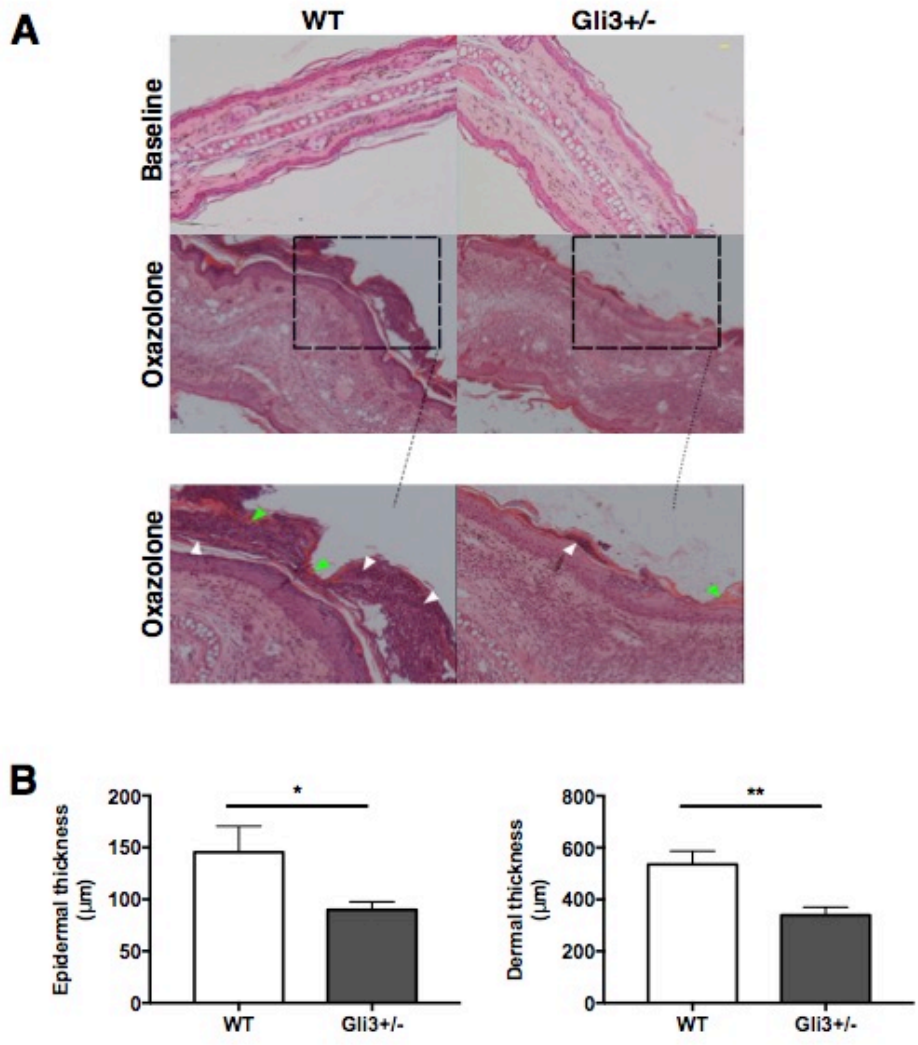


Figure 5-1: Gli3-mutation improves histopathology of Oxa-treated skin

(A) Representative H&E images of skin sections from untreated (Baseline) and Oxa-treated WT and Gli3$^{+/-}$ mice. Scale bar represents 100µm. Green arrows show hyperkeratosis while white arrows show parakeratosis.

(B) Bar charts show quantification of epidermal and dermal thickness of Oxa-treated WT treated (White bars) and Gli3$^{+/-}$ treated (Grey bars) mice.

Data were generated from two independent experiments with 4 mice per group. Plots show ± SEM. *p<0.05 and **p<0.01.

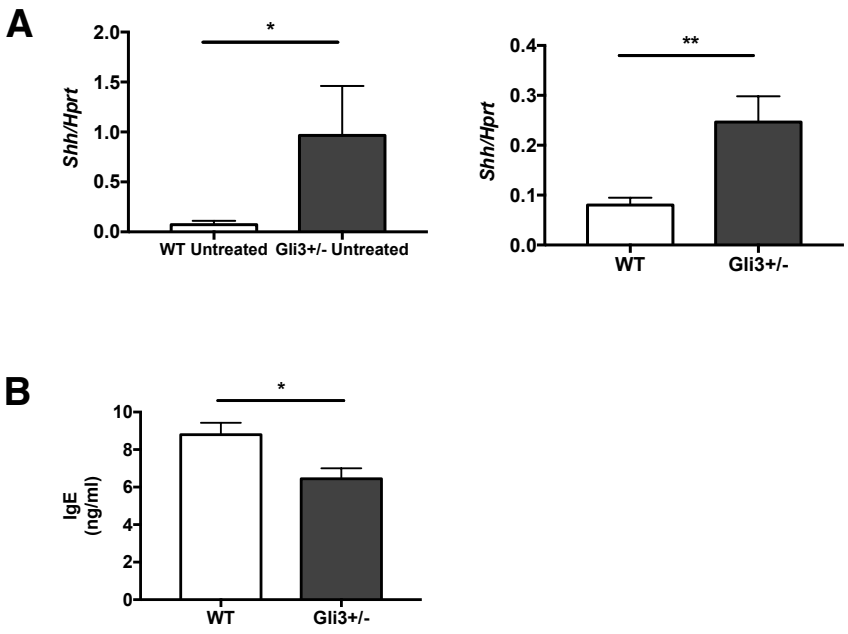


Figure 5-2: Gli3-mutation leads to increased *Shh* expression in skin and reduced IgE production

(A) mRNA expression of *Shh* as determined by quantitative PCR in untreated (Left bar chart) and Oxa-treated (Right bar chart) WT (White bars) and $Gli3^{+/-}$ (Grey bars) mice. Data were generated from two independent experiments with at least 4 mice per group.

(B) ELISA of blood serum to measure IgE levels of Oxa-treated WT and $Gli3^{+/-}$ mice. Data were generated from one experiment with seven mice per group.

Plots show mean ± SEM. *$p<0.05$ and **$p<0.01$.

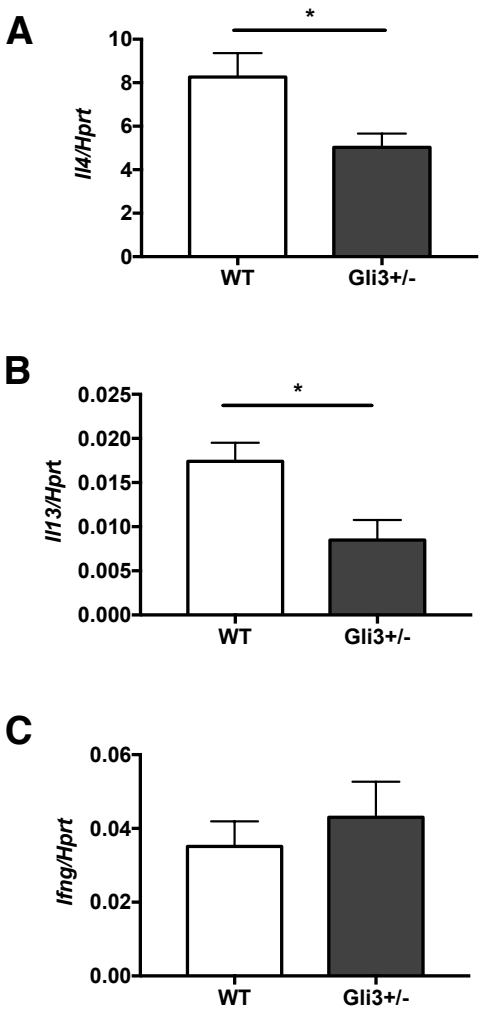


Figure 5-3: Decreased Th2 cytokine expression in skin of Gli3$^{+/-}$ treated mice

Bar charts show mRNA expression determined by quantitative PCR of **(A)** *Il4*, **(B)** *Il13* and **(C)** *Ifng* from WT (White bars) and Gli3$^{+/-}$ (Grey bars) treated homogenised skin. Data were generated from two independent experiments with 4 mice per group. Plots show mean ± SEM. *p<0.05.

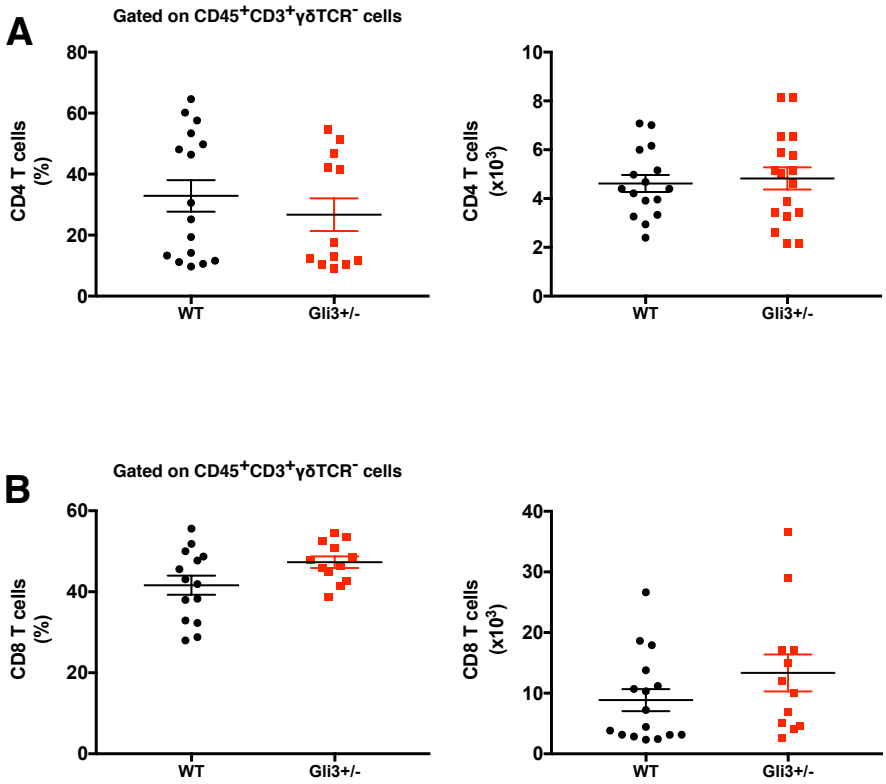
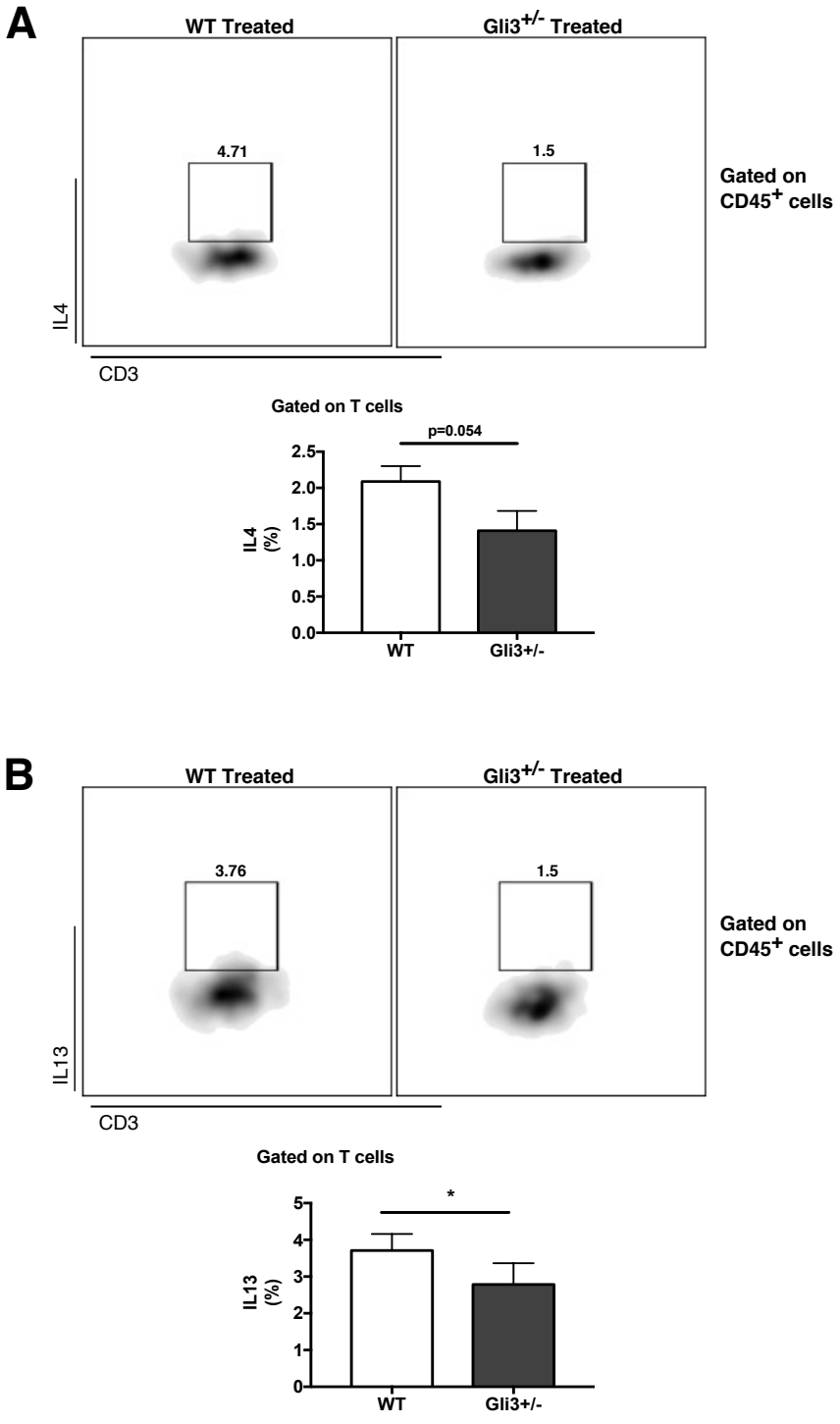


Figure 5-4: Gli3 heterozygosity does not affect T cell skin infiltration

Scatter plots represent percentage and numbers of skin **(A)** $CD4^+$ T cells and **(B)** $CD8^+$ T cells from Oxa-treated WT (black circles) and $Gli3^{+/-}$ (red squares) mice as detected by FACS analysis. Each symbol in the graph represents an individual mouse and the group mean is represented with a bar. Data were generated from two independent experiments with at least 5 mice per group.


A
WT Treated
Gli3+/- Treated
4.71
1.5
Gated on CD45+ cells
IL4
CD3
Gated on T cells
p=0.054
IL4 (%)
2.5
2.0
1.5
1.0
0.5
0.0
WT
Gli3+/-
B
WT Treated
Gli3+/- Treated
3.76
1.5
Gated on CD45+ cells
IL13
CD3
Gated on T cells
*
IL13 (%)
5
4
3
2
1
0
WT
Gli3+/-

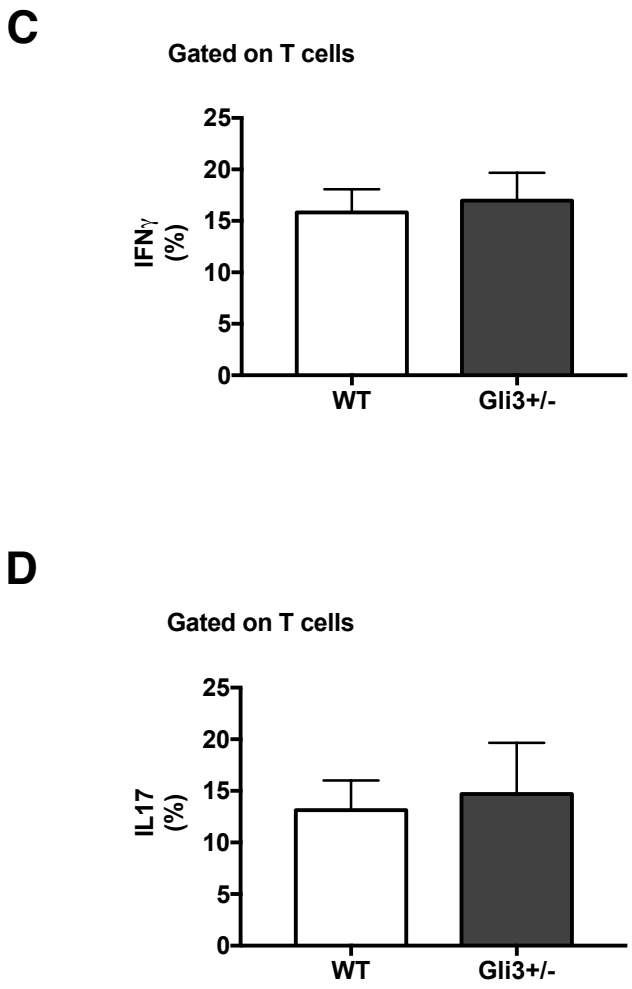


Figure 5-5: Gli3-mutation results in reduced Th2 cytokine expression in skin T cells upon Oxa-treatment

Representative density plots from Oxa-treated WT (Left) and Gli3$^{+/-}$ (Right) mice and bar charts showing **(A)** IL-4 and **(B)** IL-13 expression in skin T cells obtained by intracellular cytokine staining and measured by FACS.

Bar charts showing **(C)** IFN-γ and **(D)** IL-17 expression in skin T cells from Oxa-treated WT (White bars) and Gli3$^{+/-}$ (Grey bars) mice. Data were generated from two independent experiments with at least 5 mice per group. Plots show mean ± SEM. *$p<0.05$

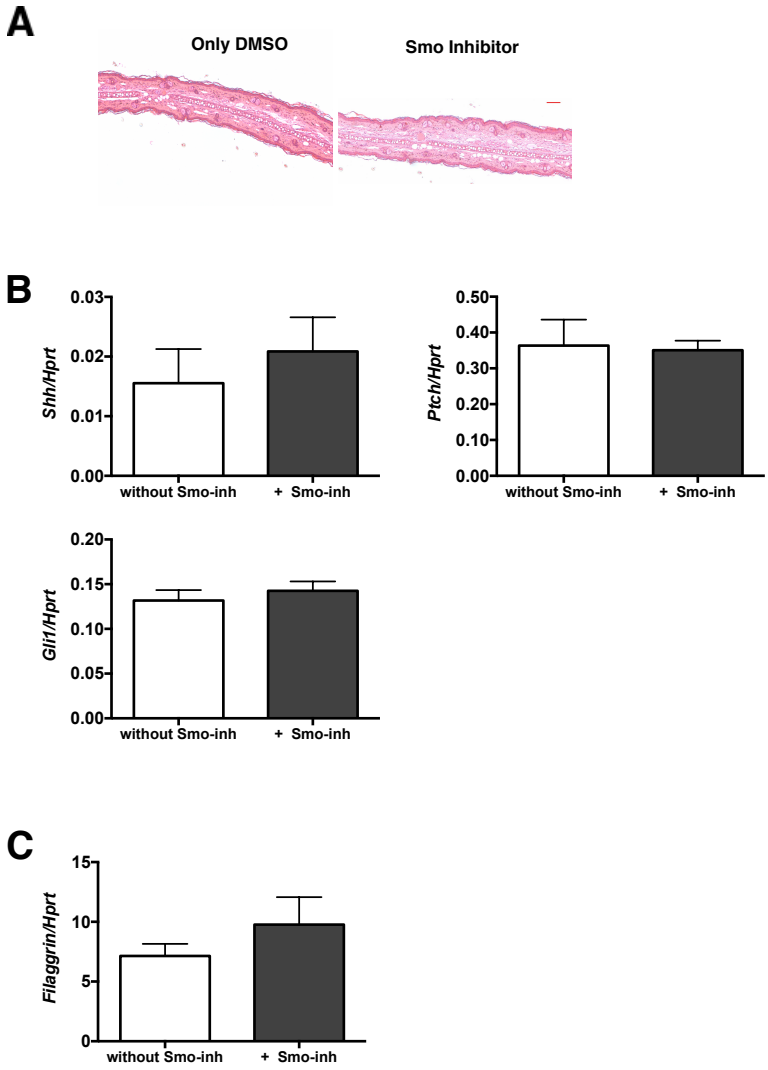


Figure 5-6: Smoothened inhibitor administration alone does not induce signs of spontaneous skin inflammation

(A) Representative images of H&E staining skin sections from DMSO-only injected (Left) and Smo-inh injected (Right) untreated mice. Scale bars represent 100µm.

mRNA expression of **(B)** *Shh*, *Ptch* and *Gli1* and **(C)** *Filaggrin* as determined by quantitative PCR from DMSO only (White bars) and Smo-inh- injected (Grey bars) untreated skin homogenates. Data were generated from one experiment with 7 mice per group. Plots show mean ± SEM.

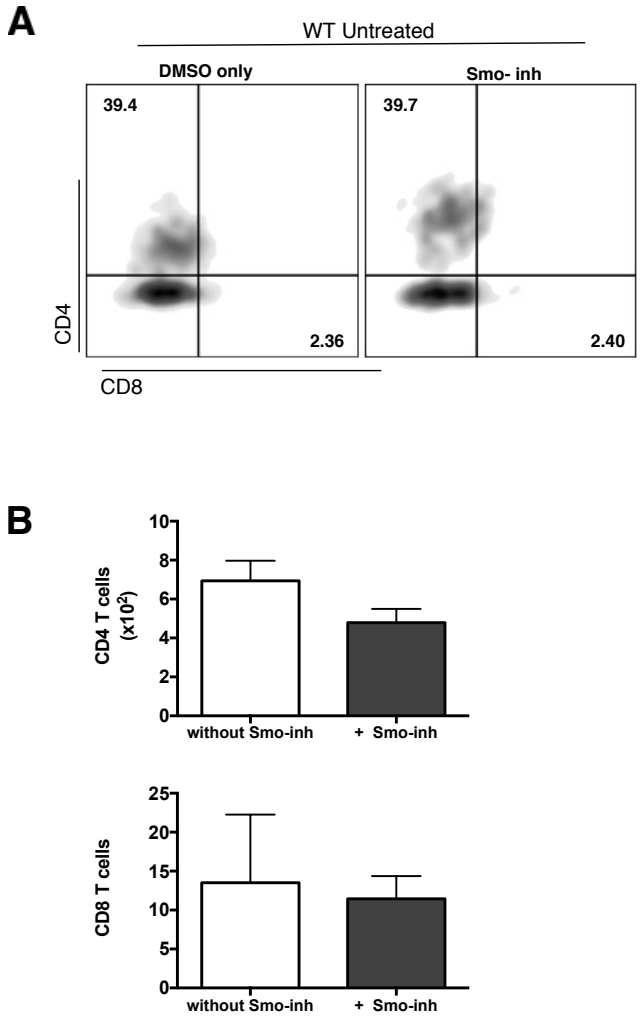


Figure 5-7: Smoothened inhibitor administration does not affect skin T cell infiltration in untreated WT skin

(B) Representative density plots of DMSO only (Left) and Smo-inh injected (Right) untreated WT skin showing percentages of $CD4^+$ and $CD8^+$ T cells.

(B) Bar charts showing numbers of skin $CD4^+$ and $CD8^+$ T cells from DMSO only (White bars) and Smo-inh injected (Grey bars) WT untreated mice.

Data were generated from one experiment with 7 mice per group. Plots show mean ± SEM.

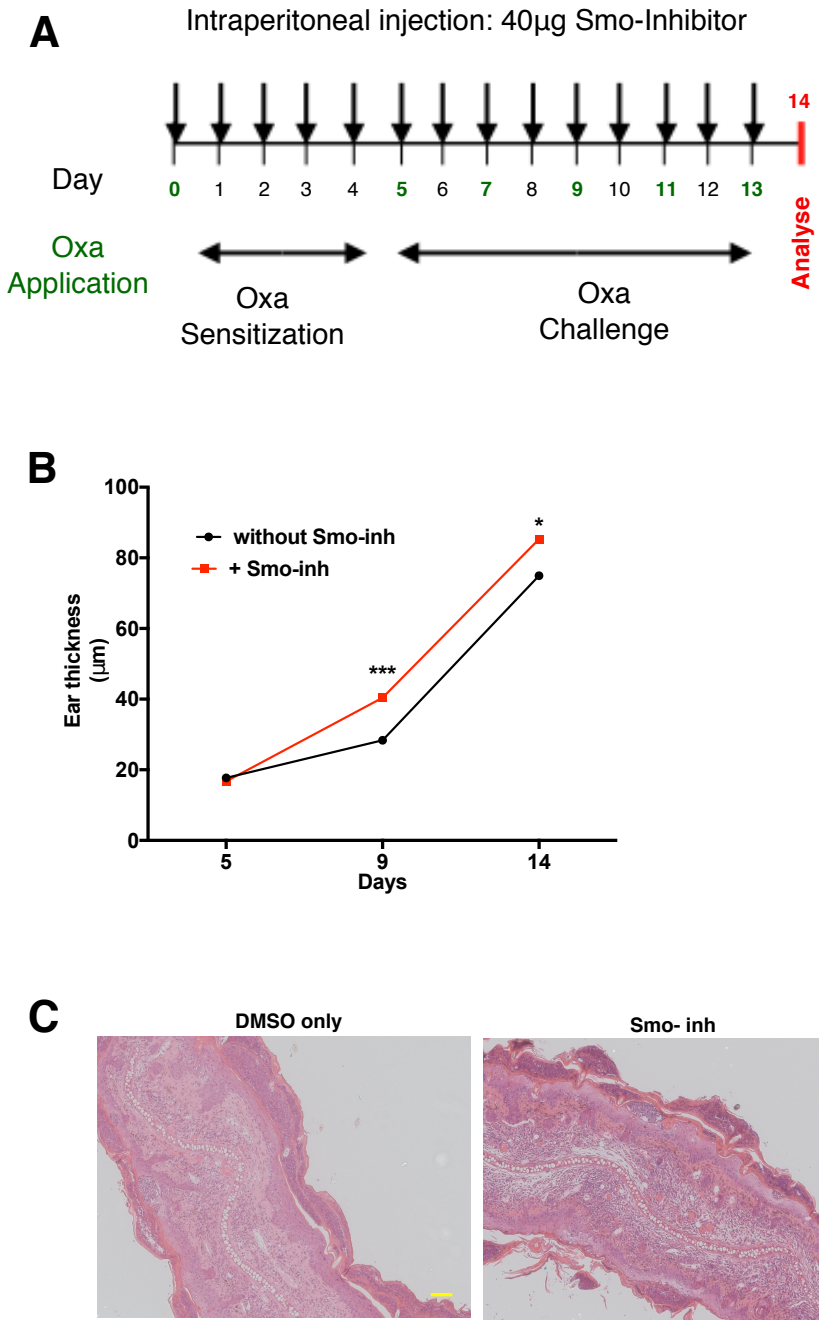


Figure 5-8: Smoothened inhibition results in increased skin inflammation in response to Oxazolone application

(A) Sensitization and challenge scheme of Oxazolone administration together with i.p. Smo-inh injection.

(B) Time course of ear thickness measurement from WT treated DMSO-only-injected (black line) and Smo-inh-injected (red line) mice.

(C) Representative images of H&E staining skin sections from DMSO (Left) and Smo-inh-injected (Right) WT treated mice. Scale bar represents 100μm.

Data were generated from one experiment with 7 mice per group. *p<0.05 and ***p<0.001.

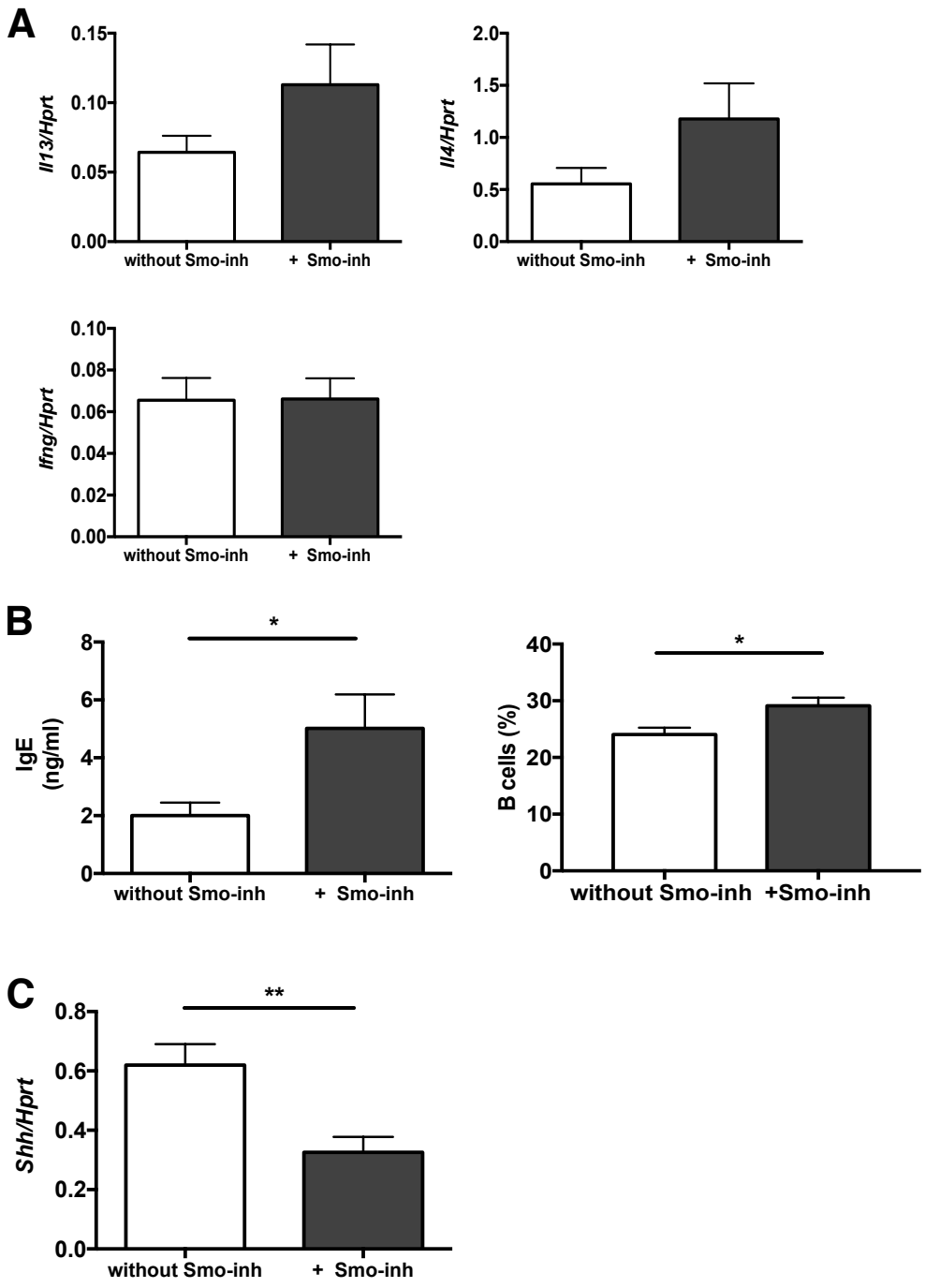
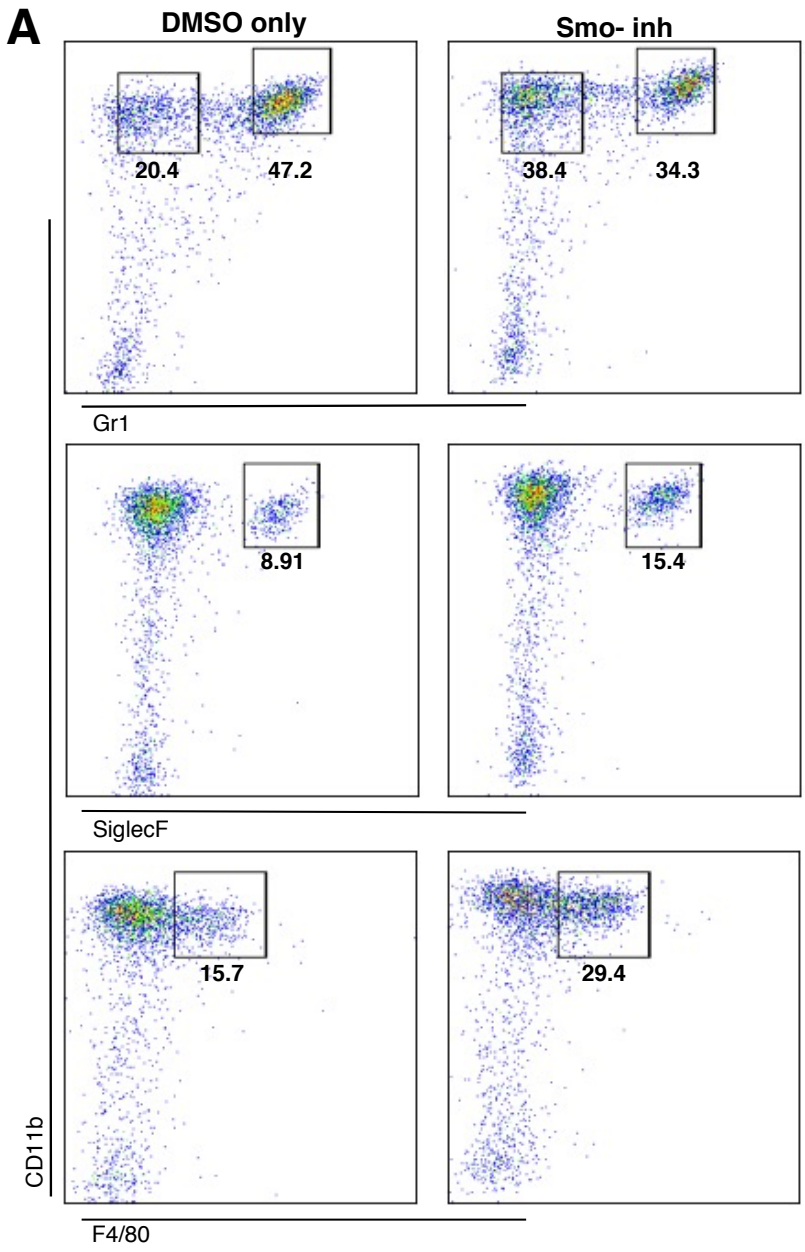


Figure 5-9: Smoothened inhibition promotes IgE expression and results in *Shh* downregulation

Bar charts show **(A)** *Il13*, *Il4*, *Ifng* and **(C)** *Shh* mRNA expression determined by quantitative PCR from DMSO-injected (White bars) and Smo-inh-injected (Grey bars) WT Oxa-treated mice.

(B) IgE blood serum secretion determined by ELISA and B cell percentage from dLNs determined by FACS analysis from DMSO-injected and Smo-inh injected WT Oxa-treated mice.

Data were generated from one experiment with 7 mice per group. Plots show mean ± SEM. *$p<0.05$ and **$p<0.01$.


A
DMSO only
Smo- inh
20.4
47.2
38.4
34.3
Gr1
8.91
15.4
SiglecF
15.7
29.4
CD11b
F4/80
B
100
80
60
40
20
0
%
**
**
*
**
without Smo-inh
+ Smo-inh
Leukocytes
Mφ
Eos
Nφ

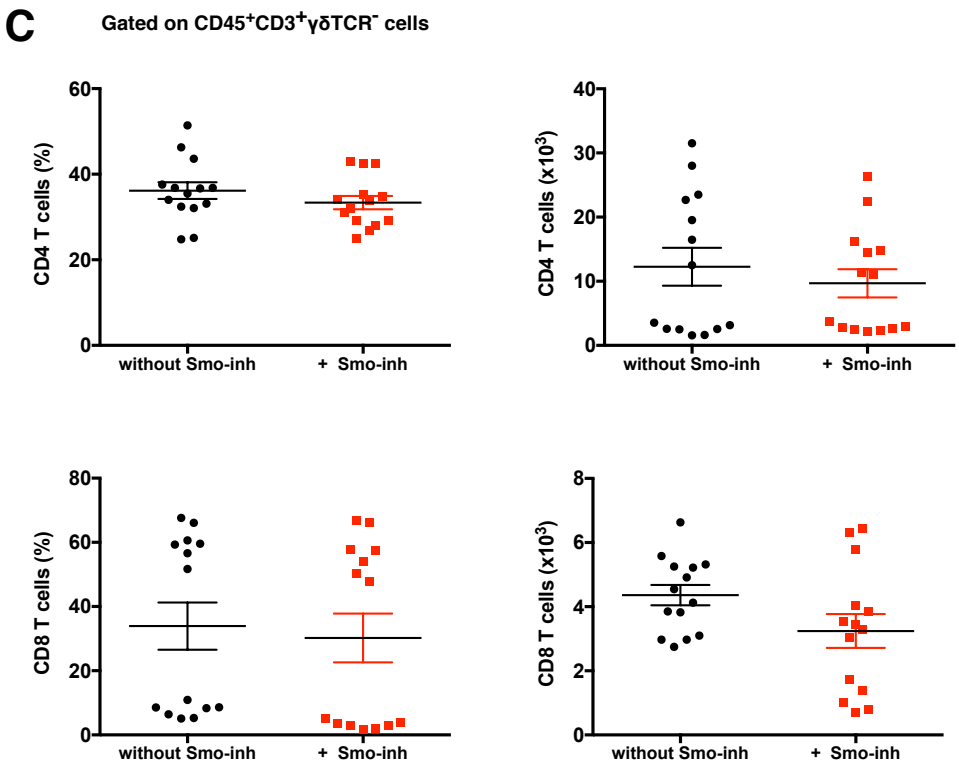


Figure 5-10: Pharmacological Smo inhibition upon Oxa-treatment alters innate immune cell but not T cell skin infiltration

(A) Representative FACS plots showing neutrophil ($CD11b^+Gr1^+$), eosinophil ($CD11b^+SiglecF^+$) and macrophage ($CD11b^+F4/80^+$) percentages of DMSO (Left) and Smo-inh-injected (Right) WT treated mice.

(B) Bar charts showing percentages of immune cell populations from DMSO-injected (White bars) and Smo-inh injected (Grey bars) WT treated mice.

Data were generated from one experiment with 7 mice per group. Plots show mean ± SEM. *$p<0.05$ and **$p<0.01$.

(C) Scatter plots show percentages and numbers of skin $CD4^+$ and $CD8^+$ T cells from DMSO-injected (black circles) and Smo-inh

injected (red squares) Oxa-treated mice. Each symbol in the graph represents an individual mouse and the group mean is represented with a bar. Data were generated from two independent experiments with at least 6 mice per group.

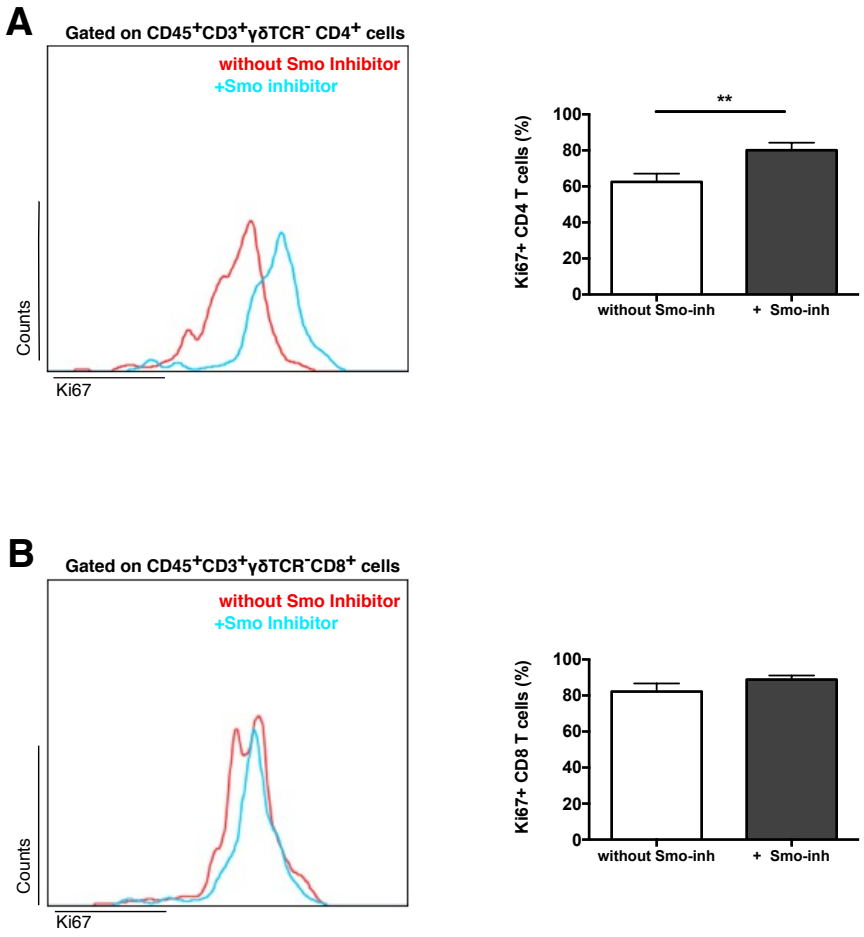


Figure 5-11: Smoothened inhibitor results in increased skin $CD4^+$ T cell proliferation upon Oxa-administration

Representative histograms of **(A)** $CD4^+$ and **(B)** $CD8^+$ Ki67 staining in skin T cells. Red line represents DMSO-injected and blue line shows Smo-inh-injected treated mice. Bar charts show percentages of $Ki67^+$ cells after DMSO injection (White bars) and Smo-inh injection (Grey bars). Data were generated from one experiment with 7 mice per group. Plots show mean ± SEM. **p<0.01.

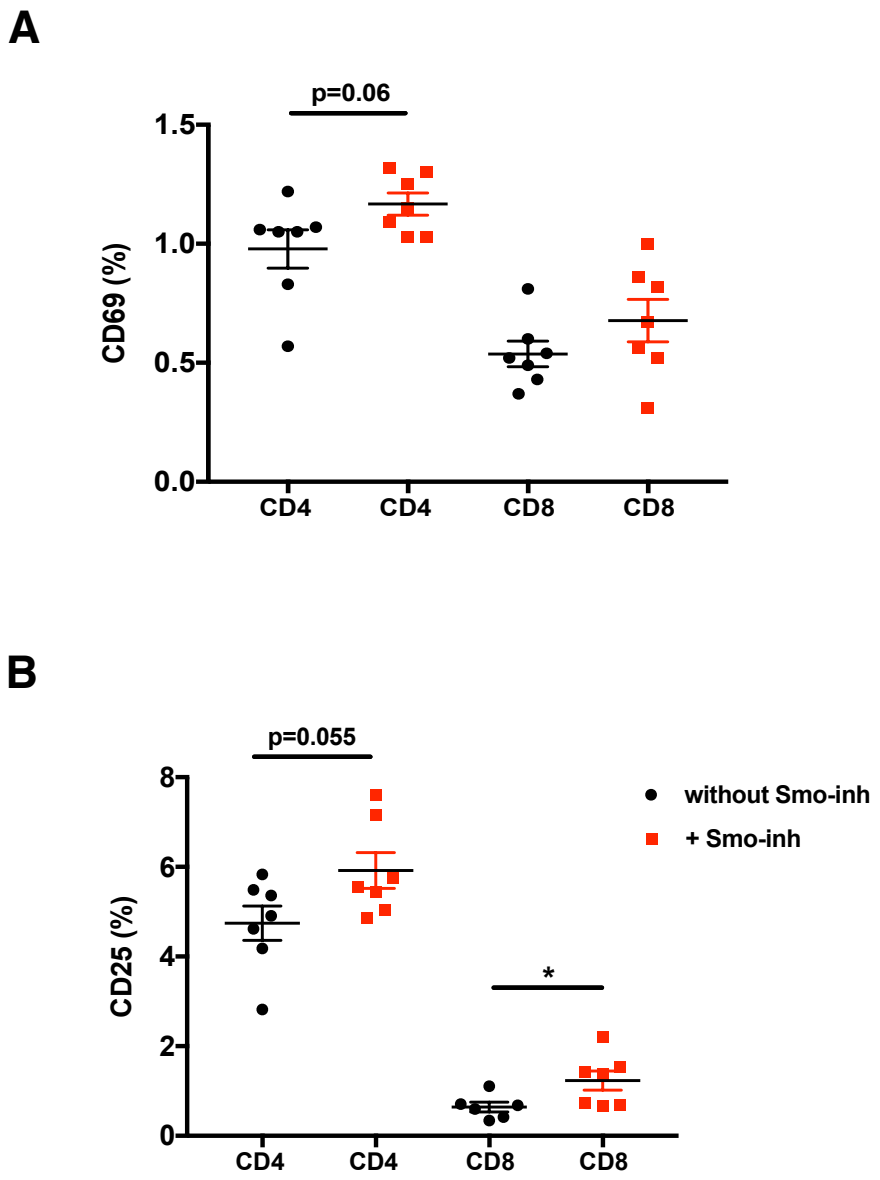


Figure 5-12: Smoothened inhibition affects early and late activation markers of peripheral T cells in Oxa-treated mice

Scatter plots show **(A)** CD69 (early) and **(B)** CD25 (late) markers on $CD4^+$ and $CD8^+$ T cell spleenocytes from DMSO-injected (black circles) and Smo-inh-injected (red squares) mice upon Oxa application. Each symbol in the graph represents an individual mouse and the group mean is represented with a bar. Data were generated from one experiment with 7 mice per group. *$p<0.05$.

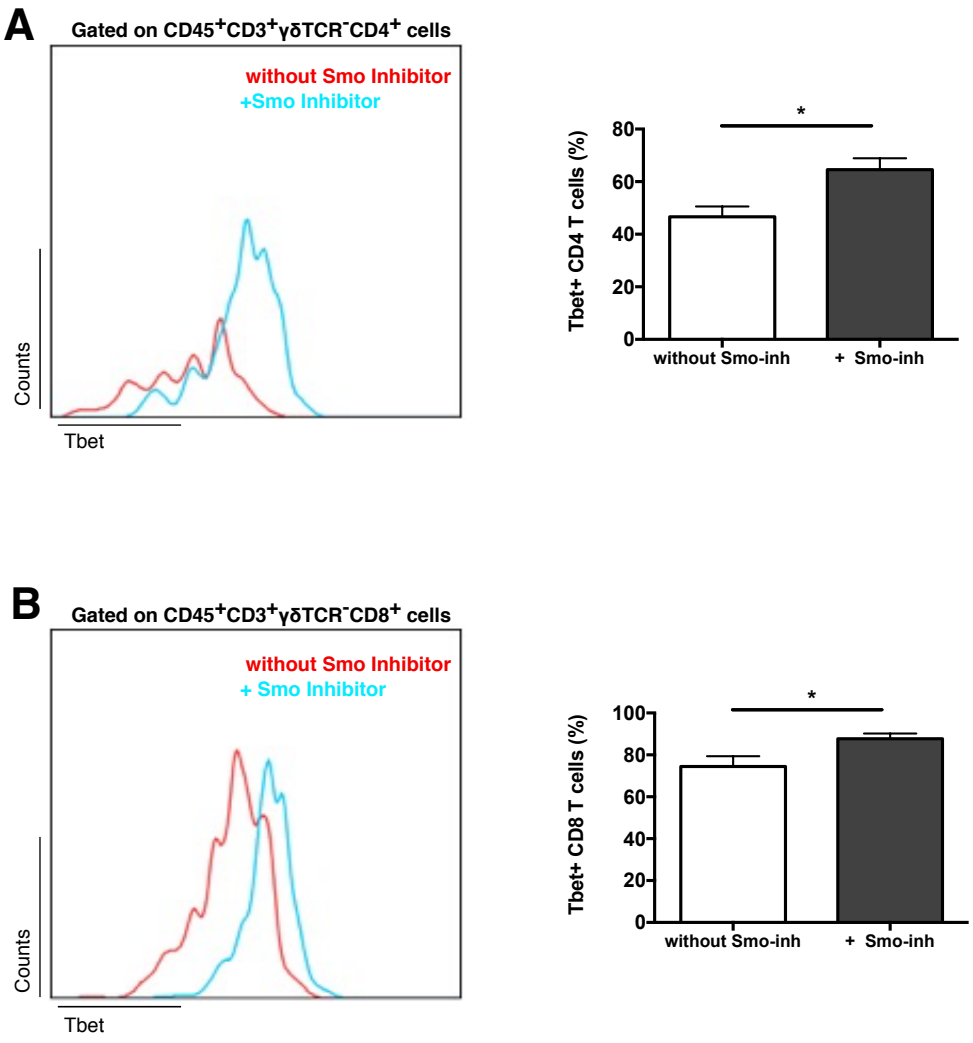


Figure 5-13: Smoothened inhibition upregulates the Tbet transcription factor in skin T cells of Oxa-treated mice

Representative histograms of Tbet expression (red line shows DMSO injected and blue line shows Smo-inh-injected Oxa-treated mice) and bar charts showing percentages of Tbet$^+$ cells in **(A)** CD4$^+$ and **(B)** CD8$^+$ skin T cell populations from DMSO injected (White bars) and Smo-inh-injected (Grey bars) Oxa-treated mice. Data were generated from one experiment with 7 mice per group. Plots represent mean ± SEM. *$p<0.05$.

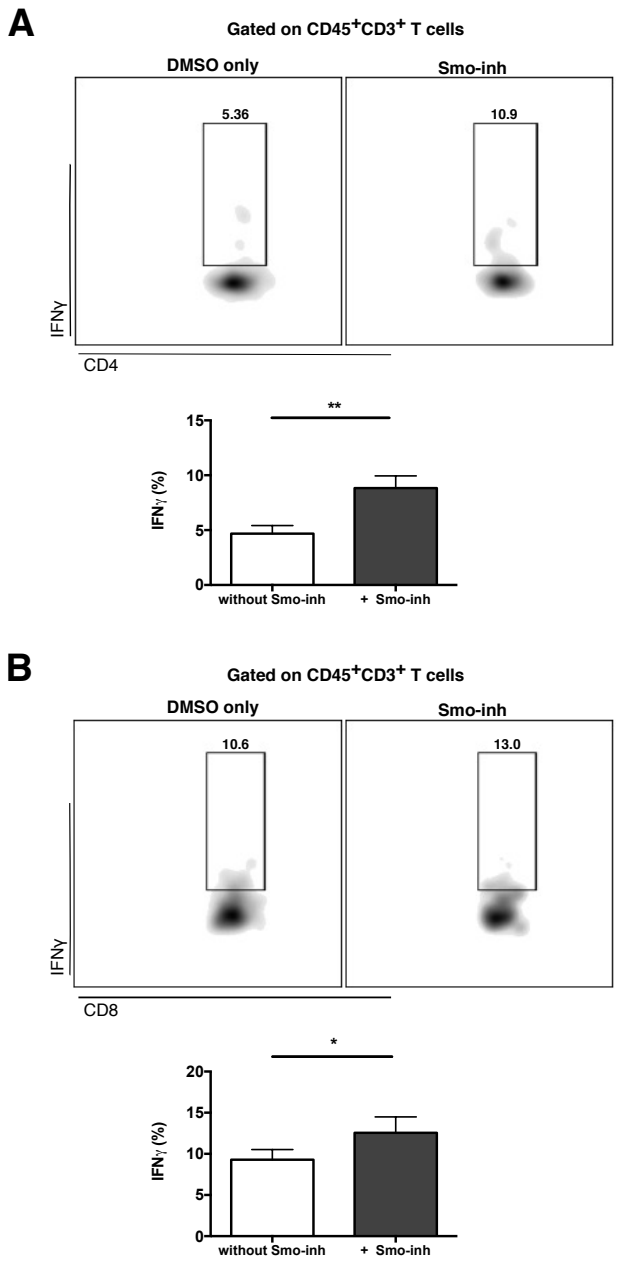


Figure 5-14: Elevated IFN-γ production by WT Oxa-treated skin T cells after Smoothened inhibitor administration

Representative density plots from DMSO injected (Left) and Smo-inh injected (Right) **(A)** $CD4^+$ and **(B)** $CD8^+$ skin IFN-γ-producing T cells. Bar charts show percentages of IFN-γ production in $CD4^+$ and $CD8^+$ T cells from Oxa- treated mice injected with DMSO (White bars) and with Smo-inh (Grey bars). Data were generated from two independent experiments with at least 6 mice per group. Plots show mean ± SEM. *p<0.05 and **p<0.01.

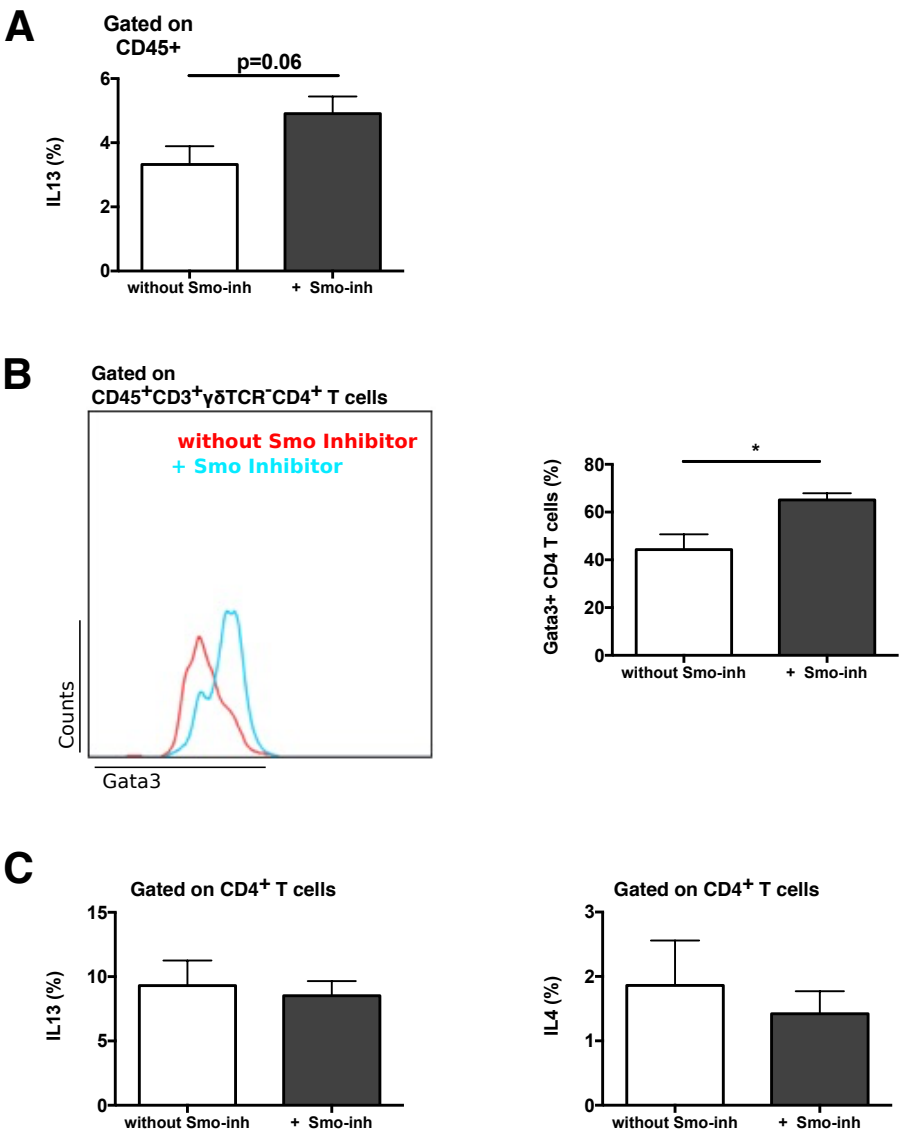


Figure 5-15: Smoothened inhibition does not affect Th2 cytokine production by skin T cells after Oxa application

(A) Bar chart showing percentages of IL-13-producing skin leukocytes ($CD45^+$) from DMSO injected (White bars) and Smo-inh-injected (Grey bars) Oxa-treated mice.

(B) Representative histograms and bar chart showing Gata3 expression on skin $CD4^+$ T cells from DMSO-treated (red line-histogram, white bar- bar chart) and Smo-inh-injected (blue line-histogram, grey bar- bar chart) WT treated mice.

(C) Bar charts showing percentages of IL-4 and IL-13-producing cells in skin $CD4^+$ T cell populations from DMSO injected and Smo-inh-injected Oxa-treated WT mice.

Data were generated from two independent experiments with at least 6 mice per group. Plots show mean ± SEM. *$p<0.05$.

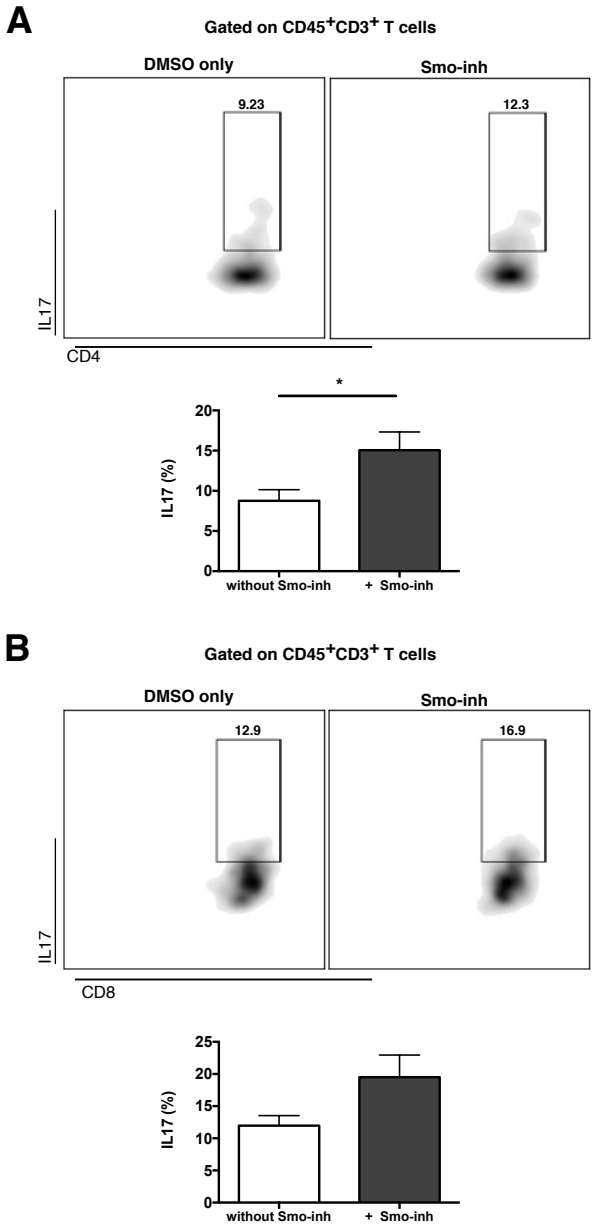


Figure 5-16: Smoothened inhibition causes an elevated Th17 response in skin T cells in Oxa-treated mice

Representative density plots from DMSO injected (Left) and Smo-inh-injected (Right) **(A)** $CD4^+$ and **(B)** $CD8^+$ skin IL-17-producing T cells. Bar charts show percentages of IL-17^+ cells in $CD4^+$ and $CD8^+$ T cells from Oxa-treated mice injected with DMSO (White bars) and with Smo-inh (Grey bars). Data were generated from two independent experiments with at least 6 mice per group. Plots show mean ± SEM. *$p<0.05$.

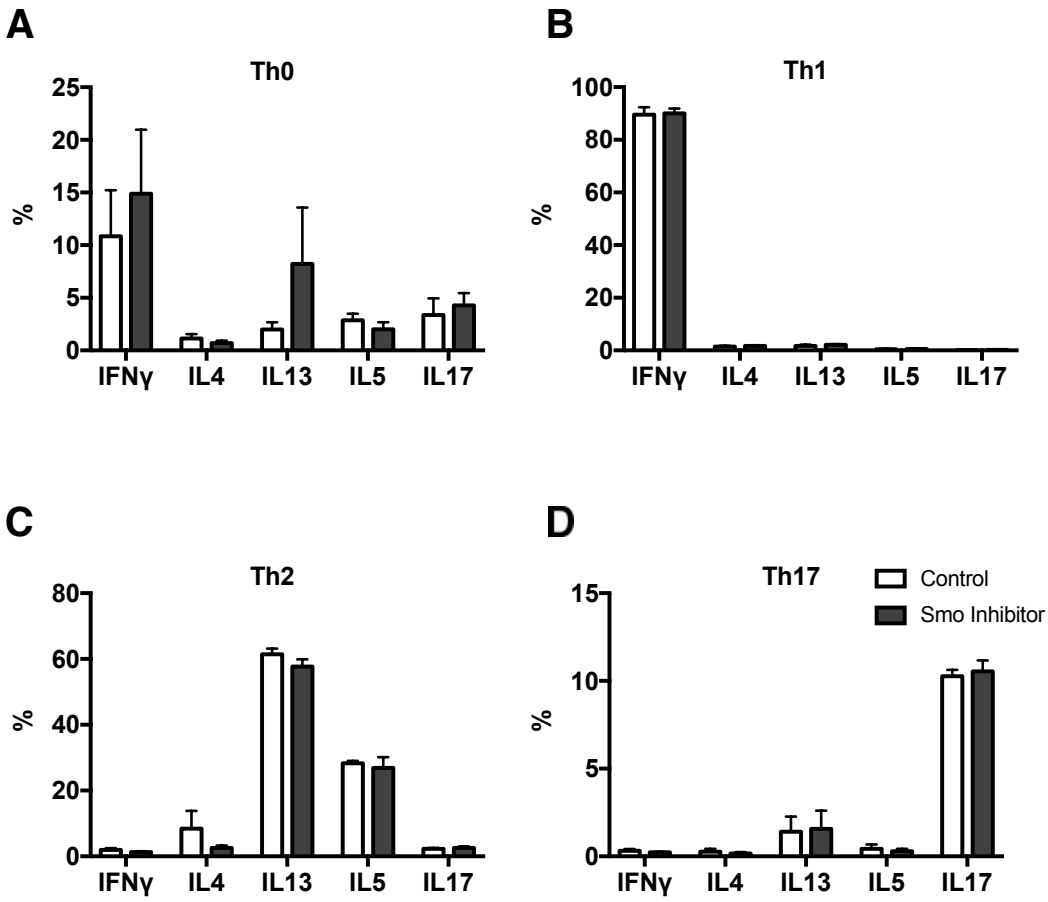


Figure 5-17: Smoothened inhibitor does not significantly influence $CD4^+$ T cell differentiation *in vitro*

Bar charts showing cytokine production of $CD4^+$ T cells isolated from spleen and polarised under **(A)** Th0, **(B)** Th1, **(C)** Th2 and **(D)** Th17 conditions. White bars show T cells cultured with DMSO while grey bars show T cells cultured with Smo-inh. Data were generated from one experiment with 4 mice per condition and group.

5.4 Discussion

In this chapter I have shown that Gli3 mutation rescues skin from dermatitis-like inflammation, as $Gli3^{+/-}$ skin had signs of moderate inflammation with reduced hyperkeratosis and parakeratosis compared to WT mice. Furthermore, the reduced IgE blood serum levels, the downregulated *Il4* and *Il13* skin transcripts and IL-4- and IL-13 production by skin T cells, observed in Gli3-mutant mice, suggested that these mice exhibited a downregulated Th2 response compared with their WT littermates. The equivalent levels of IFN-γ expression in both groups as detected by q-PCR and flow cytometry in skin T cells, in combination with the differences in Th2 responses indicated that the mice were still in the acute phase and had not entered the chronic phase of skin inflammation at the time point the experiment was terminated. When testing Hh pathway components, I confirmed elevated *Shh* levels in both untreated and treated $Gli3^{+/-}$ skin compared to WT controls. Although this result was expected, given that many studies support that Gli3 repression upregulates Shh signalling expression in a plethora of tissues (Hager-Theodorides et al., 2005, Hager-Theodorides et al., 2009, Solanki et al., 2017, Solanki et al., 2018, te Welscher et al., 2002, Wang et al., 2000), it had not to my knowledge previously been shown in skin tissue. Since it is known that Gli3- and Shh- have opposite phenotypes in many different tissues, it would be interesting to test the response of $Shh^{+/-}$ mice after Oxa-induced skin inflammation in the future.

Upon Smo-inh treatment, the untreated mice did not show any signs of spontaneous inflammation, indicating that inhibition of Smo could not alone

induce dermatitis-like skin inflammation. Surprisingly none of the tested Hh pathway components were diminished upon Smo-inh administration (Figure **5-6 B**). A possible explanation could be that the effect of inhibition of Smo is only detectable under inflammatory state when the pathway is upregulated. So far, treatment with PF-04449913 is reported to reduce expression of Hh signalling components downstream of Smo in tumours (Minami et al., 2017, Sadarangani et al., 2015, Wagner et al., 2015). In the experiments with Oxa-treated mice, I only tested *Shh* expression and detected a significant decrease in its expression (Figure **5-9 A**). Smo-inhibitor has been shown to inhibit Gli1 expression (Minami et al., 2017, Wagner et al., 2015) and Gli1 itself is a Hh target gene and acts only as a Hh pathway activator (Park et al., 2000). Therefore, Gli1 downregulation could create a negative loop causing Shh downregulation upon Smo-inh injection in Oxa-treated mice. Further research should be done in order to test the rest of Hh signalling components in Oxa-treated Smo-inh-injected mice.

Hh pathway signalling can be via canonical or non-canonical pathways. The canonical pathway acts via Smo transmembrane protein while non-canonical can be either Gli- or Smo-independent (Riobo and Manning, 2007, Teglund and Toftgard, 2010, Teperino et al., 2012, Teperino et al., 2014). The fact that Smoothened inhibition exacerbated skin inflammation and diminished *Shh* expression indicates that the canonical Smo-dependent Hh pathway is implicated in the disease. Another important finding was that Smo-inh does not act directly on $CD4^+$ T cells during T cell

polarization *in vitro*, as there were no significant differences in key T helper cytokine production compared with vehicle-only cultured cells.

In both Gli3 and Smo-inh experiments, skin $CD4^+$ and $CD8^+$ T cells were not altered in terms of percentages and numbers. However, in Smo-inh experiments, mice injected with the inhibitor displayed an elevated proportion of leukocytes and further analysis revealed increased innate immune cells such as macrophages and eosinophils. Also, upon Smo-inhibition, skin $CD4^+$ T cell proliferation was elevated, but I did not observe an increase in their numbers. That could be due to the fact that although cells proliferate more, there is also a high number of cells dying, which results in no difference in the final numbers of $CD4^+$ T cells. In order to clarify this hypothesis, more experiments using apoptotic markers should be done.

From the results I propose that Smoothened inhibition aggravates atopic dermatitis skin inflammation, and does not directly affect skin T cells, but alters Shh expression in skin epithelium or in non-T cell populations and also directly affects the innate immune cells. The crosstalk between innate immune cells and T cells as well as the altered Shh signals that T cells receive through their Patched receptor could then affect their activation and T cell cytokine production.

Overall, I have shown that unconditional Gli3 mutation increased *Shh* expression in skin tissue and the mice exhibited ameliorated skin inflammation, while systemic Smo inhibition, repressed *Shh* skin expression exaggerating skin inflammation, which was characterised by elevated T cell responses, although Smo-inh did not have a direct effect on

$CD4^+$ T cells. It would be interesting to test how do other Gli transcription factors affect skin inflammation and T cells. In the following chapter I present the data from experiments done on transgenic mice which conditionally overexpress or inhibit Gli2 specifically on T cells.

Finally, PF-04449913 compound is widely used in cancer research. Skin of patients administered with this compound may receive the added benefit of an increased immune response against tumour.

Chapter 6

Chapter 6: The role of conditional Gli2 modulation in chronic atopic dermatitis and skin inflammation

6.1 Introduction

The Gli2 member of the Glioma-associated oncogene family of transcription factors acts as an *in vivo* activator of transcription (Bai et al., 2002) and is important for the initiation of Hh signalling pathway. However, Gli2 can be processed to act as either activator or repressor of transcription in the presence or absence of the pathway respectively (Aza-Blanc et al., 2000). Gli2 has multiple roles in skin cancer as well as in immune T cell development and differentiation, which are described below.

6.1.1 The Gli2 transcription factor in skin

In normal skin, Gli2 is expressed in interfollicular epidermis and outer root sheath of hair follicles (Ikram et al., 2004) with a well-established role in hair follicle development (Bai et al., 2002, Mill et al., 2003). Gli2 is essential for hair follicle development by mediating Shh expression and cyclins D1 and D2, while its absence arrests cell proliferation (Mill et al., 2003). Its activity in keratinocytes is regulated by Kif7 and Su(Fu) (Li et al., 2012). Moreover, Gli2 is shown to have an inhibitory activity in human epidermis, as it represses genes that promote epidermal differentiation or expressed by fully differentiated keratinocytes (Regl et al., 2004).

Aberrant Gli2 expression is reported by many independent groups in a series of cancers, including BCC (Epstein, 2008). Its abnormal expression is maintained by inactivation of regulatory Hh pathway components or by non-canonical Hh signalling pathways (Javelaud et al., 2011, Li et al.,

2011). Studies in mice have shown that dysregulated Gli2 expression in epithelium is sufficient to maintain tumour growth, leading to malformations with BCC features (Grachtchouk et al., 2000, Hutchin et al., 2005) and its overexpression in mouse hair follicle stem cells drives BCC (Grachtchouk et al., 2011). Constitutive expression of Gli2 in tumours directly induces Gli1 expression by binding to its promoter, causing increased proliferation of human keratinocytes, which is observed in BCC, tumour invasion and metastatic processes (Ikram et al., 2004, Javelaud et al., 2012, Regl et al., 2004). Finally, a recent study reports TGF-β-driven Gli2 upregulation which is responsible for drug resistance in some patients with skin melanoma (Faiao-Flores et al., 2017).

6.1.2 *The Gli2 transcription factor and immunity*

Gli2 is expressed in both foetal and adult thymocytes (Outram et al., 2000, El Andaloussi et al., 2006). The Gli2 transcription factor in T cells has been reported to affect thymocyte development at different stages (Furmanski et al., 2012, Rowbotham et al., 2007, Rowbotham et al., 2008, Rowbotham et al., 2009). In peripheral $CD4^+$ T cells, overexpression of Gli2A suppresses their activation (Rowbotham et al., 2007, Furmanski et al., 2015), while inhibition of Gli2 increases their activation and proliferation via modulation of TCR signalling (Rowbotham et al., 2008). Furthermore, in the context of inflammation, Gli2-mediated Hh transcription promotes differentiation of naïve $CD4^+$ T cells towards Th2 cells leading to exacerbation of asthma in a mouse model of allergic airway disease (Furmanski et al., 2013, Standing et al., 2017).

6.1.3 *Transgenic mouse models of Gli2-mediated transcription*

In order to perform my experiments, I used two transgenic models: Gli2ΔN2 and Gli2ΔC2 mice. Gli2ΔN2 is a mouse constructed by our group (Rowbotham et al., 2007). The N truncated form of Gli2 acts as an activator of transcription only, independently of the upstream signals received by the pathway. This form is expressed under the control of Lck promoter, allowing T cells in the mouse to constitutively express the Gli2 activator. Gli1, which is a target gene of Gli2 (Dai et al., 1999, Ikram et al., 2004), showed a 20-times higher expression in sorted CD4 single positive thymocytes, confirming the T cell intrinsic activation of the Hh signalling pathway in these mice. Therefore, Gli2ΔN2 mice mimic the transcriptional events that happen upon Hh pathway activation, allowing the study of the effects of pathway activation specifically in T cells.

Gli2ΔC2 mice were also generated by our group (Rowbotham et al., 2008). These mice express the C-truncated form of Gli2 under the Lck promoter, which acts as a strong repressor of transcription. *Patch1* expression, which is itself also a Hh pathway target gene (Johnson et al., 1995, Ingham et al., 1991), was downregulated, validating that the Gli2ΔC2 mice successfully repress Hh signalling. Thus, T cells constitutively express the Gli2R, and are unable to respond transcriptionally upon Hh pathway activation, allowing the study of the effects of autonomous T cell inhibition of the pathway.

6.2 Aim

Previously I showed that Gli3 mutation led to Shh upregulation in mouse skin and rescued mice from skin inflammation. The opposite results were observed with systemic pharmacological inhibition of Smo, suggesting that Shh protects mice from atopic dermatitis and skin inflammation. Since I have shown that systemic Hh modulation affects skin inflammation, in this chapter I aim to test the hypothesis that T cell intrinsic Hh signalling has an impact on the disease. Therefore, I will use our Gli2ΔN2 and Gli2ΔC2 transgenic mice described above, to test whether **1)** conditional Gli2 overexpression or constitutive inhibition of normal Gli2 activity specifically in T cells, affects induction of chronic atopic dermatitis, if **2)** conditional inhibition of Gli2-mediated transcription exacerbates inflammation and if **3)** constitutive overexpression of Gli2 ameliorates atopic dermatitis. Ear skin histology will be examined by H&E staining in steady state conditions and upon Oxa-induced dermatitis. Furthermore, by using the Immunoplex technique I will measure the expression of various cytokines from skin supernatants. Since my main focus is on $CD4^+$ and $CD8^+$ T cell populations, I will assess their differences in proportions and numbers by FACS analysis. I will also evaluate Gli2 impact on activation in peripherally induced T cell populations from dLNs and spleen. Finally, cytokine production will be tested in skin T cells in both mouse strains.

6.3 Results

6.3.1 Constitutive Gli2 expression in T cells restrains dermatitis-like inflammation

Our group has previously shown that constitutive overexpression of the activator form of Gli (GliA) in T cells exacerbates allergic asthma by

upregulating Gata3 expression and promoting IL-4 production (Furmanski et al., 2013). In order to evaluate the role of the Gli2 transcription factor in skin inflammation, I induced dermatitis in Gli2ΔN2 mice which constitutively express the activator form of Gli2 under the Lck promoter and investigated skin histology by H&E staining. Skin sections of untreated WT (**Left**) and Gli2ΔN2 (**Right**) mice were first examined and I did not observe any signs of spontaneous dermatitis in the transgenic mice compared to the WT controls as shown in the representative images (Figure **6-1 A**; **Top panel**). However, after induction of dermatitis although both skin sections were characterised by dermal infiltration and ear thickening (Figure **6-1 A**; **Bottom panel**), Oxa-treated WT mice suffered more severe inflammation, with aberrant epidermal thickening and cellular infiltration (**Left**), whereas Oxa-treated Gli2ΔN2 counterparts displayed less epidermal thickening, without any signs of parakeratosis (**Right**).

I also quantified the skin thickening by measuring epidermal and dermal thickness of untreated and treated mice. As expected epidermal and dermal thickness of untreated WT and Gli2ΔN2 mice was not distinguishable, however both epidermal and dermal thickening were significantly reduced in the Oxa-treated transgenic mice compared to the WT littermates (Figure **6-1 B**) indicating less skin inflammation. The kinetics of inflammation was determined by a time-course experiment, which confirmed that throughout the Oxa-application Gli2ΔN2 mice had significantly decreased ear thickness (Figure **6-1 C, Red line**), compared to WT ear skin (**Black line**) suggesting delayed or reduced induction of skin inflammation.

In order to further examine a possible explanation for the milder dermatitis symptoms in Gli2ΔN2 mice, I tested *Filaggrin* expression by qPCR on skin homogenates, where I observed a threefold increase in expression of the terminal differentiation marker in the transgenic mice compared to the WT (0.4362 ± 0.1061 vs 0.1553 ± 0.06836, $p<0.05$; Figure **6-2 A**). As described in the introduction, Th2 cytokines such as IL-4 and IL-13 boost skin disruption by downregulating Filaggrin (Howell et al., 2009). In agreement with the increased Filaggrin expression in the skin homogenates, *Il4* transcript expression was significantly reduced in Oxa-treated skin of Gli2ΔN2 compared to WT dermatitis mice (2 ± 0.3758 vs 6.056 ± 1.003, $p<0.05$). *Il13* expression however did not show any differences in the examined groups, but Gli2ΔN2 skin was characterised by increased *Il13* production. qPCR also revealed a threefold decrease in *Ifng* expression (0.009051 ± 0.0009154 vs 0.02784 ± 0.00412, $p<0.05$) in the transgenic mice (Figure **6-2 B**), suggesting that the Gli2ΔN2 mice might not display features of chronic dermatitis, in line with the ameliorated skin inflammation which was previously described.

Cytokines secreted by skin were measured with the Immunoplex technique, using skin supernatants from control and Oxa-treated WT and Gli2ΔN2 mice. IFN-γ levels were not detected in untreated skin of both groups, while levels in Oxa-treated Gli2ΔN2 mice were reduced compared to Oxa-treated WT skin (Figure **6-3 A**). Furthermore, IL-13 expression was detected only in WT untreated skin while no expression was measured in the untreated Gli2ΔN2 mice. As expected, IL-13 expression was upregulated in WT treated compared to WT control mice. In Oxa-treated mice, IL-13 levels

were significantly lower in Gli2ΔN2 skin compared to the WT (Figure **6-3 B**).

IL-1β is a proinflammatory cytokine which is implicated in eczema and is a marker of skin injury (Mizutani et al., 1991). In line with previous findings, its levels were halved in Gli2ΔN2 Oxa-treated skin compared to WT and no expression was detected in any of the untreated experimental groups (Figure **6-3 C**). I also tested IL-6 expression, which is a pro-inflammatory cytokine expressed by keratinocytes reported to inhibit epidermal terminal differentiation via NF-κB signalling (Son et al., 2014, Son et al., 2016). Surprisingly, no differences were obvious in skin of treated groups and in fact its levels were significantly heightened in WT untreated compared to WT treated skin. In addition, there was significantly lower expression in untreated skin of Gli2ΔN2 mice compared to WT-untreated mice (Figure **6-3 D**). Finally, TNF-α expression was analysed in skin supernatants. TNF-α has a key pathogenic role in atopic dermatitis (Kondo and Sauder, 1997) as this pro-inflammatory cytokine affects epidermal barrier function (Danso et al., 2014) and many TNF-α polymorphisms are related to the disease (Behniafard et al., 2012). Furthermore, TNF-α is reported to drive IL-1β production by mast cells causing skin disease (Nakamura et al., 2012). Its expression was significantly elevated in skin of both treated experimental groups compared to the healthy controls, however there was a significantly decreased TNF-α induction in Oxa-treated Gli2ΔN2 compared to WT mice (Figure **6-3 E**).

Taken together, the results showed that constitutive expression of Gli2A in T cells alone does not provoke any signs of spontaneous inflammation in

the skin of untreated mice, but on the induction of dermatitis, it is sufficient to dampen down the inflammatory response in skin, by reducing both the transcription and secretion of pro-inflammatory cytokines from the whole skin, which leads to milder skin injury, as measured by *Filaggrin* levels.

6.3.2 *Gli2-mediated transcription in T cells affects peripheral and skin CD4⁺ and CD8⁺ T cell populations*

Since I previously observed marked dermal infiltration in treated skin sections, I decided to examine skin innate and adaptive immune cells in treated WT and Gli2ΔN2 mice. First, I analysed skin for macrophages, neutrophils and eosinophils, which are increased during the acute phase of the disease. Although proportions and numbers of macrophages were higher in Oxa-treated Gli2ΔN2 mice compared to WT Oxa-treated skin, the differences were not significant between the two groups. Moreover, eosinophils were similar in terms of percentage and numbers in Oxa-treated skin of Gli2ΔN2 and WT mice. On the other hand, skin of Oxa-treated Gli2ΔN2 mice was characterised by a significantly increased infiltration of neutrophils compared to the Oxa-treated WT mice (37.76 ± 3.505 vs 27.12 ± 1.523, $p<0.05$) (Figure **6-4 A**).

Analysis of skin $CD4^+$ and $CD8^+$ T cell percentages revealed that skin $CD4^+$ T cells were almost doubled (61.92 ± 2.94 vs 37.23 ± 4.933, $p<0.001$) while skin $CD8^+$ T cell percentage was almost halved in Oxa-treated Gli2ΔN2 treated compared to WT (24.5 ± 3.405 vs 50.87 ± 3.354, $p<0.0001$) (Figure **6-4 B**). Numbers showed no difference in skin $CD4^+$ T cells in any of the groups, however there was a substantial reduction in the number of $CD8^+$

T cells present in skin of Oxa-treated Gli2ΔN2 compared to WT mice (3.095 ± 0.6377 vs 7.283 ± 1.123, $p<0.01$) (Figure **6-4 C**).

Since $CD4^+$ and $CD8^+$ T cells migrate to skin from lymph nodes when skin is inflamed, I tested them, their activation status, along with naïve and memory subsets in dLNs upon Oxa-induced inflammation. I found that both $CD4^+$ and $CD8^+$ T cells were markedly decreased in Oxa-treated dLNs of Gli2ΔN2 mice compared to the Oxa-treated littermates (Figure **6-5 A**). Naïve T cells are cells that are non-primed, recirculating in secondary lymphoid organs and blood and are characterised in mice as $CD62L^+CD44^-$, while memory T cells emerge after the body has encountered and interacted with a pathogen and are grouped as T effector (T_{eff}) cells characterised for the following markers: $CD62L^-CD44^+$ and T central memory (T_{cm}) cells characterised as $CD62L^+CD44^+$ in mice. Recently T_{eff} and T_{cm} cell were shown to be increased in patients with AD compared to healthy controls (Czarnowicki et al., 2017). After induction of dermatitis, I tested dLN $CD4^+$ and $CD8^+$ T cell phenotypes, staining them with the markers mentioned above. Representative FACS plots in Figure **6-5 B**, show naïve and memory T cells in $CD4^+$ (**Top panel**) and $CD8^+$ T cells (**Lower panel**) from Oxa-treated WT and Gli2ΔN2 mice. In Gli2ΔN2, dLNs significantly increased proportions of naïve cells but decreased populations of effector memory and central memory $CD4^+$ T cells were observed compared to WT littermates (Figure **6-5 B**). In $CD8^+$ T cells although no difference was detected in percentage of naïve $CD8^+$ T cells, both effector and central memory $CD8^+$ populations were markedly lower in Gli2ΔN2 compared to the WT group (Figure **6-5 B**).

To assess activation of peripherally-induced T cells in dLNs and spleen, I stained $CD4^+$ and $CD8^+$ T cells with early (CD69) and late (CD25) activation markers. CD69 expression in dLNs of the experimental groups was not altered in $CD4^+$ T cells however the levels were strikingly higher in $CD8^+$ T cells from Gli2ΔN2 compared to WT mice (Figure **6-6 A**). Interestingly expression of the activation marker CD69 in spleen of $CD4^+$ and $CD8^+$ T cells was markedly upregulated in the Oxa-treated transgenic group compared to the WT mice (Figure **6-6 B**). $CD25^+CD4^+$ T cells were significantly increased in Oxa-treated Gli2ΔN2 mice in both dLNs and spleen, while there was no difference in $CD8^+CD25^+$ cells between the groups in either dLNs or spleen, and in fact CD25 expression in $CD8^+$ T cells was barely detectable (Figures **6-6 C** and **6-6 D**).

6.3.3 *Gli2 activity impairs cytokine production by skin activated $CD4^+$ and $CD8^+$ T cells*

Next, I tested the capability of skin T cells from WT and Gli2ΔN2 mice to produce Th1, Th2 and Th17 cytokines after Oxa-treatment. IFN-γ production was evaluated in skin $CD4^+$ and $CD8^+$ T cells. As shown in representative FACS plots in Figure **6-7 A**, $CD4^+$ T cells in Gli2ΔN2 mice produced significantly less IFN-γ compared to WT mice (5.88% vs 11.7%). In line with the lower percentage in the transgenic mice, there was a threefold reduction in numbers of IFN-γ–producing $CD4^+$ T cells in Gli2ΔN2 mice compared to WT mice (439.7 ± 53.56 vs 1126 ± 384.1, $p<0.05$). Proportions of IFN-γ–producing $CD8^+$ T cells were also markedly lower in Gli2ΔN2 (representative FACS plots in Figure **6-7 B**) and quantification of

the percentages, showed that the numbers of IFN-γ$^+$CD8$^+$ T cells were significantly higher in Oxa-treated WT skin compared to Gli2ΔN2.

Then I examined the capacity of skin T cells to produce IL-17. Lower percentages of skin IL-17$^+$CD4$^+$ T cells were identified in Oxa-treated Gli2ΔN2 mice and although the numbers of IL-17$^+$CD4$^+$ T cells were also reduced, the difference was not significant when compared to WT treated skin (Figure **6-8 A**). Intracellular IL-17 expression in CD8$^+$ T cells of both groups, as shown in the representative FACS plots in Figure **6-8 B** was much higher compared to CD4$^+$ T cells. Quantification of the previous results showed that IL-17$^+$CD8$^+$ T cells were diminished in Oxa-treated Gli2ΔN2 treated compared to WT mice (1360 ± 479.4 vs 3469 ± 827.7, $p<0.05$).

Finally, Th2 cytokines were examined in skin CD4$^+$ T cells. The percentage of IL-13$^+$CD4$^+$ T cells was significantly lower in Oxa-treated Gli2ΔN2 mice (Representative FACS plots in Figure **6-9 A**), and likewise their numbers were reduced to half compared to WT mice (464.3 ± 74.43 vs 1079 ± 312.8, $p<0.05$). In addition, expression of intracellular IL-4 and IL-5 cytokines was investigated, but the numbers of CD4$^+$ T cells producing these cytokines were not different between the experimental groups (Figure **6-9 B**).

6.3.4 Inhibition of Hh-mediated transcription via constitutive inhibition of Gli2-mediated transcription in T cells is sufficient to worsen symptoms of dermatitis and skin inflammation

Having shown that constitutive overexpression of GliA in T cells, resulted in protection against dermatitis, I aimed to test if constitutive inhibition of Gli2-mediated transcription would have the opposite outcome. Therefore, I

used the Gli2ΔC2 transgenic mice, which as described above, inhibit physiological Hh-mediated transcription in all T cells, using the Lck-promoter. First, I performed H&E histology of skin sections of untreated and Oxa-treated WT and Gli2ΔC2 mice. Baseline controls of both groups (**Top panel**; WT: **Left**, Gli2ΔC2: **Right**, Figure **6-10 A**) had normal histological architecture, characterised by normal dermal cellular infiltration and lack of cellular infiltration in epidermis. However, H&E staining of lesional skin sections after Oxa-treatment (**Bottom panel**) showed signs of inflammation characterised by aberrant dermal infiltration and increased epidermal hyperplasia in both groups. The Oxa-treated Gli2ΔC2 group displayed features of more severe skin pathology as the mice had more obvious hyperkeratosis and parakeratosis signs compared to the WT Oxa-treated littermates.

To confirm this, I analysed the kinetics of skin thickening by measuring ear thickness during the Oxa protocol (Figure **6-10 B**). Although ear thickness was similar in both groups at the initiation of the experiment, after repeated Oxa-applications, Gli2ΔC2 ear thickness was consistently higher than the WT ear thickness. At the end of the experiment, I observed an almost two-fold increase in the transgenic group compared to WT littermates.

To further examine the impact of physiological Hh-mediated transcription in skin pathology, I analysed the expression of *Filaggrin* by qPCR, where I observed a marked downregulation in Oxa-treated mice Gli2ΔC2 compared to WT (Figure **6-11 A**). Additionally, and in agreement with the reduced expression of the terminal differentiation marker, *Il13* expression was strikingly higher in the transgenic group (0.2412 ± 0.03953 vs 0.08618 ±

0.01451, p<0.001), and *Il4* expression followed the same trend. *Ifng* expression in Oxa-treated Gli2ΔC2 skin was also elevated, although the difference was not statistically significant in comparison with the WT group (Figure **6-11 B**).

Next, I compared protein expression in control and Oxa-treated skin supernatants by the Immunoplex technique. IFN-γ expression was not detected in the healthy controls. Upon Oxa-application it was induced in both groups and it was significantly elevated in Gli2ΔC2 compared to WT littermates (Figure **6-12 A**). Moreover, Th2 cytokine expression was evaluated. IL-4 protein was not detected in non-inflamed skin in either groups, whereas upon Oxa-treatment it was expressed in WT and Gli2ΔC2 skin and its levels were similar (Figure **6-12 B**). IL-5 protein was present in healthy WT skin, while it was not detected in control Gli2ΔC2 mice, but its expression was doubled after Oxa-application in WT skin and Gli2ΔC2 skin (Figure **6-12 C**). IL-13 secretion was also measured however it was not detected in any of the samples.

IL-6 pro-inflammatory cytokine was characterised by low expression in healthy controls, while its levels were increased in inflamed skin of both experimental groups. Furthermore, Oxa-treated Gli2ΔC2 skin had a significantly higher expression of IL-6 compared to WT treated mice (Figure **6-12 D**). Finally, TNF-α measurement revealed a similar expression pattern with IL-6. In control skin its expression was barely detected and was similar in all mice, while upon Oxa-treatment it was substantially upregulated in WT and Gli2ΔC2 mice and the Oxa-treated Gli2ΔC2 group was

characterised by significantly elevated expression compared to WT treated mice (Figure **6-12 E**).

Overall the findings so far indicate that constitutive inhibition of Gli2-mediated transcription in T cells alone is not sufficient to provoke spontaneous skin inflammation. However, upon Oxa-induced dermatitis, it was sufficient to promote skin inflammation, as detected by enhanced ear thickness and elevated pro-inflammatory cytokine expression in skin of Gli2ΔC2 mice compared to WT.

6.3.5 *Inhibition of Gli2-mediated Hh signalling increases T cell numbers and is responsible for their activated phenotype*

Since I observed increased dermal infiltration in Oxa-treated mice, I examined the possible differences in T cell percentages and numbers present in Oxa-treated WT and Gli2ΔC2 skin. FACS analysis revealed that the proportion of $CD4^+$ T cells was significantly higher in Oxa-treated Gli2ΔC2 compared to WT skin (45.02 ± 2.156 vs 39.12 ± 1.745, $p<0.05$). Similar results were obtained for skin $CD8^+$ T cells where the percentage in Gli2ΔC2 mice was almost doubled (41.25 ± 2.553 vs 28.12 ± 3.555, $p<0.01$) (Figure **6-13 A**).

I quantified the results, and interestingly found out that there were no differences in numbers in skin $CD4^+$ T cells in the experimental groups. However, there was a threefold increase in the number of $CD8^+$ T cells obtained from the skin of Gli2ΔC2 mice compared to the Oxa-treated WT control (128565 ± 33153 vs 48651 ± 20297, $p<0.05$) (Figure **6-13 B**).

Additionally, I analysed the phenotypes of the peripherally-induced T cells in the dLNs. Consistent with the results from the skin, the percentages of

$CD4^+$ T cells were equivalent, whereas there was a significantly higher percentage of $CD8^+$ T cells in Oxa-treated Gli2ΔC2 compared to Oxa-treated WT dLNs (Figure **6-14 A**).

I then evaluated T cells for early and late activation, using CD69 and CD25 markers. $CD4^+$ T cells were more activated in the Gli2ΔC2 mice compared to the WT dLNs as indicated by the representative histogram (**Top histogram**; Blue line:Gli2ΔC2, Red line:WT) and the difference was markedly significant. The same phenotype was observed for early activation of $CD8^+$ T cells, where proportions of $CD8^+CD69^+$ were significantly increased (Figure **6-14 B**). Consistent with the previous results, late activation of $CD4^+$ T cells in Gli2ΔC2 treated dLNs was also higher compared to treated WT dLNs (6.358 ± 0.2487 vs 5.651 ± 0.2076, $p<0.05$). However, $CD8^+$ T cells did not significantly differ in CD25 expression in the experimental groups (Figure **6-14 C**).

Finally, I evaluated dLNs T cells for naïve and memory subtypes. $CD4^+$ T cells were characterised by a significantly decreased proportion of naïve cells, whereas memory $CD4^+$ T cells were markedly increased in Oxa-treated Gli2ΔC2 mice compared to WT controls. Regarding $CD8^+$ T cells subsets, no significant differences were observed between the transgenic and WT mice (Figure **6-14 D**).

6.3.6 *Constitutive inhibition of Gli2-mediated transcription increases the capacity of skin T cells to produce pro-inflammatory cytokines*

After confirming that T cell activation is increased in Gli2ΔC2 mice, I compared these mice with their WT counterparts in terms of the cytokines that skin T cells produced. I first tested IFN-γ expression on $CD4^+$ T cells,

and I found that there was a significant twofold increase in the percentage of $CD4^+IFN\text{-}\gamma^+$ T cells in Gli2ΔC2 mice and in line with this, $CD4^+IFN\text{-}\gamma^+$ T cell numbers were doubled (275.5 ± 36.11 vs 148.2 ± 26.07, $p<0.05$) compared to WT littermates (Figure **6-15 A**). Then I examined $CD8^+$ T cells for IFN-γ production (Figure **6-15 B**) and I observed overall higher production of this cytokine in $CD8^+$ compared to $CD4^+$ T cells. As expected, the proportion of $CD8^+IFN\text{-}\gamma^+$ T cells was significantly elevated in Gli2ΔC2 mice, and there was a substantial elevation in numbers of $CD8^+IFN\text{-}\gamma^+$ T cells in this strain compared to the WT skin (920.3 ± 150.5 vs 596.3 ± 71.85, $p<0.05$).

Furthermore, I tested Th17 cytokines in skin of these mice, by measuring IL-17 production. As shown in Figure **6-16**, both $CD4^+$ and $CD8^+$ T cells were characterised by high expression of IL-17. $CD4^+IL\text{-}17^+$ T cell percentages were significantly higher in Gli2ΔC2 mice compared to WT treated controls, and although the numbers of $CD4^+IL\text{-}17^+$ T cells were also increased, the differences were not statistically significant (Figure **6-16 A**). On the other hand, $CD8^+$ T cells of both Gli2ΔC2 and WT mice, showed similar percentages and numbers of $IL\text{-}17^+$ cells in skin (Figure **6-16 B**).

Finally, Th2 cytokines (IL-4, IL-5 and IL-13) were examined on $CD4^+$ T cells. Although there was a higher proportion and higher numbers of $CD4^+IL\text{-}13^+$ T cells in the Oxa-treated transgenic mice compared to WT littermates, the differences were not significant (Figure **6-17 A**). Examination of IL-4 and IL-5-producing $CD4^+$ T cells did not reveal any significant differences between the two groups, although numbers were elevated in Gli2ΔC2 mice (Figure **6-17 B**). Thus, cytokine examination, highlighted a substantial

difference between the two groups in terms of IFN-γ production, while other cytokines examined although highly produced did not show any differences in skin of Gli2ΔC2 and WT Oxa-treated mice.

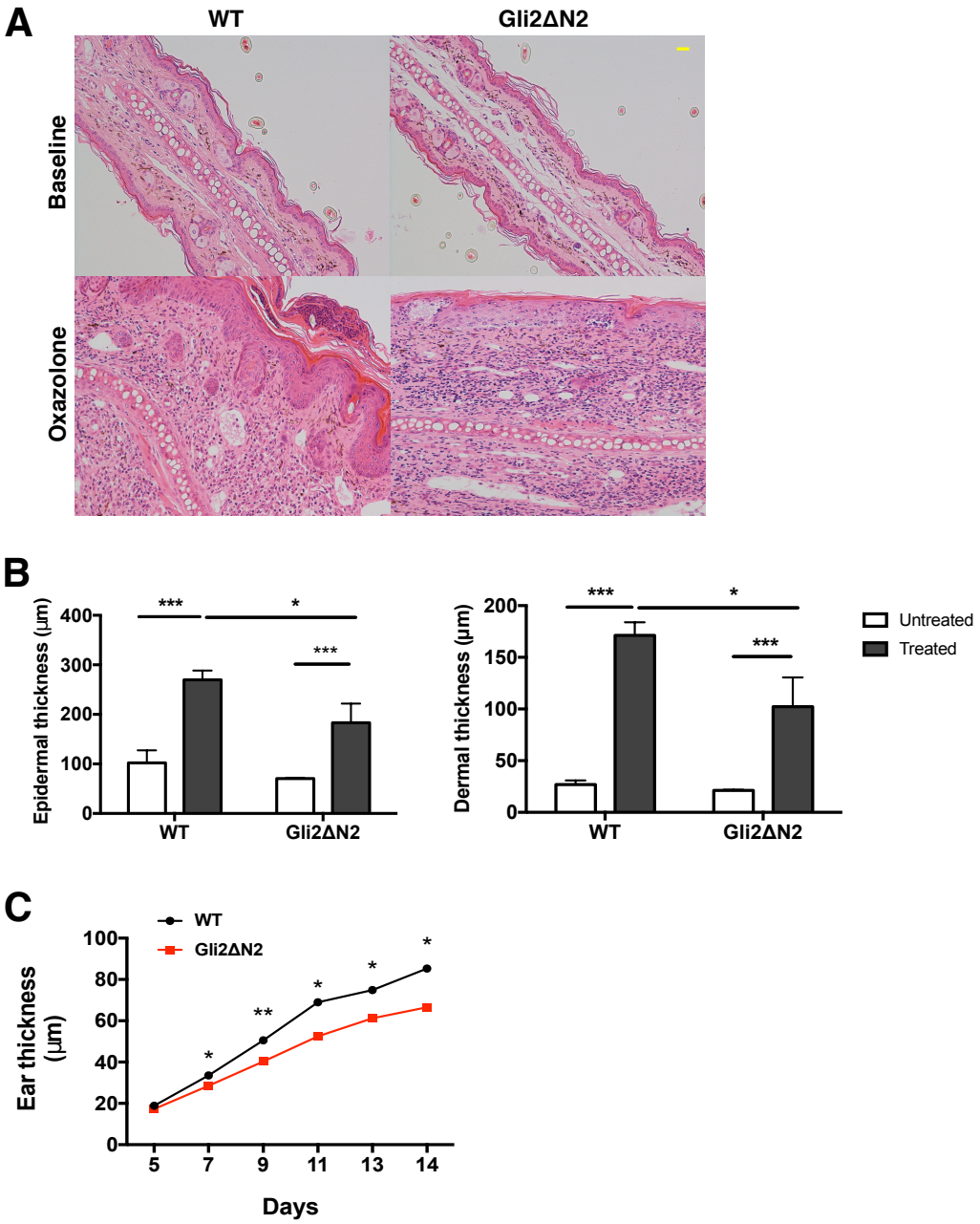


Figure 6-1: Skin histology reveals ameliorated symptoms of atopic dermatitis in Gli2ΔN2 mice

(A) Representative H&E images of skin sections from untreated (Baseline) and treated (Oxazolone) WT (Left) and Gli2ΔN2 (Right) mice. Scale bar represents 100µm.

(B) Bar charts show quantification of epidermal and dermal thickness of WT and Gli2ΔN2 untreated (White bars) and Oxa-treated (Grey bars) mice, when the experiment was terminated.

(C) Time course of ear thickness measurement from Oxa-treated WT treated (Black line) and Gli2ΔN2 (Red line) mice.

Data were generated from two independent experiments with 4 mice per group. Plots show mean ± SEM. *p<0.05, **p<0.01 and ***p<0.001.

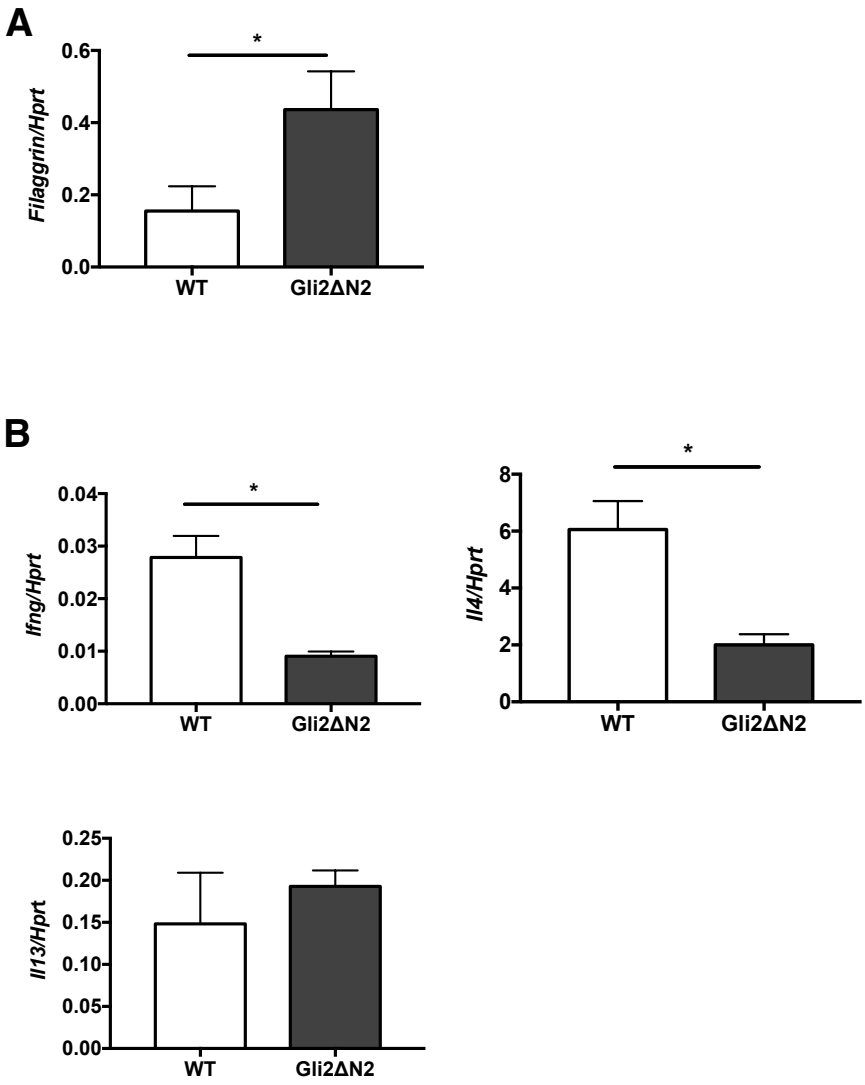


Figure 6-2: Gli2-mediated transcription causes decreased barrier defect and cytokine expression upon Oxa-treatment

Bar charts show mRNA expression of **(A)** *Filaggrin*, **(B)** *Ifng*, *Il13* and *Il4* cytokines as determined by quantitative PCR from skin homogenates from Oxa-treated WT (White bars) and Gli2ΔN2 (Grey bars) mice. Data were generated from 2 independent experiments with at least 6 mice per group. Plots show mean ± SEM. *p<0.05.

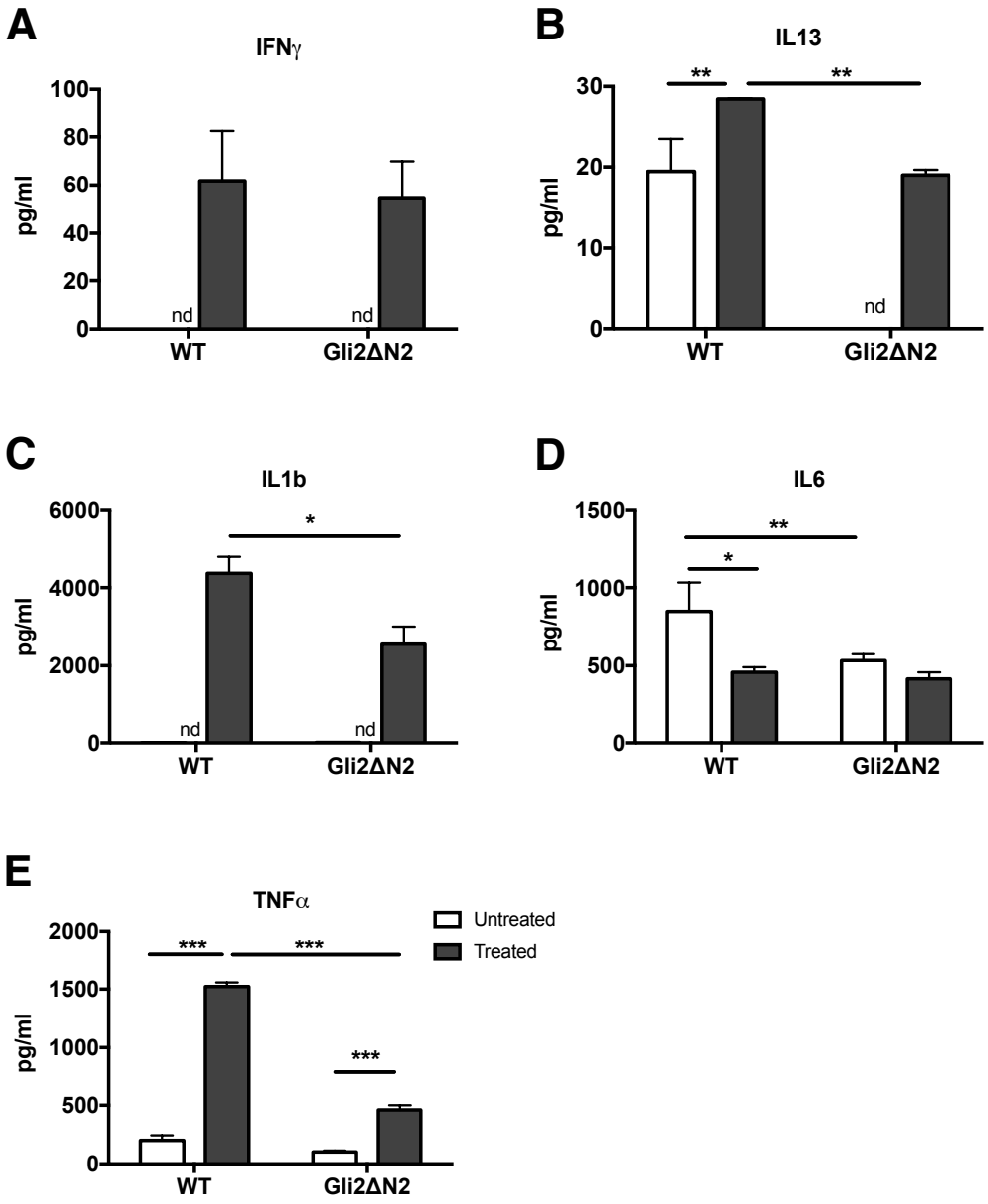


Figure 6-3: Diminished levels of pro-inflammatory cytokines in the skin of Gli2ΔN2 treated mice

Measurement of **(A)** IFN-γ, **(B)** IL-13, **(C)** IL1-β, **(D)** IL-6 and **(E)** TNF-α cytokine concentrations in skin supernatants from untreated (White bars) and Oxa-treated (Grey bars) WT and Gli2ΔN2 mice. Data were generated from two independent experiments with at least 3 mice per group. Plots show mean ± SEM. 2way ANOVA statistical test was performed. n.d.: not detected, *p<0.05, **p<0.01 and ***p<0.001.

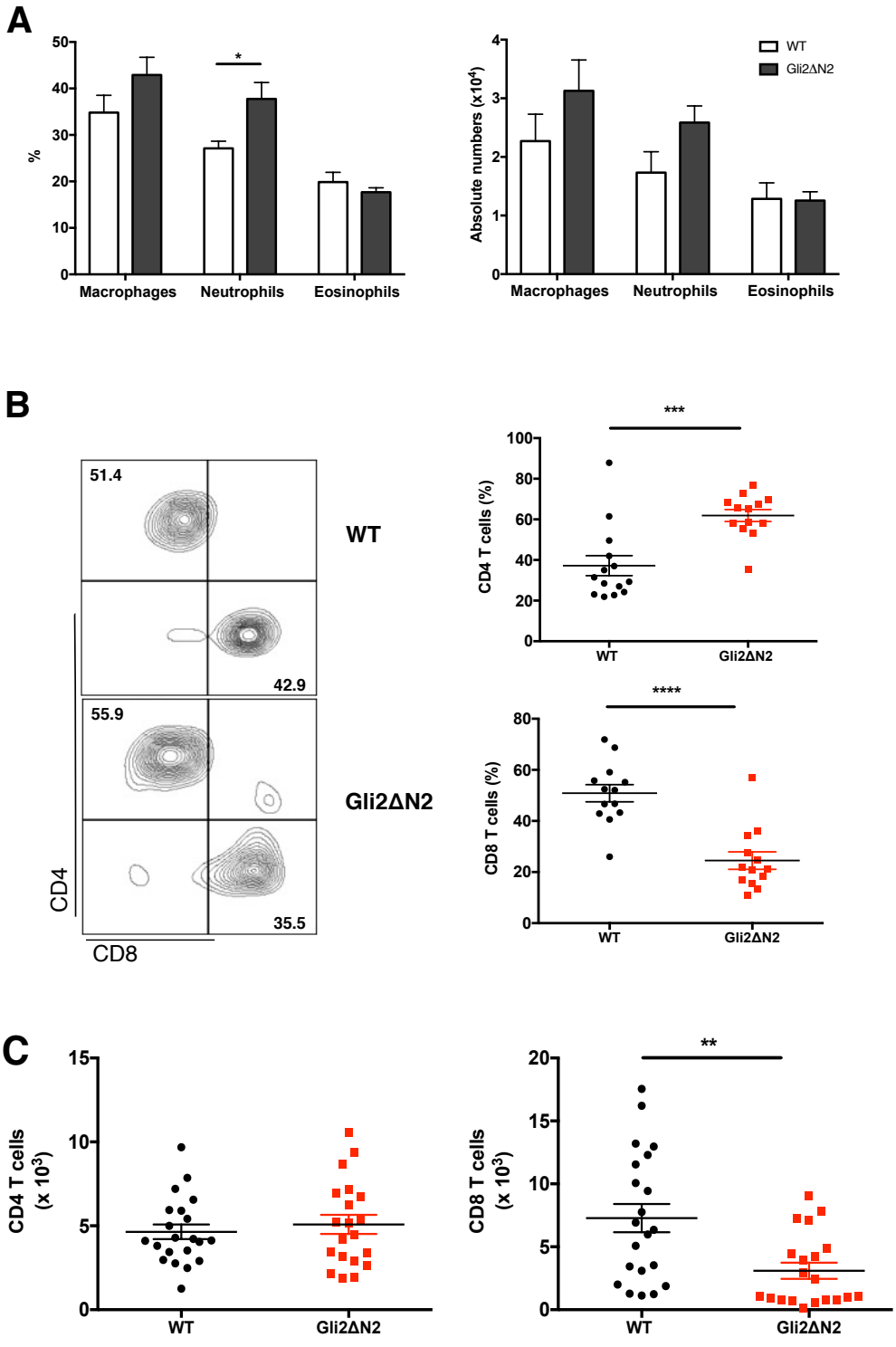


Figure 6-4: Gli2-mediated transcription in T cells, affects the skin immune cell populations after induction of dermatitis

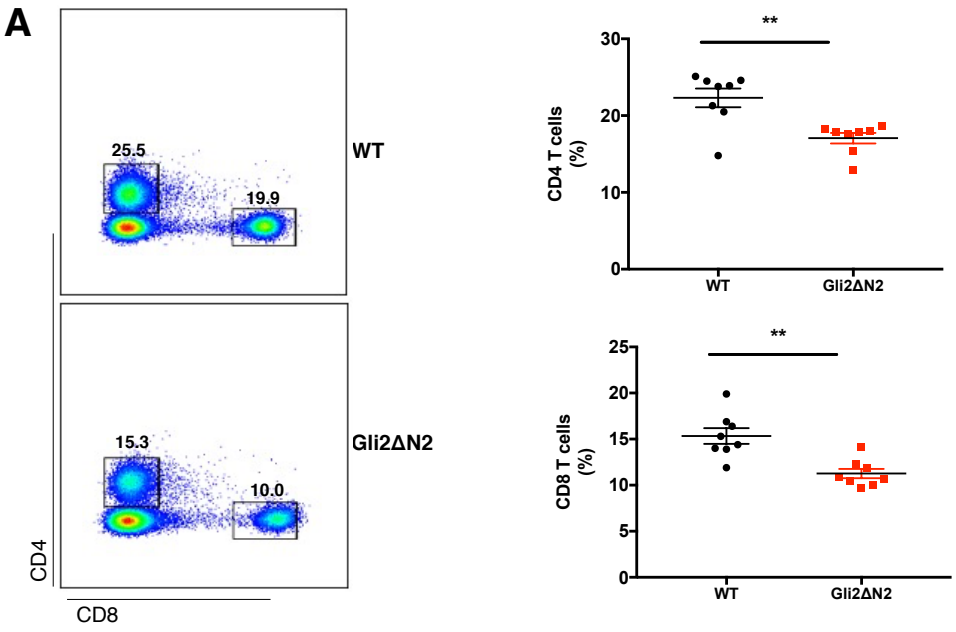
(A) Bar charts show the percentages and numbers of innate immune cells in skin from Oxa-treated WT (White bars) and Gli2ΔN2 (Grey

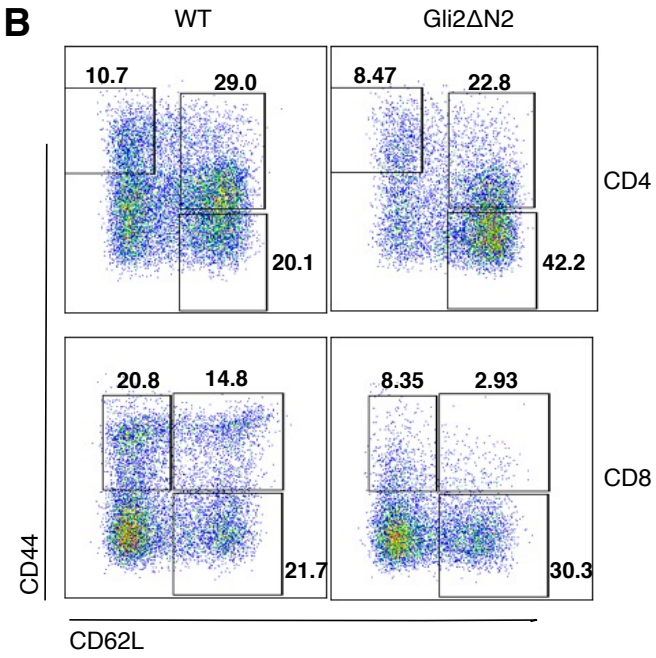
bars) mice. Data were generated from one experiment with 7 mice per group.

(B) Representative contour plots from Oxa-treated WT (Top) and Gli2ΔN2 (Bottom) mice. Scatter plots showing the percentages of skin $CD4^+$ T cells and $CD8^+$ T cells from Oxa-treated WT (black circles) and Gli2ΔN2 (red squares).

(C) Scatter plots represent numbers of skin $CD4^+$ T cells and $CD8^+$ T cells from Oxa-treated WT and Gli2ΔN2 mice.

For **(B)** and **(C)** each symbol in the graph represents an individual mouse and the group mean is represented with a bar. Data were generated from two independent experiments with at least 6 mice per group. *$p<0.05$, **$p<0.01$, ***$p<0.001$ and ****$p<0.0001$.


A
25.5
19.9
WT
15.3
10.0
Gli2ΔN2
CD4
CD8
**
CD4 T cells (%)
30
20
10
0
WT
Gli2ΔN2
**
CD8 T cells (%)
25
20
15
10
5
0
WT
Gli2ΔN2


B
WT
Gli2ΔN2
10.7
29.0
20.1
8.47
22.8
42.2
CD4
20.8
14.8
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8.35
2.93
30.3
CD8
CD44
CD62L

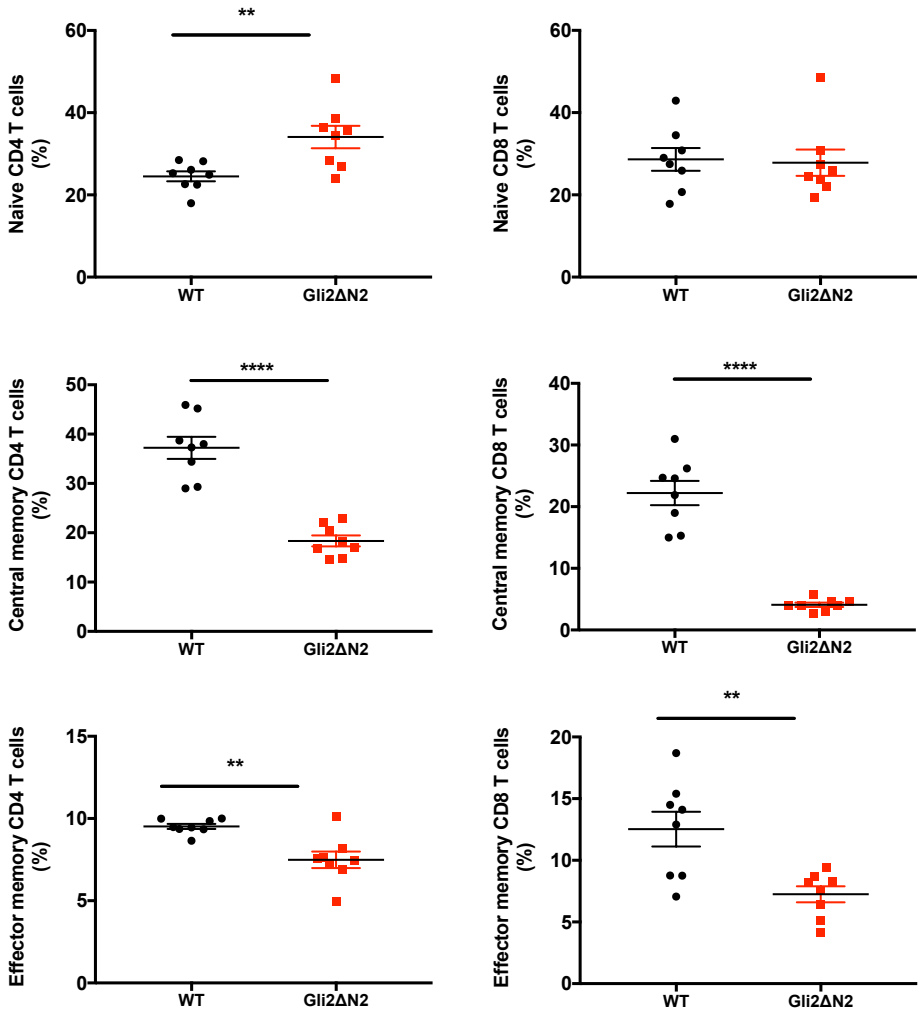


Figure 6-5: Gli2-mediated transcription in T cells affects peripheral T cells and their subtypes upon induction of dermatitis-like skin inflammation

(A) Representative FACS plots of dLNs from WT (Top) and Gli2ΔN2 (Bottom) treated mice and percentages of $CD4^+$ and $CD8^+$ T cells from WT and Gli2ΔN2 dLNs.

(B) Representative FACS plots of naïve and memory staining on $CD4^+$ T cells (Top) and $CD8^+$ T cells (Bottom) from WT (Left) and Gli2ΔN2 (Right) treated dLNs. Percentages of naïve and memory $CD4^+$ T cells and $CD8^+$ T cells from WT (Black circles) and Gli2ΔN2 (Red squares) dLNs.

Each symbol in the graphs represents an individual mouse and the group mean is represented with a bar. Data were generated from two independent experiments with at least 4 mice per group. **$p<0.01$.

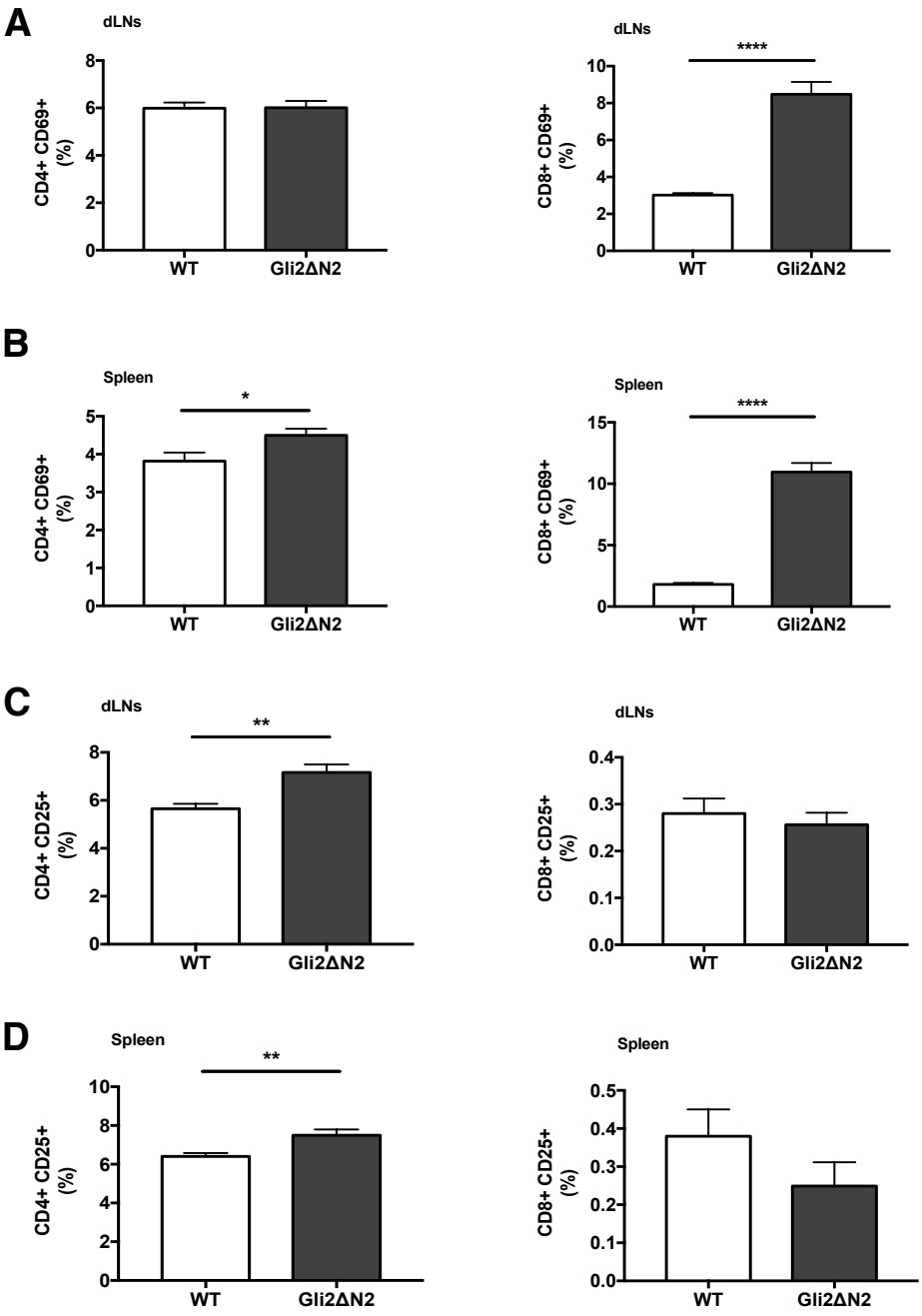


Figure 6-6: Constitutive Gli2 expression in T cells affects the activation status of peripheral T cells.

Bar charts show **(A, B)** early and **(C, D)** late activation of $CD4^+$ T cells and $CD8^+$ T cells in dLNs and spleen from Oxa-treated WT (White bars) and Gli2ΔN2 (Grey bars) mice. Data were generated from two independent experiments with at least 4 mice per group. *p<0.05, **p<0.01 and ****p<0.0001.

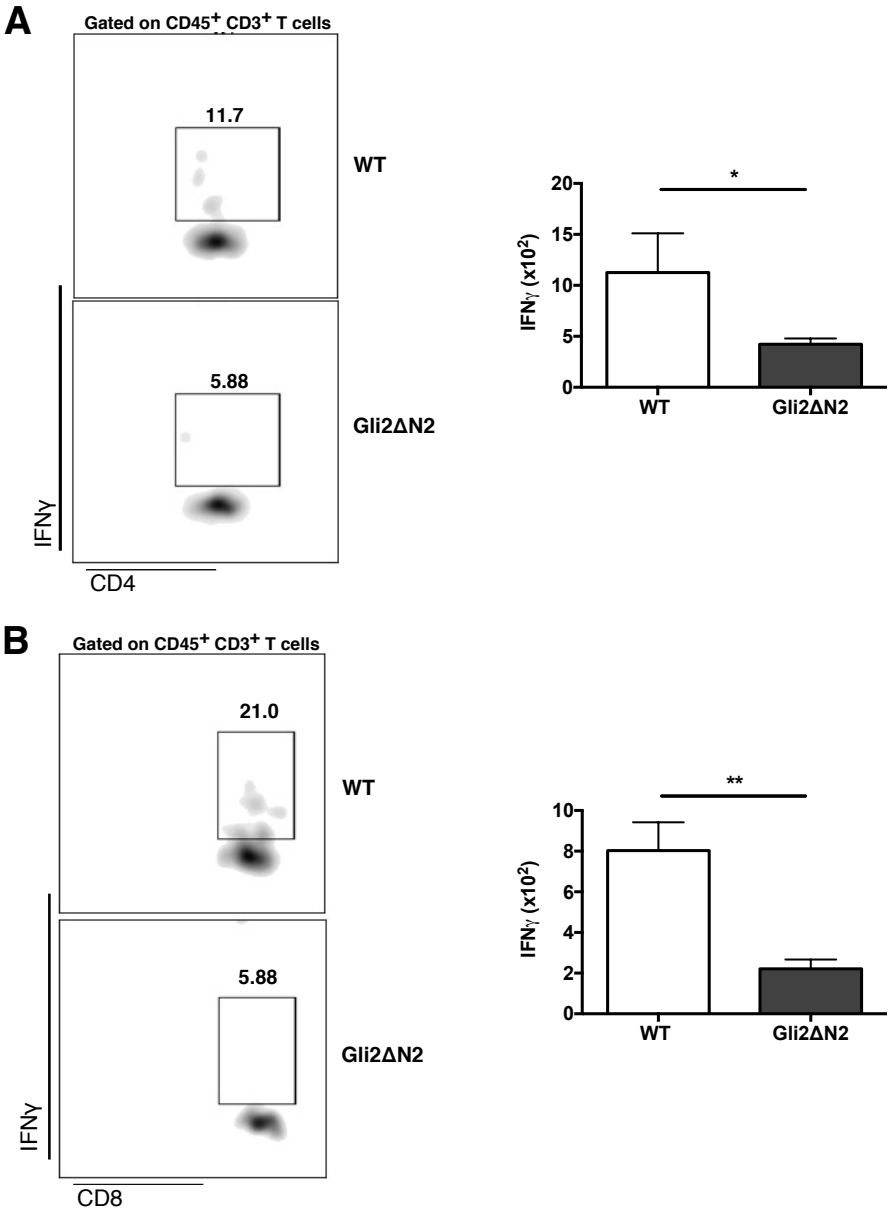


Figure 6-7: Impaired production of IFN-γ in skin T cells in Oxa-treated Gli2ΔN2 mice

Representative density plots of **(A)** IFN-γ producing $CD4^+$ T cells and **(B)** IFN-γ producing $CD8^+$ T cells from WT and Oxa-treated Gli2ΔN2 mice. Bar charts represent numbers of **(A)** IFN-γ producing $CD4^+$ T cells and **(B)** IFN-γ producing $CD8^+$ T cells from WT (White bars) and Gli2ΔN2 (Grey bars) mice. Data were generated from two independent experiments with at least 7 mice per group. Plots show mean ± SEM. *p<0.05 and **p<0.01.

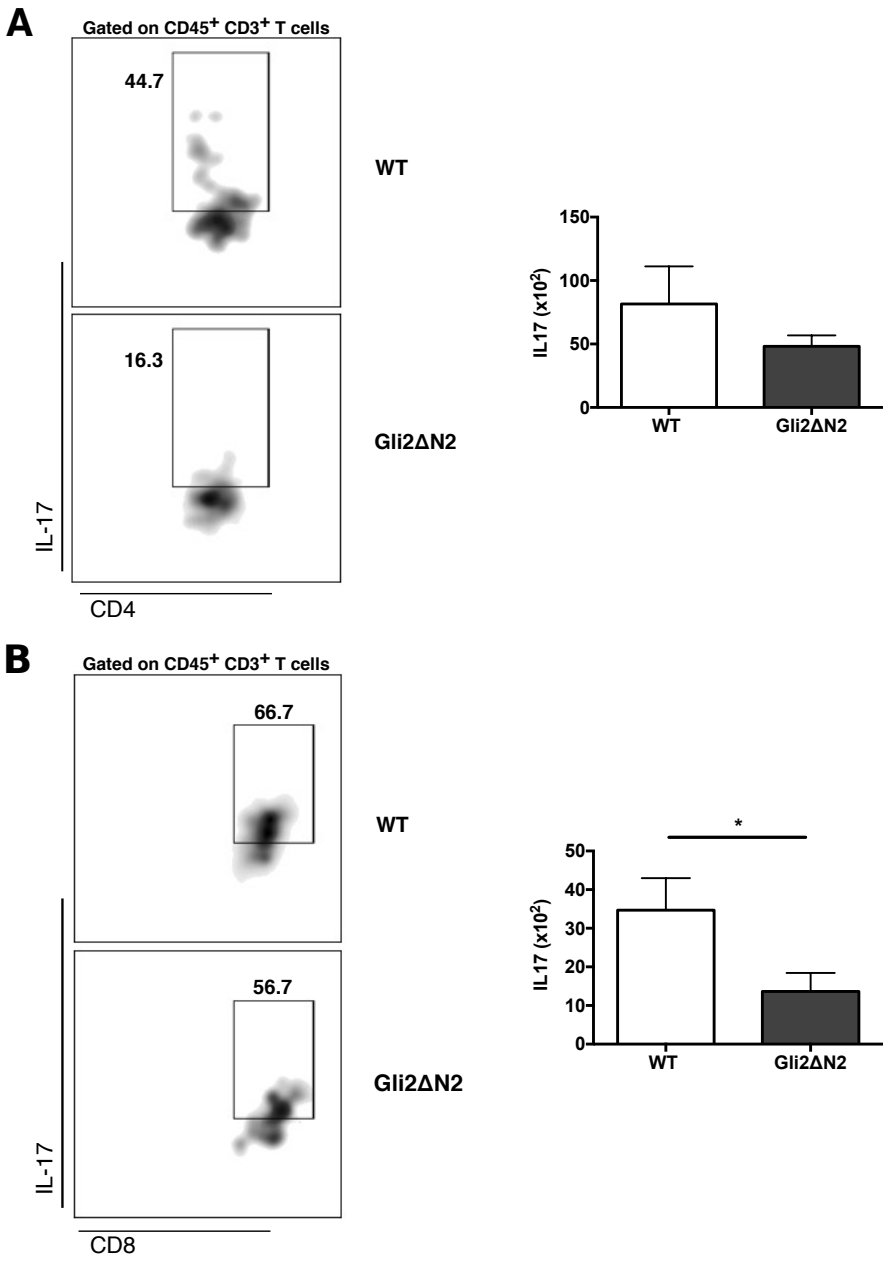


Figure 6-8: Gli2ΔN2 skin T cells have reduced capacity to produce IL-17 on induction of atopic dermatitis

Representative density plots of **(A)** IL-17 producing $CD4^+$ T cells and **(B)** IL-17 producing $CD8^+$ T cells from WT and Gli2ΔN2 treated mice. Bar charts represent numbers of **(A)** IL-17 producing $CD4^+$ T cells and **(B)** IL-17 producing $CD8^+$ T cells from Oxa-treated WT (White bars) and Gli2ΔN2 (Grey bars) mice. Data were generated from two independent experiments with at least 7 mice per group. Plots show mean ± SEM. *$p<0.05$.

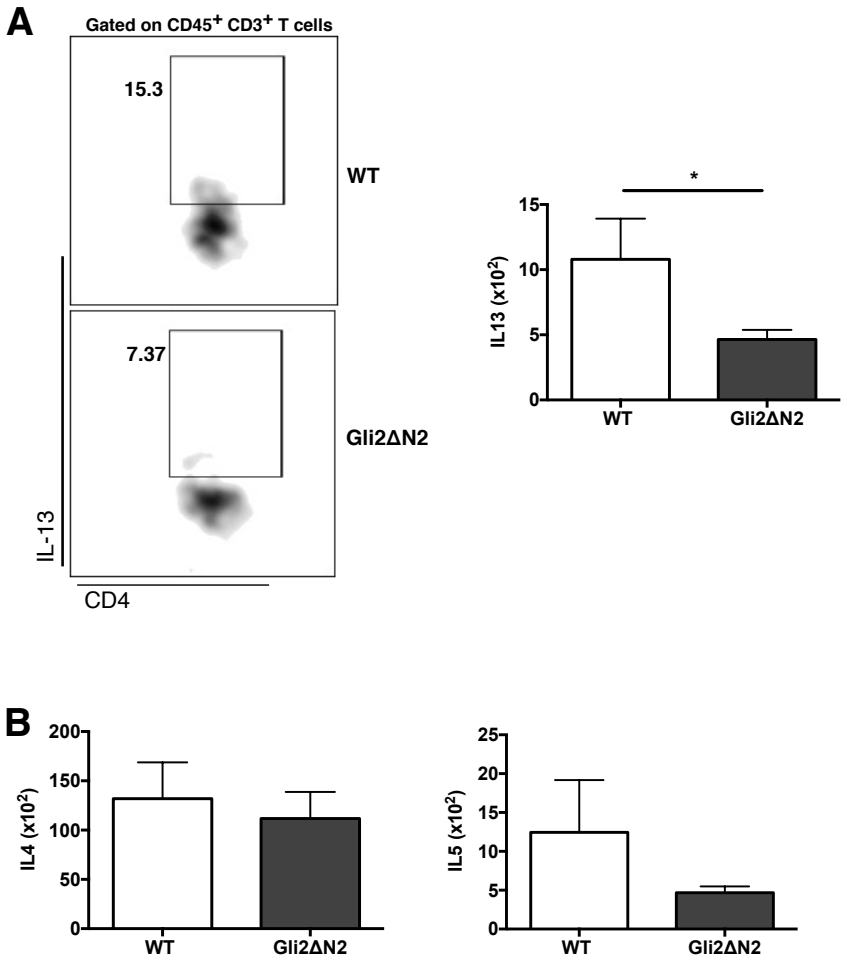


Figure 6-9: Conditional Gli2-mediated transcription in T cells, reduces IL-13- producing $CD4^+$ T cells in skin of Oxa-treated mice

(A) Representative density plots of IL-13-producing $CD4^+$ T cells from Oxa-treated WT (Top) and Gli2ΔN2 (Bottom) treated mice.

Bar charts represent numbers of **(A)** IL-13-, **(B)** IL-4- and IL-5-producing skin $CD4^+$ T cells from Oxa-treated WT (White bars) and Gli2ΔN2 (Grey bars) mice. Data were generated from two independent experiments with at least 7 mice per group. Plots show mean ± SEM. *$p<0.05$.

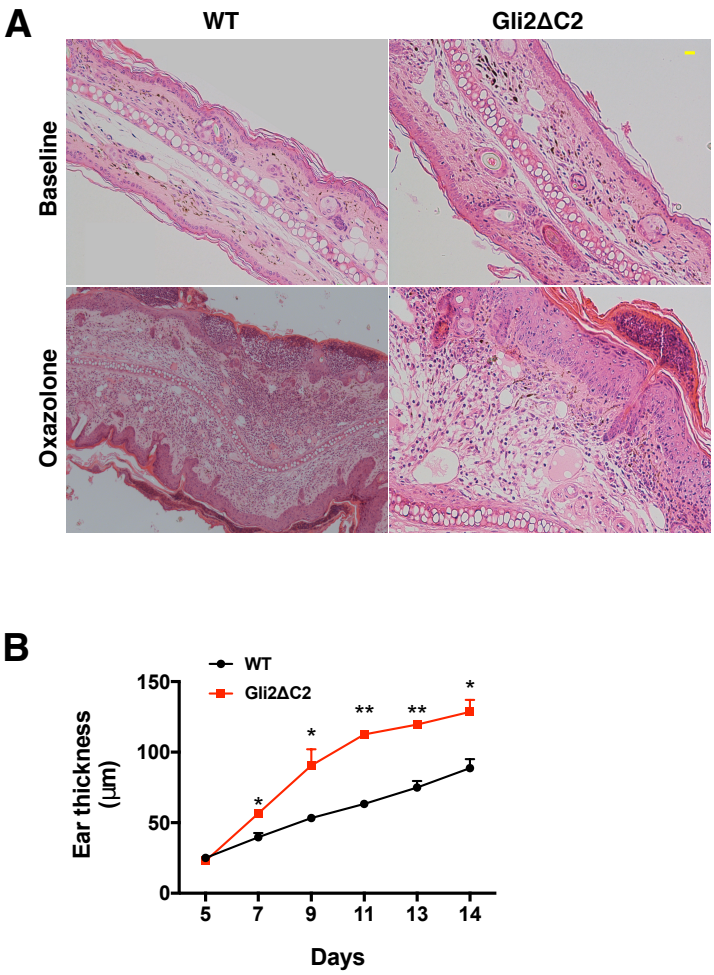


Figure 6-10: Skin structure is deteriorated upon induction of chronic atopic dermatitis in Gli2ΔC2 mice

(A) Representative H&E images of skin sections from untreated (Baseline) and Oxa-treated WT (Left) and Gli2ΔC2 (Right) mice. Scale bar represents 100μm.

(B) Time course of ear thickness from Oxa-treated WT (Black line) and Gli2ΔC2 (Red line) mice.

Data were generated from two independent experiments with 4 mice per group. *p<0.05 and **p<0.01.

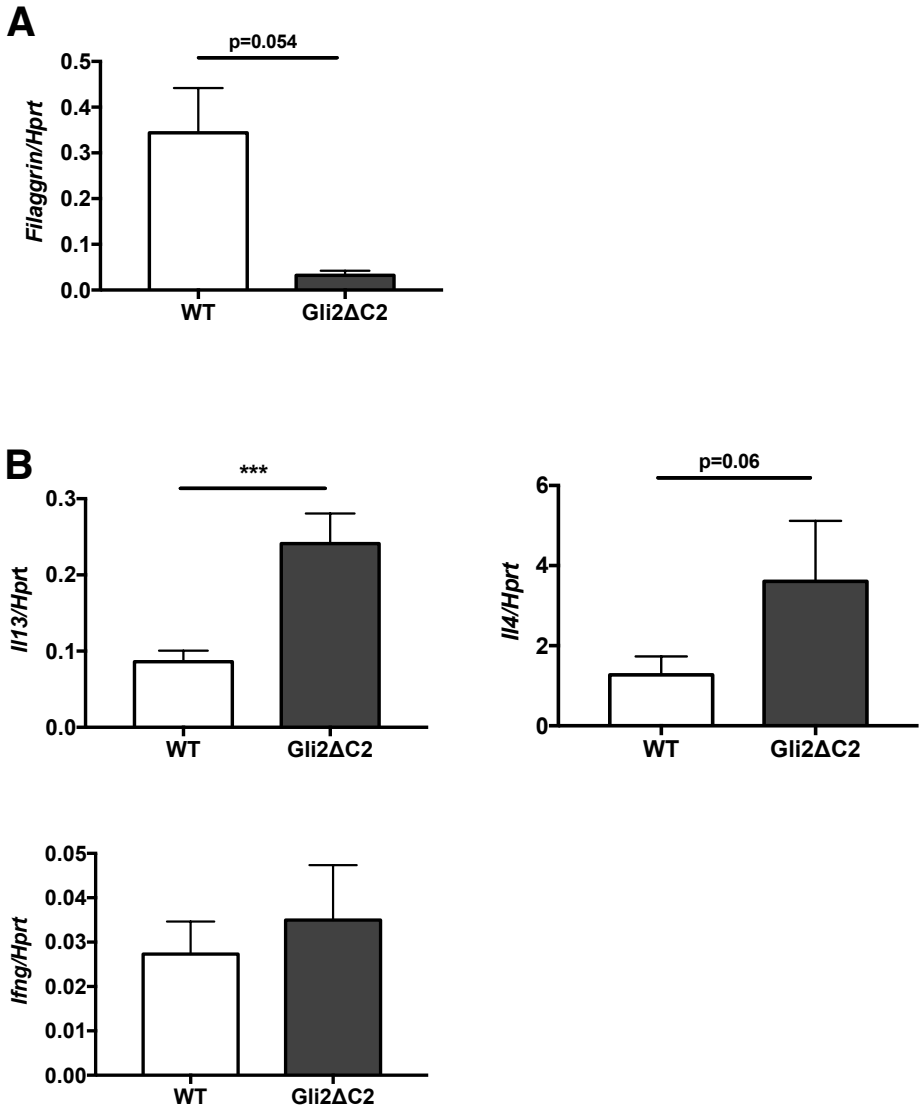


Figure 6-11: Impaired barrier function and increased cytokine expression in skin of Gli2ΔC2 mice on induction of atopic dermatitis

Bar charts show **(A)** *Filaggrin*, **(B)** *Il13*, *Il4* and *Ifng* transcript expression in skin homogenates from Oxa-treated WT (White bars) and Gli2ΔC2 (Grey bars) mice as determined by qPCR. Data were generated from two independent experiments from 3-7 mice per group. Plots show mean ± SEM. ***p<0.001.

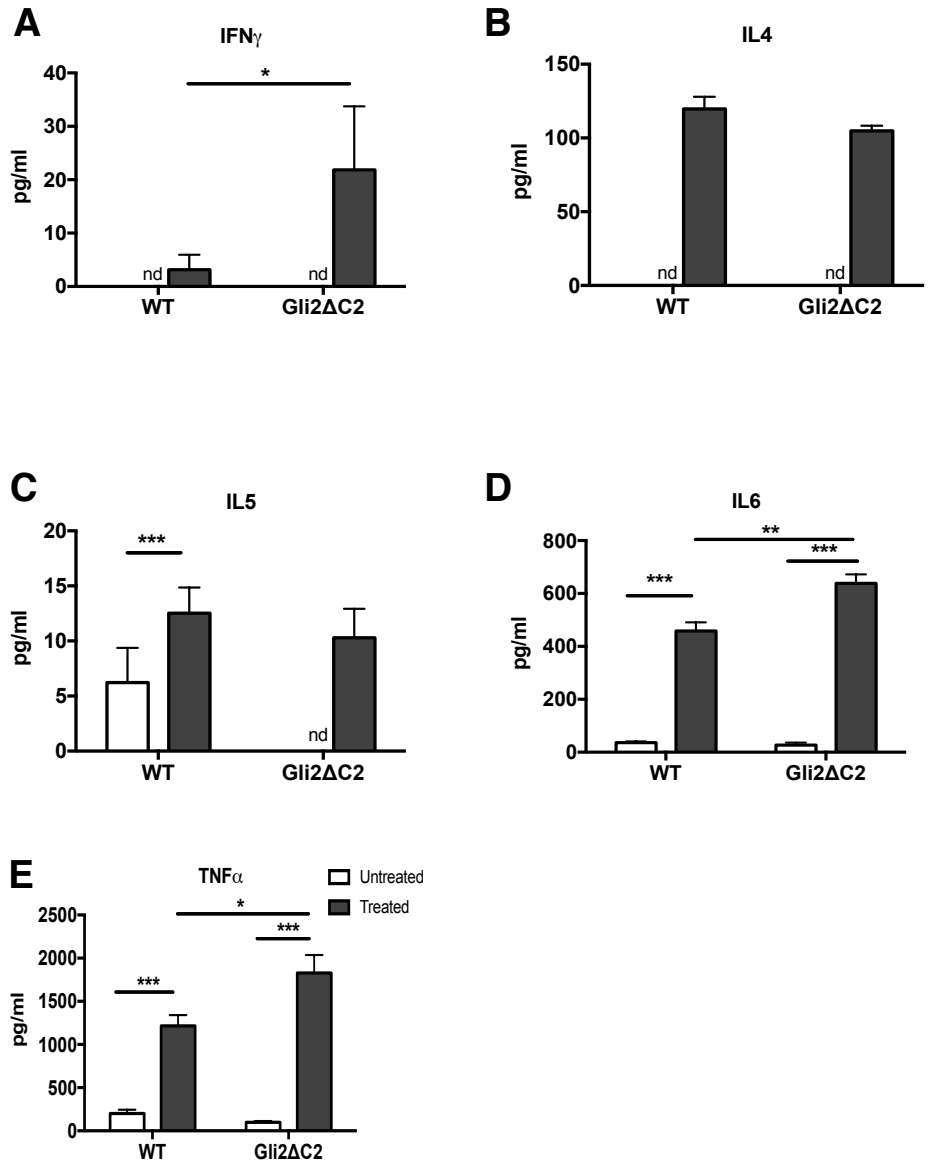


Figure 6-12: Inhibition of Gli2-mediated Hh signalling in T cells leads to increased secretion of pro-inflammatory cytokines in skin supernatants

Measurement of **(A)** IFN-γ, **(B)** IL-4, **(C)** IL-5, **(D)** IL-6 and **(E)** TNF-α cytokine concentrations in skin supernatants from untreated (White bars) and Oxa-treated (Grey bars) WT and Gli2ΔC2 mice. Data were generated from two independent experiments with at least 3 mice per group. Plots show mean ± SEM. 2way ANOVA statistical test was performed. n.d.: not detected, *$p<0.05$, **$p<0.01$ and ***$p<0.001$.

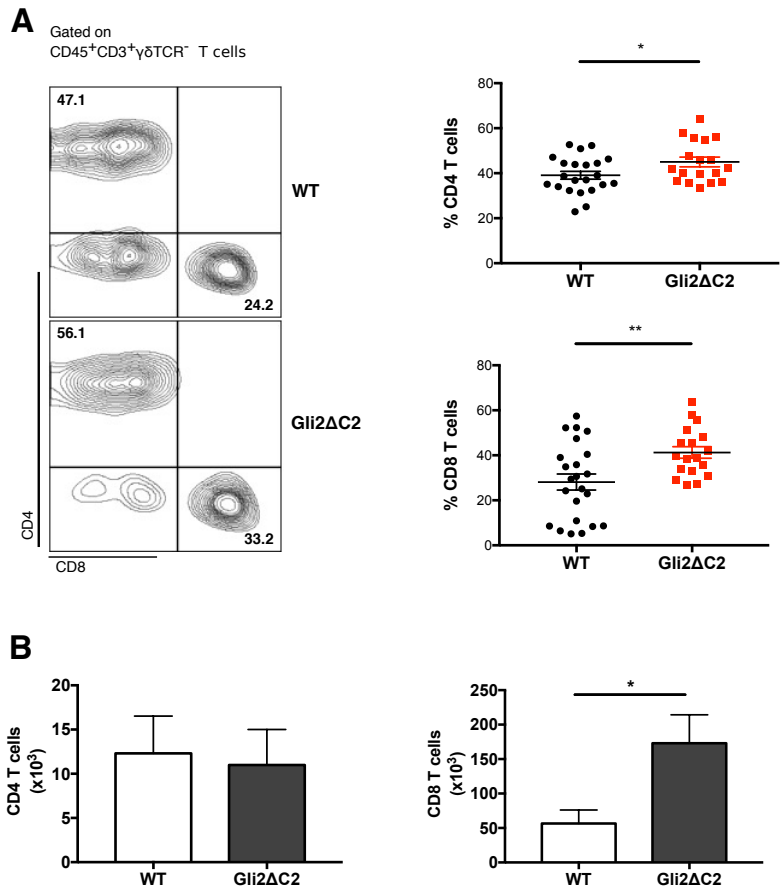
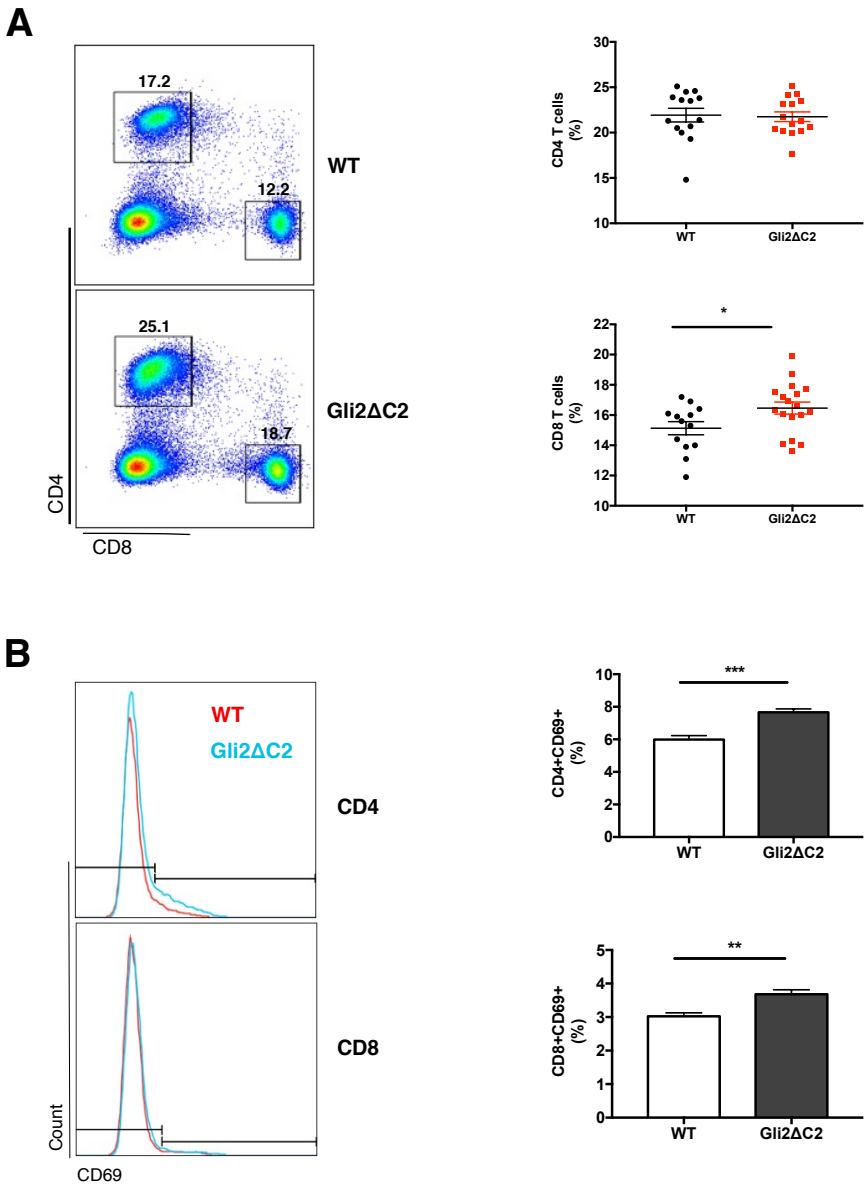


Figure 6-13: Increased T cell skin infiltration upon induction of atopic dermatitis in Gli2ΔC2 mice

(A) Representative contour plots of skin $CD4^+$ T cells and $CD8^+$ T cells from WT (Top) and Gli2ΔC2 (Bottom) treated mice. Scatter plots show percentages of Oxa-treated skin $CD4^+$ and $CD8^+$ T cells from WT (black circles) and Gli2ΔC2 (red squares) mice. Each symbol in the graph represents an individual mouse and the group mean is represented with a bar.

(B) Graphs show numbers of Oxa-treated skin $CD4^+$ and $CD8^+$ T cells from WT (White) and Gli2ΔC2 (Grey) mice. Plots shown mean ±SEM.

Data were generated from two independent experiments with at least 6 mice per group. *p<0.05 and **p<0.01.


A
17.2
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WT
25.1
18.7
Gli2ΔC2
CD4
CD8
CD4 T cells (%)
CD8 T cells (%)
WT
Gli2ΔC2
*
B
WT
Gli2ΔC2
CD4
CD8
Count
CD69
CD4+CD69+ (%)
CD8+CD69+ (%)
***
**

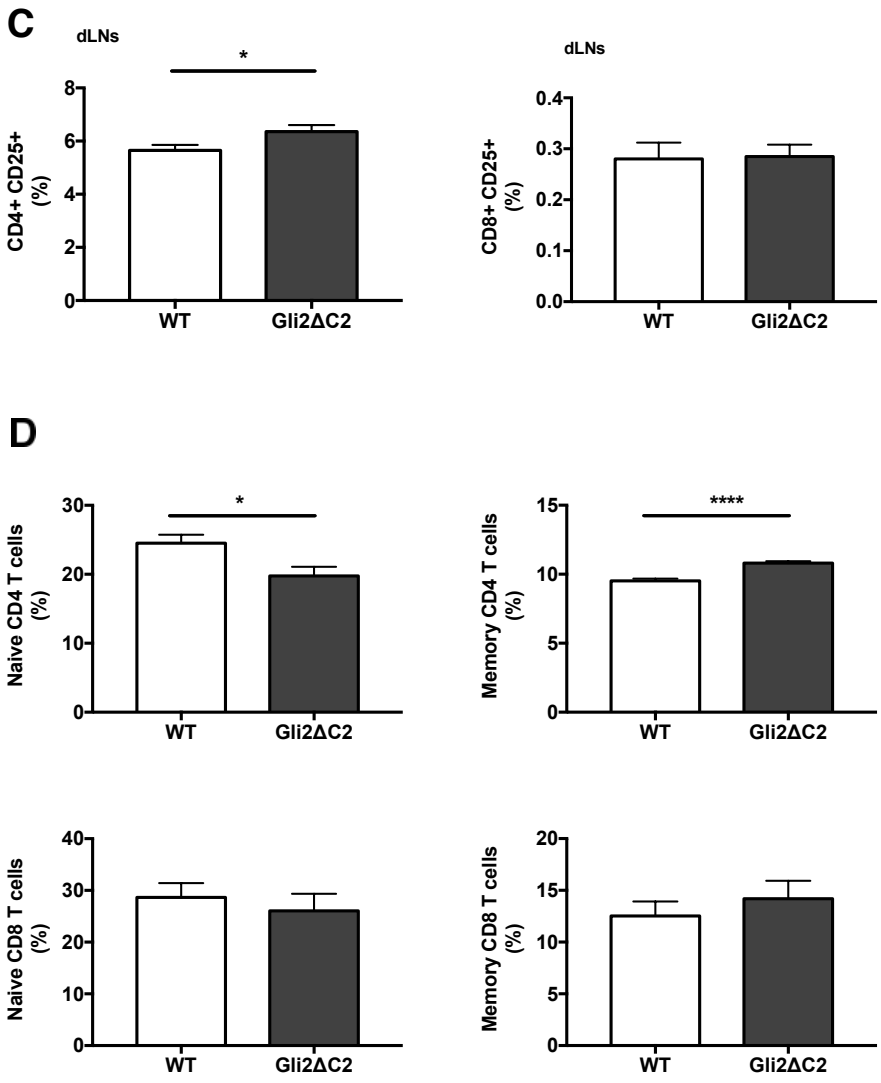


Figure 6-14: Constitutive inhibition of Gli2-mediated transcription affects the number and activation state of peripherally-induced T cells upon dermatitis induction

(A) Representative FACS plots of $CD4^+$ T cells and $CD8^+$ T cells from WT (Top) and Gli2ΔC2 (Bottom) dLNs. Scatter plots show percentages of $CD4^+$ and $CD8^+$ T cells from Oxa-treated skin $CD4^+$ and $CD8^+$ T cells from WT (black circles) and Gli2ΔC2 (red squares) mice. Each symbol in the graph represents an individual mouse and the group mean is represented with a bar.

(B) Representative histograms of $CD4^+CD69^+$ T cells (Top) and $CD8^+CD69^+$ T cells (Bottom) from dLNs. Red line represents WT and blue line Gli2ΔC2 treated mice.

Bar charts show percentages of $CD4^+$ and $CD8^+$ T cell **(B)** early activation, **(C)** late activation, **(D)** naïve and memory $CD4^+$ T cells and $CD8^+$ T cells from WT (White bars) and Gli2ΔC2 (Grey bars) treated dLNs. Plots show mean ± SEM.

Data were generated from two independent experiments with at least 6 mice per group. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

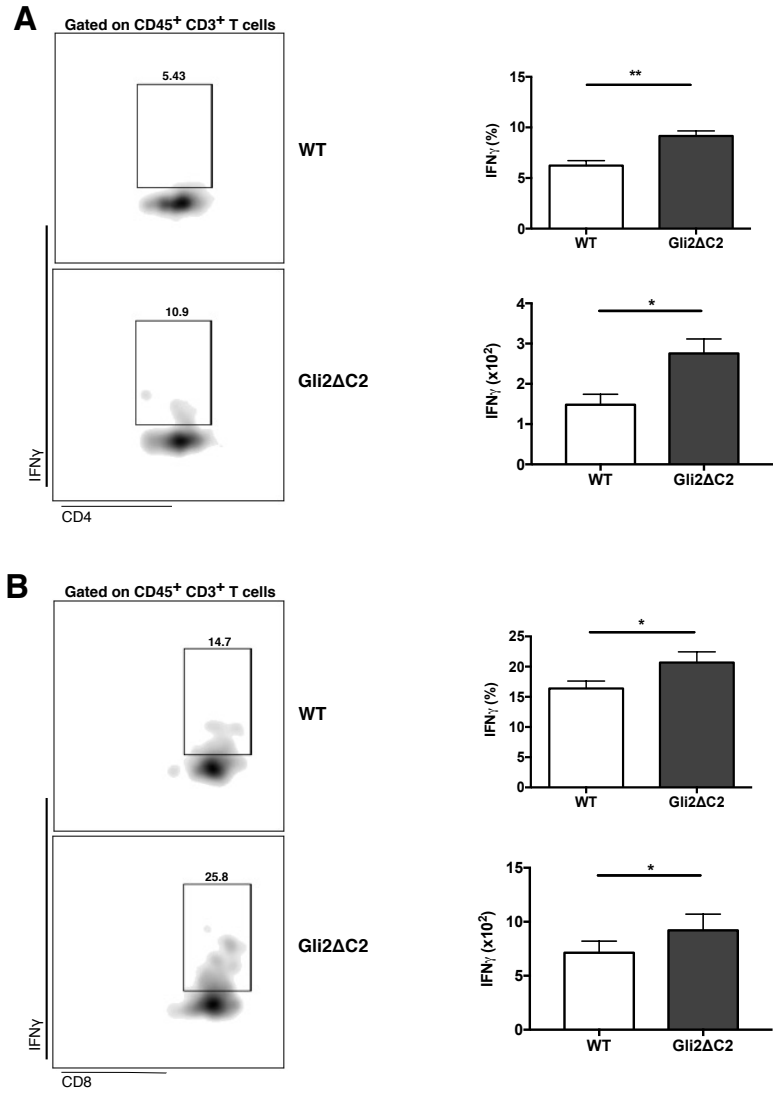


Figure 6-15: Elevated IFN-γ production by skin T cells when Gli2-mediated Hh transcription is inhibited on unduction of atopic dermatitis

Representative density plots of **(A)** IFN-γ producing $CD4^+$ T cells and **(B)** IFN-γ producing $CD8^+$ T cells from WT and Gli2ΔC2 treated mice. Bar charts represent percentages and numbers of **(A)** IFN-γ producing $CD4^+$ T cells and **(B)** IFN-γ producing $CD8^+$ T cells from WT (White bars) and Gli2ΔC2 (Grey bars) treated mice. Data were generated from two independent experiments with at least 7 mice per group. Plots show mean ± SEM. *p<0.05 and **p<0.01.

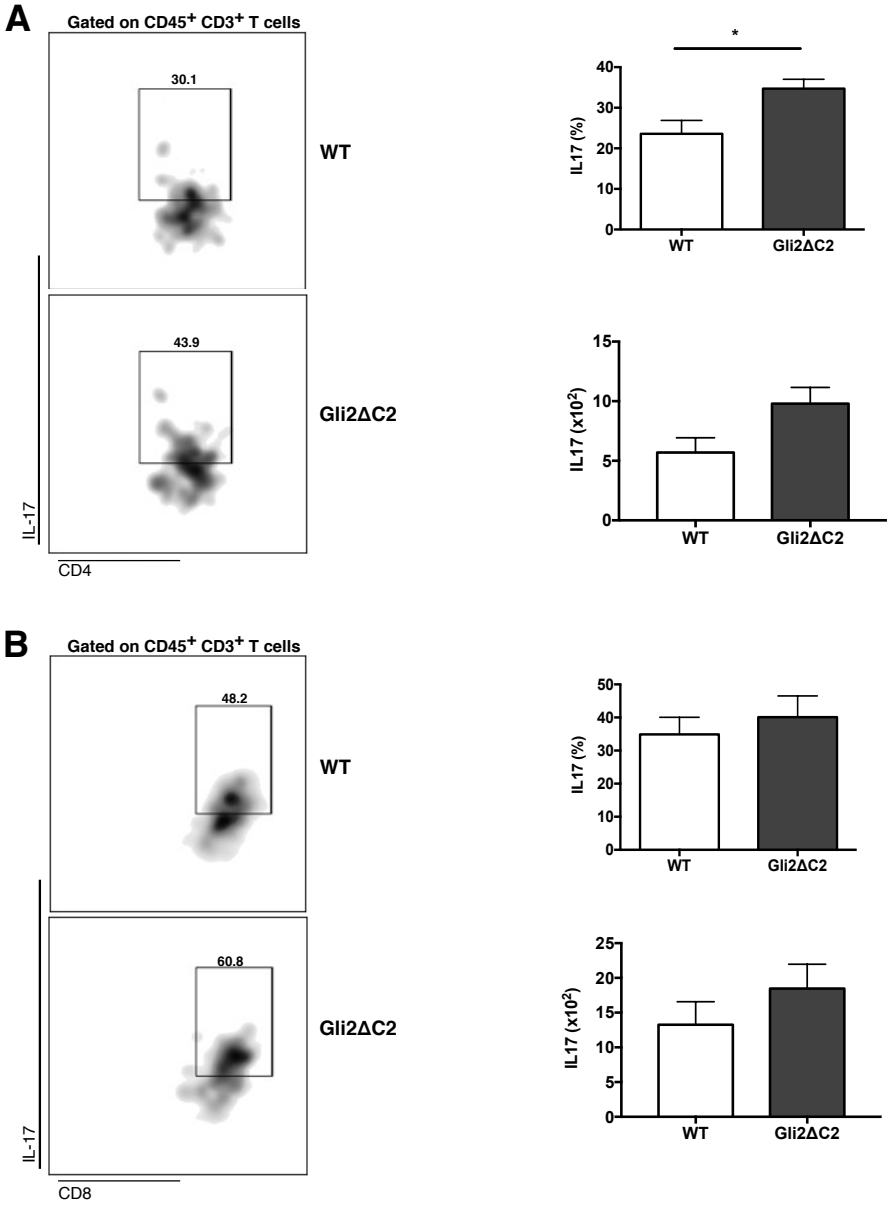


Figure 6-16: IL-17 production by skin T cells is a consequence of Gli2-mediated inhibition of Hh signalling in T cells

Representative density plots of **(A)** IL-17 producing $CD4^+$ T cells and **(B)** IL-17 producing $CD8^+$ T cells from Oxa-treated WT and Gli2ΔC2 mice. Bar charts represent percentages and numbers of **(A)** IL-17 producing $CD4^+$ T cells and **(B)** IL-17 producing $CD8^+$ T cells from WT (White bars) and Gli2ΔC2 (Grey bars) mice. Data were generated from two independent experiments with at least 7 mice per group. Plots show mean ± SEM. *p<0.05.

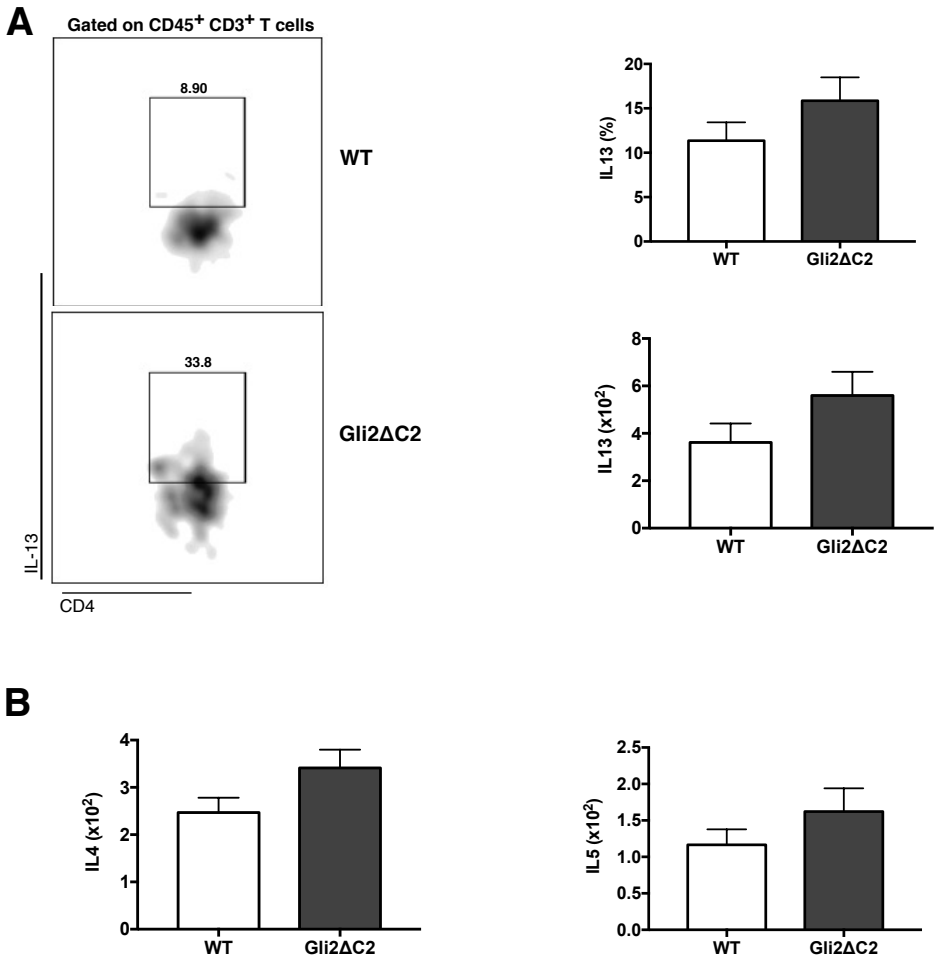


Figure 6-17: Constitutive inhibition of Gli2-mediated transcription in T cells does not affect the capacity of skin CD4$^+$ T cells to produce Th2 cytokines after induction of atopic dermatitis

(A) Representative density plots of IL-13-producing CD4$^+$ T cells from WT (Top) and Gli2ΔC2 (Bottom) treated mice. Bar charts represent percentages and numbers of IL-13-producing skin CD4$^+$ T cells from WT (White bars) and Gli2ΔC2 (Grey bars) treated mice.

(B) Bar charts show numbers of IL-4- and IL-5-producing skin CD4$^+$ T cells from WT and Gli2ΔC2.

Data were generated from two independent experiments with at least 7 mice per group. Plots show mean ± SEM.

6.4 Discussion

In this chapter I have shown that the Gli2 transcription factor specifically expressed in T cells under conditions of skin inflammation led to a reduction in inflammation, showing that T cell autonomous Hh-mediated transcription can modulate skin inflammation. The data are supported by skin histology in Oxa-treated Gli2ΔN2 mice which revealed ameliorated clinical image of inflamed skin, characterised by reduced ear thickening and improved skin structure, as confirmed by increased expression of the terminal differentiation marker *Filaggrin* and decline in Th1 and Th2 cytokine transcript expression. When cytokine secretion was examined in the skin supernatants, diminished secretion of pro-inflammatory cytokines was detected and skin $CD4^+$ and $CD8^+$ T cells were characterised by impaired Th1, Th2 and Th17 production. In contrast, inhibition of Gli2-mediated transcription in T cells exaggerated the pathology of dermatitis in skin of Gli2ΔC2 mice, causing worsening of features of chronic atopic dermatitis. Skin of Oxa-treated Gli2ΔC2 mice showed severe hyperkeratosis and parakeratosis, elevated pro-inflammatory expression in skin supernatants and increased IFN-γ production by skin T cells. However, none of the untreated transgenic mice developed signs of spontaneous dermatitis, indicating that inhibition of Gli2 is not sufficient on its own to provoke skin barrier defects.

Aberrant Gli2 activation is reported to be involved in many cancers, as described previously. Studies have shown that constitutive Gli2 expression under the keratin-5 promoter is linked with skin cancers in mice (Sheng et al., 2002), and basal cell carcinoma-like abnormalities in pigs (McCalla-

Martin et al., 2010) but it is important to note that we have not observed T cell cancer development in any of the Gli2ΔN2 mice.

Examination of $CD4^+$ T cells from dLNs showed reduced T cell levels in Gli2ΔN2 mice with more naïve and less memory cells, indicative of reduced inflammation, while in Gli2ΔC2 mice I found the opposite phenotypes for these cells. These data are different from a recent study published by our group, showing that although overexpression of Gli2A in T cells increased percentages of naïve T cells, the transcription factor did not affect the proportions of memory and effector cells (Furmanski et al., 2015). The experiments were performed in mice under steady state conditions and not under conditions of inflammation, as is the case for the data presented here. Furthermore, early activation of $CD4^+$ T cells was upregulated in both Gli2ΔN2 and Gli2ΔC2 mice compared to the WT controls, which for Gli2ΔN2 mice, again is in contrast to the same previous study (Furmanski et al., 2015), where Gli2-mediated Hh expression in T cells reduced early $CD4^+$ T cell activation. However, the CD69 marker that I used as a marker for early activation, has been shown to be present in $Foxp3^+$ T_{regs} and is indispensable for T_{reg} suppression function and protection against development of inflammation (Cortes et al., 2014). Therefore, the increased $CD4^+CD69^+$ proportion in the Gli2ΔN2 mice compared to WT littermates could be attributed to T_{reg} population, since the populations is identified as $CD4^+CD25^+Foxp3^+$ subset.

Regarding $CD8^+$ T cells I have shown that overexpression of Gli2A led to reduced proportions of memory phenotype cells, non-altered naïve percentages, and increased early activation marker expression in

secondary lymphoid tissues, whereas conditional Gli2-inhibition increased overall $CD8^+$ T cell percentage and their early activation, consistent with a previous study where Gli2-mediated Hh signalling inhibition increased $CD8^+$ T cell activation and proliferation (Rowbotham et al., 2008).

Published data has evaluated the role of Hh signalling in CD8 thymocyte development (Hager-Theodorides et al., 2009, Furmanski et al., 2012, Rowbotham et al., 2007). Our experiments showed that Gli2-mediated inhibition of Hh signalling increased the capability of $CD8^+$ T cells to produce IFN-γ. This finding is of importance given that Hh ligands are expressed in many tumours and that $CD8^+$ T cell cytotoxicity is important for the anti-tumour response. By inhibiting Gli2 activity in T cells, we could increase cytotoxicity of $CD8^+$ T cells, and that could be used as a beneficial tool to achieve tumour regression. Since higher levels of IFN-γ-producing $CD8^+$ T cells were observed in my experiments, it would be interesting in the future to test how the Gli2ΔC2 mice modulate the tumour microenvironment and by focusing on $CD8^+$ T cell populations evaluate if they are more potent in induction of tumour regression.

My findings show that Gli2-mediated activation of the Hh signalling pathway rescues skin against the inflammation of atopic dermatitis. Interestingly, recent studies using asthmatic models have showed that Hh signalling is upregulated in asthmatic lungs and promotes Th2 differentiation via IL-4 and Gata3 upregulation (Furmanski et al., 2013, Standing et al., 2017). These differences highlight the possibility that the Hh pathway has different mechanisms of action, dependent on the tissue context where the pathway is expressed.

I have shown that Gli2-mediated Hh modulation affects pro-inflammatory cytokine production in the skin. It would be interesting to examine anti-inflammatory cytokines such as IL-10, and test the hypothesis that in Gli2ΔN2 mice, where we observed decreased levels of pro-inflammatory cytokines, IL-10 will be higher whereas in Gli2ΔC2 mice there will be the opposite outcome. T_{reg} populations are the major source of IL-10 production, therefore it would be also of interest to evaluate this population in skin of the transgenic and WT mice. As shown in Figure **6-4 A** I found increased percentages skin $CD4^+$ T cell populations while in figures **6-6 C** and **6-6 D** increased proportions of $CD4^+CD25^+$ T cells in dLNs and spleen are present in Gli2ΔN2 mice, although there is less inflammation in these mice. The increased percentages in skin $CD4^+$ T cells could be due to T_{reg} populations, while the increased CD25 expression observed in the lymphoid tissues could be again because T_{reg} cells are included in the $CD4^+CD25^+$ subset. In order to elucidate these observations, I need to do further experiments on skin and peripheral T_{reg} populations and examine if Hh signalling pathway has an impact on them.

Finally, the transcriptional mechanisms behind the elevated inflammation in Gli2ΔC2 mice and impaired inflammation in Gli2ΔN2 mice, should be tested. In the following chapter, I present my findings regarding the transcriptional changes in these mice and I propose a possible mechanism via which Gli2-mediated Hh modulation could control dermatitis-like skin inflammation.

Chapter 7

Chapter 7: The transcriptional mechanisms that control Oxazolone-induced skin inflammation

7.1 Introduction

7.1.1 Transcriptional mechanisms implicated in regulatory T cells

Immune regulation is essential for tolerance against harmless and self-antigens. T_{regs} are important mediators of tissue homeostasis and modulate excessive inflammatory responses.

Naturally occurring regulatory T cells are $CD4^+$ T cells, which constantly express the IL-2ra chain CD25 and the Foxp3 transcription factor. Foxp3 deficiency has been linked to immune dysregulation, polyendocrinopathy, enteropathy and X-linked syndrome (Bennett et al., 2001, Fontenot et al., 2003, Hori et al., 2003, Sakaguchi et al., 1995). The suppressive activity of T_{regs} depends on demethylation of Foxp3 DNA and its expression is regulated by a series of transcription factors including the Foxo, Smad, AP1, cREL and Stat5 (Lee and Lee, 2018).

Certain activation markers such as Klrg1, and effector molecules such as Ctla4 and Il10, give the T_{regs} their activated phenotype which is responsible for their immunosuppressive phenotype (Cheng et al., 2012, Feuerer et al., 2010, Rubtsov et al., 2008, Tang and Bluestone, 2008). TGF-β cytokine, which is vital for generation and maintenance of T_{reg} populations, exists in three isoforms, (TGF-β1, TGF-β2, TGF-β3) and TGF-β1 is the most common (Dardalhon et al., 2008a, Dardalhon et al., 2008b, Konkel et al., 2014, Liu et al., 2008, Roberts et al., 1981) and restrains immune activation as it is one of the dominant mechanisms of immune suppressive function

of T_{reg} populations (Frimpong-Boateng et al., 2010, Konkel et al., 2017, Mempel et al., 2006, Shen et al., 2011, Takimoto et al., 2010).

Initiation of TGF-β pathway requires binding of TGF-β to its high-affinity receptor, TGF-βRII, which will phosphorylate a second receptor TGF-βRI (Heldin et al., 1997). The receptors then activate Smad2 and Smad3 proteins by phosphorylation allowing them to create a complex with Smad4 protein. Upon its translocation to the nucleus the whole complex regulates transcription (Huse et al., 2001, Wahl, 2007). However, in order to be able to deliver its suppressive effect, and before its binding to its receptor, TGF-β must first acquire its active form (Tran, 2012). The premature form of TGF-β is the pre-pro-TGF-β which is formed of the latency associated peptide (LAP) and the mature TGF-β. This form is unable to bind to its receptor, as LAP surrounds TGF-β, forming the latent TGF-β (Tran, 2012). Latent TGF-β exists in three forms: the small latent form where LAP is linked to TGF-β, the large latent form where the small latent form is bound to latent TGF-β binding protein and the membrane latent form, where the small latent form is linked with glycoprotein A (Stockis et al., 2009, Tran et al., 2009a, Tran et al., 2009b). Conformational changes of LAP upon post-transcriptional modifications, allow it to expose TGF-β and become active TGF-β (Annes et al., 2003, Lawrence, 2001, Li and Flavell, 2008).

TGF-β in low concentrations favours the Th17 axis, however when highly expressed, it promotes T_{reg} differentiation (Zhou et al., 2008). Skin is abundant in TGF-β, which mediates CD103 expression on T cells, a cytokine which helps T_{reg} migration to non-lymphoid tissues and localisation

at the sites of inflammation, upon interaction with keratinocytes (Huehn et al., 2004, Suffia et al., 2005, Watanabe et al., 2015).

Although the role of developmental pathways in T_{reg} development and function is not widely studied, recently it has been demonstrated that peripheral T_{reg} function is controlled by intrinsic Notch signalling, where activation of canonical Notch signalling impaired T_{reg} function, increased their apoptosis and enabled Th1 and Tc1 cell responses having a detrimental role in the context of inflammation (Charbonnier et al., 2015). In addition, Ptgs2, which is a target gene of Hh signalling orchestrates Shh-dependent T_{reg} expansion upon *Mycobacteria* infection (Holla et al., 2016) and Wnt signalling reduces T_{reg} suppressive function in arthritis patients and in mice upon induction of an arthritis model via upregulation of Wnt3a signalling component (van Loosdregt et al., 2013).

7.1.2 *Regulatory T cells in skin homeostasis and atopic dermatitis*

As discussed in detail in the introduction, skin resident T_{regs} characterised as $CD4^+CD25^+Foxp3^+$, are abundant in skin dermis (Heath and Carbone, 2013, Panduro et al., 2016). Activation of skin resident T_{regs} is of importance for the maintenance of skin homeostasis and their presence in skin under skin inflammatory conditions is controversial. Some studies show that their numbers are increased in AD skin (Caproni et al., 2006, Schnopp et al., 2007), others suggest that the population is not altered in AD skin lesions (Malhotra et al., 2018, Szegedi et al., 2009) and finally some findings show a decrease in skin T_{reg} numbers which contributes to exacerbation of skin inflammatory disorders (Verhagen et al., 2006).

Recent findings investigating the role of T_{regs} in skin of atopic dermatitis lesions have shown that the population loses its immunosuppressive functions in AD lesions and that these cells are directly controlled by signals sent from keratinocytes (Cardona et al., 2006, Kashiwagi et al., 2017, Sanchez Rodriguez et al., 2014, Verhagen et al., 2006). However, when T_{regs} acquire an activated phenotype they are potent inhibitors of dermatitis and skin inflammation (Kashiwagi et al., 2017, Malhotra et al., 2018).

7.2 Aim

The transcriptional mechanisms that control T_{reg} development and function have been widely studied. However, the relationship between Hedgehog signalling and T_{reg} function remains elusive. In this chapter I will evaluate the transcriptional changes that take place in skin $CD4^+$ T cells of Oxa-treated Gli2ΔN2 and Gli2ΔC2 transgenic mice. First, I will FACS sort skin $CD4^+$ T cell populations and I will use RNA-sequencing (RNA-seq) in order to analyse the genome wide transcriptional changes. Furthermore, I will examine if overexpression or inhibition of Gli2 in skin T cells correlates with differences in T_{reg} populations and if it affects their ability to regulate the induction of Oxa-induced skin inflammation. I will do *in vitro* experiments in order to identify if key genes uncovered in the RNA-sequencing analysis, play a role in regulation of Oxa-induced dermatitis. Finally, I will propose a mechanism through which the Gli2 transcription factor in skin T cells, regulates dermatitis-like skin inflammation.

7.3 Results

7.3.1 *Gli2-inhibition in skin CD4$^+$ T cells favours the expression of genes related to inflammation and migration compared to WT samples*

In order to investigate the molecular mechanisms of the modulation of skin inflammation by Gli2-mediated Hh pathway activation, first I sorted skin CD4$^+$ T cells from Oxa-treated ear skin. In order to do that I followed the gating strategy that is described in Figure **3.5** in Chapter 3 and skin CD4$^+$ T cells were defined as negatively stained for the viability dye and CD45$^+$CD3$^+$γδTCR$^-$CD4$^+$CD8$^-$. The limitation of sorting skin CD4$^+$ T cell populations was that the number of skin CD4$^+$ T cells that I managed to get from one mice was very low. Therefore, I treated both mouse ears with Oxazolone, and I pooled three mice in one sample in order to be able to sort skin CD4$^+$ T cells. In this way I created two sample groups for WT, Gli2ΔN2 and Gli2ΔC2 mice. After sorting the populations, samples were prepared and sent for RNA-seq which was carried out by UCL genomics.

To investigate the datasets in a non-biased way, PCA analysis was performed on the six samples (2WT, 2 Gli2ΔN2 and 2 Gli2ΔC2). PCA segregated the datasets by genotype on PC1 and PC2 axes, which are the ones that contribute most to the variability of the datasets (Figure **7-1 A**). WT and Gli2ΔN2 samples were clearly separated on PC1 axis, while WT and Gli2ΔC2 were separated on PC2 axis.

I then wanted to investigate genes which contribute most to the PCA variability in WT and Gli2ΔC2 datasets as well as WT and Gli2ΔC2 groups. Therefore, I examined PC1 and PC2 axes individually. PC2 axis contributes to 20% of data variability and separated WT (blue circles) from Gli2ΔC2

(green triangles) genotypes. The analysis revealed that genes that contributed strongly to PC2 included Th1 genes like *Stat4*, *Ifgnr1*, *Tbx21*, which are strongly upregulated in Oxa-treated skin $CD4^+$ T cells from Gli2ΔC2 mice (as that is represented by negative scores in PC2 axis) compared to WT mice. In addition, *Pclb3* which is a Th2 pathway inhibitor (Ando et al., 2014) was highly expressed in skin $CD4^+$ T cells from treated WT mice. In contrast, immune regulatory genes such as *Il10ra* were downregulated in Gli2ΔC2 mice compared to WT mice (Figure **7-1 B**).

WT (blue circles) against Gli2ΔN2 (red squares) groups were plotted in PC1 axis, which accounted for 33% of variability in the datasets. The PC1 axis again reflected differences in Th1 pathway genes like *Stat4*, *Ifgnr1*, *Tbx21*, which were strongly downregulated in skin $CD4^+$ T cells from Oxa-treated Gli2ΔN2 mice (negative scores in PC1 axis) compared to the WT groups. On the other hand, differences were also highlighted in *Pclb3* and *Foxp3* genes, which were highly expressed in Gli2ΔN2 mice compared to treated $CD4^+$ T cells of the WT group. (Figure **7-1 C**).

To understand better the transcriptome wide differences in our data when Gli2-mediated transcription was inhibited in T cells, we next intersected the differentially expressed genes (DEG) identified by Ebayes statistics ($p<0.05$) with the genes that were selected in PCA analysis. 3000 genes (with lowest and highest scores) that contributed most to the PC2 axis were intersected with the 1500 most significant DEG. From the intersection, 796 genes were identified, as represented in the Venn diagram (Figure **7-2 A**). The intersected genes were subsequently clustered in a heat map, which represents the genes downregulated (green colour) and upregulated (red

colour). This analysis showed that Hh pathway genes and known targets (*Hus1*, *Stmn1*, *Kif7*, *Smo*) (Chung et al., 2010, Lau et al., 2017, Leonard et al., 2008, Pak and Segal, 2016) were downregulated in skin $CD4^+$ T cells from Oxa-treated Gli2ΔC2 samples compared to the WT group. The analysis also uncovered upregulation of inflammation (*Tnfrsf9*, *Il1a*, *Il12rb*), migration as well as migratory receptor genes (*Ccr9*, *Cxcl16*, *Ccl20*, *Ccl24*) and inhibitors of immune regulation (*Crbn*) (Min et al., 2016), which were upregulated in Gli2ΔC2 compared to WT mice (Figure **7-2 B**).

Additionally, in order to evaluate the transcriptional profile of skin $CD4^+$ T cells when Gli2-mediated transcription was increased in T cells, 3000 genes (with highest and lowest scores) that most contributed to the PC1 axis, were intersected with 2500 DEG genes (Ebayes statistics, $p<0.05$), and 1681 genes were identified by this intersection as shown in the Venn diagram in Figure **7-3 A**. These genes were clustered in a heat map, which is shown in Figure **7-3 B**. The heat map revealed Hh signalling components and target genes (*Ptch1*, *Gli2*, *Mafb*) (Lu et al., 2015) which were upregulated in Gli2ΔN2 samples compared to WT. Furthermore, immune regulatory genes (*Tgfb1*, *Il10*, *Il10rb*, *Klrg1*, *Cd44*) (Kashiwagi et al., 2017) and *Jund*, involved in T cell proliferation (Meixner et al., 2004) were also upregulated in the Gli2ΔN2 transgenic mice compared to WT. In contrast *Tgif1*, a negative regulator of TGF-β (Wang et al., 2017a), inflammatory (*Stat4*, *Il4ra*) and migratory (*Ccr7*) genes were downregulated in the Gli2ΔN2 datasets compared to WT. *Dusp1*, which encodes a signalling molecule was less expressed in Gli2ΔN2 samples compared to WT. Of note

Ptgs2 gene which is both a Hh signalling target gene and a positive regulator of T_{regs} (Holla et al., 2016) was upregulated in Gli2ΔN2 mice.

Shh and *Gli3* expression were not detected in skin $CD4^+$ T cells in any of the genotypes, consistent with the previous findings that *Shh* and *Gli3* were expressed in epithelial cells in different tissues but not detected in T cells (El Andaloussi et al., 2006, Furmanski et al., 2013, Hager-Theodorides et al., 2009, Saldana et al., 2016, Solanki et al., 2017, Solanki et al., 2018, Woo et al., 2012). Furthermore, *Ihh* and *Dhh* expression were not detected in WT and Gli2ΔC2 samples and their expression was very low in the Gli2ΔN2 group.

7.3.2 *Gli2-mediated transcription in skin $CD4^+$ T cells upregulates genes that are related to immune regulatory function compared to samples with Gli2-inhibition*

Since the previous RNA-seq data analysis showed that in Gli2ΔN2 samples genes related with inflammatory pathway were less expressed, I compared RNA-seq datasets of skin $CD4^+$ T cells between Gli2ΔN2 and Gli2ΔC2 samples. The analysis revealed 1453 DEG with higher expression and 1155 DEG with lower expression in Gli2ΔN2 compared to Gli2ΔC2 groups. Interestingly genes involved in T_{reg} function such as *Klrg1*, *Il10*, *Tgfb1*, *Ebl3*, *Fgl2* and *Areg* (Kashiwagi et al., 2017) were significantly upregulated in Gli2ΔN2 (blue circles) compared to Gli2ΔC2 (red squares) samples (Ebayes statistics, $p<0.05$; **Figure 7-4 A**). In contrast *Tgif1*, a negative regulator of Tgfb1, *Crem* and *Crbn* expression were significantly upregulated in Gli2ΔC2 group compared to Gli2ΔN2 samples. Taken together the data indicate that Gli2-driven Hh-mediated transcription

enhanced the expression of genes that are involved in immune regulation and function.

I also examined the expression levels of Hh pathway component genes which are downstream Gli2 or Hh pathway targets. As expected, Hh signalling components like *Ptch1*, *Smo*, *Gsk3b*, *Prkar1b*, *Ptgs2* and *Gli2* genes were significantly higher in Gli2ΔN2 samples compared to Gli2ΔC2 groups, whereas inhibitors of the Hh pathway such as *Numb* and *Csnk1e* were significantly higher in Gli2ΔC2 samples compared to Gli2ΔN2 (Figure **7-4 B**).

Chemokines and chemokine receptors orchestrate migration, which is increased in inflammation helping the trafficking of immune cells towards the site of inflammation. Therefore, I additionally examined genes related to migration and inflammation. *Ccr4* gene which is expressed by skin-homing T cells and had been related to Th2 responses and AD (Andrew et al., 2001, Campbell et al., 1999, Soler et al., 2003, Yamamoto et al., 2000) and *Ccr7* which is expressed by naïve and central memory T cells and controls migration of immune cells to secondary lymphoid organs (Forster et al., 2008) were significantly higher in Gli2ΔC2 samples compared to Gli2ΔN2 (Figure **7-4 B**).

Furthermore, I examined genes related with inflammation and Th1, Th2 and Th17 responses. I found that genes related with inflammatory pathways (*Myd88*, *Jak1*, *Sp110*, *Ripk2*), Th1 responses (*Ifngr1*, *Tbx21*, *Stat4*, *Runx3*), Th2 responses (*Stat6*, *Stat5a*, *Sta5b*, *Il4ra*), and Th17 responses (*Runx1*) were significantly upregulated in Gli2ΔC2 samples compared to Gli2ΔN2 group. In contrast *Plcb3*, *Il1r2* a negative regulator of

inflammation, *Jund* and *Dusp1* have significantly higher expression in Gli2ΔN2 compared to Gli2ΔC2 datasets (Figure **7-4 B**).

Thus, the genome wide transcriptional profile of Gli2ΔN2 and Gli2ΔC2 skin $CD4^+$ T cells showed that this population in Gli2ΔN2 mice was characterised by increased Hh signalling pathway and reduced expression of genes related to inflammation, which is consistent with the ameliorated skin inflammation that was observed in Gli2ΔN2 mice upon Oxa-treatment. Interestingly, Gli2ΔN2 samples showed enhanced expression of immune regulatory genes compared to Gli2ΔC2 samples, so I decided to evaluate and compare the T_{reg} cell numbers and function in these mice.

7.3.3 *Gli2-mediated transcription is critical for elevated numbers and increased activation of skin and peripheral T_{reg} populations and upregulates TGF-β*

Next, I examined the skin T_{reg} populations in the transgenic mice and in the $Gli3^{+/-}$ mutants. First, I induced skin inflammation in the WT and Gli2ΔN2 mice and I analysed the skin T_{reg} populations. Figure **7-5 A** shows representative contour plots of $CD4^+CD25^+Foxp3^+$ skin T_{regs} from the two experimental groups. I observed an outstanding upregulation in the percentages of skin T_{regs} in Oxa-treated Gli2ΔN2 mice compared to WT controls (31.51 ± 2.044 vs 19.58 ± 1.749, $p<0.0001$). Quantification of the data revealed significantly augmented skin T_{reg} numbers in the transgenic mice compared to the littermate controls. In order to evaluate if overall Shh upregulation also increases the T_{reg} population in the Oxa-treated skin, I examined WT and $Gli3^{+/-}$ mice, which as described in Chapter **5**, have increased *Shh* expression in the skin. Consistent with the previous finding, I observed increased proportions of skin T_{reg} cells in Oxa-treated $Gli3^{+/-}$

mice compared to the WT group. When I examined T_{reg} populations for IL-10 expression, the mutant mice, were characterised by increased IL-10 expression compared to the WT, however the difference was not significant (Figure **7-5 B**). Taken together the results showed that elevated *Shh* expression in skin, is sufficient to increase the T_{reg} populations under skin inflammatory conditions and this increase is Gli2-driven.

Therefore, I decided to test if Oxa-treated Gli2ΔC2 mice, had decreased T_{reg} populations. I stained skin T cells for $CD4^+CD25^+Foxp3^+$ as shown in the representative contour plots in Figure **7-5 C**, and I found that the T_{reg} proportions in the Oxa-treated Gli2ΔC2 transgenic mice, were significantly lower than WT (32.49 ± 1.659 vs 36.65 ± 1.914, $p<0.05$). However, the number of T_{regs} was not altered between Oxa-treated WT and Gli2ΔC2 groups (Figure **7-5 C**).

After confirming the differences in skin T_{reg} populations, I wanted to examine whether Gli2-driven transcription alters peripheral T_{reg} cells. I isolated skin dLNs from Gli2ΔN2, WT and Gli2ΔC2 Oxa-treated ears and examined the proportions of T_{regs}. I found that Gli2ΔN2 dLNs had increased proportions of T_{reg} cells compared to WT, although the difference was not significant ($p=0.06$). When comparing WT with Gli2ΔC2 dLNs, I found that the Gli2ΔC2 transgenic group had significantly decreased percentages of T_{reg} populations compared to WT (9.549 ± 0.3295 vs 11.97 ± 0.5835, $p<0.01$). Gli2ΔC2 mice showed also severely reduced T_{reg} percentages compared to the Gli2ΔN2 mice (9.549 ± 0.3295 vs 13.33 ± 0.3483, $p<0.0001$) (Figure **7-6 A**). In order to evaluate the functionality of T_{reg} populations, present in the skin dLNs under inflammatory conditions, I

examined T_{reg} population for activation and immunosuppressive markers, such as CD44, CTLA-4, Klrg1 and Ki67. I found that although Gli2ΔN2 and WT T_{reg} populations had similar levels of CD44 and CTLA-4 expression, Gli2ΔC2 dLN T_{regs} had significantly lower levels of both CD44 and CTLA-4 compared to WT and Gli2ΔN2 mice. Interestingly, Klrg1 staining revealed a strong upregulation in Gli2ΔN2 T_{regs} compared to WT mice (22.43 ± 1.507 vs 16.02 ± 1.055, $p<0.01$) and a significant downregulation of the marker in Gli2ΔC2 T_{reg} populations (10 ± 0.8984 vs 16.02 ± 1.055, $p<0.01$), which was consistent with the higher *Krlg1* expression observed in skin $CD4^+$ T cells from Gli2ΔC2 samples compared to Gli2ΔC2 samples as revealed by RNA-seq data (Figure **7-4 A**). Ki67 staining showed significant upregulation in Gli2ΔN2 T_{regs} compared to the T_{regs} from WT mice, while there was no obvious difference between WT and Gli2ΔC2 samples (Figure **7-6 B**). Taken together the data showed that inhibition of Gli2-mediated transcription reduced the skin and peripheral T_{reg} populations and affected their functionality as that was measured by activation, co-inhibitory and immunosuppressive markers.

In order to confirm the previous results, I performed an *in vitro* suppression assay using $CD4^+$ T cells and T_{reg} populations isolated from Oxa-treated WT and Gli2ΔC2 mice. T_{reg} populations were co-cultured in different ratios with $CD4^+$ T cells stained with CFSE, and $CD4^+$ T cell proliferation was measured by evaluating CFSE expression. As shown in representative histograms in Figure **7-7 A**, $CD4^+$ T cell proliferation of WT and Gli2ΔC2 $CD4^+$ T cells was identical, without addition of T_{regs} in the culture (left histogram). However, when I co-cultured WT $CD4^+$ T cells with either WT

T_{regs} (red line, Right histogram) or Gli2ΔC2 T_{regs} (blue line, right histogram), I observed more proliferation in $CD4^+$ T cells co-cultured with Gli2ΔC2 T_{regs} compared to $CD4^+$ T cells co-cultured with WT T_{regs}. Furthermore, I examined the percentages of non-proliferating $CD4^+$ T cells when these were co-cultured with different ratios of WT and Gli2ΔC2 T_{regs}. I showed a significant upregulation in non-proliferating $CD4^+$ T cells when these were cultured with WT T_{regs} in 1:1 ratio, compared to non-proliferating $CD4^+$ T cells co-cultured with Gli2ΔC2 T_{regs} in 1:1 ratio (Figure **7-7 B**). The results indicated an impaired ability of Gli2ΔC2 T_{reg} population to suppress *in vitro* $CD4^+$ T cell proliferation, which was in agreement with the reduced functionality of Gli2ΔC2 T_{reg} populations, as determined by flow cytometry staining of activation and immunoregulatory T_{reg} markers (Figure **7-6**).

As *Tgfb1* and *Tgif1* were differentially expressed between Gli2ΔN2 and Gli2ΔC2 samples (Figure **7-4 A**) and TGF-β signals for the induction and maintenance of T_{reg} cell populations (Konkel et al., 2017, Tran, 2012), I evaluated TGF-β levels by surface staining against anti-latency associated peptide (LAP) in skin cell populations from Oxa-treated Gli2ΔN2, WT and Gli2ΔC2 mice. LAP is derived by cleavage of the N-terminal of TGF-β precursor protein and is held on the cell surface membrane where it forms the inactive latent TGF-β preventing this way the non-regulated activation of TGF-β receptors (Keski-Oja et al., 2004, Miyazono et al., 1993). LAP expression on $CD4^+CD25^+$ population enhances its suppressive potential in a TGF-β-dependent manner, while LAP-expressing T_{regs} are more potent in suppressing experimental autoimmune encephalitis compared to T_{regs} lacking LAP expression (Chen et al., 2008, Ochi et al., 2006). I first tested

skin non-T cell leukocyte populations ($CD45^+CD3^-$) and showed that although Gli2ΔN2 cells did not have a significant difference in LAP expression compared to WT samples, Oxa-treated Gli2ΔC2 samples showed a striking downregulation of LAP expression compared to WT (2.36 ± 0.4266 vs 66.88 ±5.727, $p<0.0001$). When I examined $CD4^+$ non-T_{reg} cell populations for LAP expression, I observed that Gli2ΔN2 cells had a significantly elevated LAP expression compared to WT littermates, whereas Oxa-treated Gli2ΔC2 mice displayed significantly reduced LAP expression compared to the WT group (31.56 ± 2.7861 vs 62.42 ± 1.9722, $p<0.0001$) (Figure **7-8 A**). Furthermore, I analysed LAP expression on T_{reg} population from the three Oxa-treated genotypes. I observed increased proportions of LAP expression on T_{regs} from Oxa-treated Gli2ΔN2 mice and decreased proportions of LAP-expressing T_{reg} populations in Oxa-treated Gli2ΔC2 compared to Oxa-treated WT groups, shown in representative histograms in Figure **7-8 B**. Quantification of the data showed a twofold increase in numbers of LAP-expressing T_{regs} in Gli2ΔN2 samples compared to the WT littermate group (0.6447 ± 0.08821 vs 0.3407 ± 0.03598, $p<0.05$), while these numbers were three times lower in Gli2ΔC2 samples compared to the WT littermates (0.5437 ± 0.1133 vs 1.788 ±0.2647, $p<0.001$) (Figure **7-8 B**). In order to confirm that the LAP expression corresponds to active TGF-β, I measured its levels by ELISA in supernatants from WT and Gli2ΔC2 samples, which were collected from the suppression assay experiment described above. Gli2ΔC2 spleenocytes secreted significantly lower active TGF-β compared to the WT samples (Fig **7-8 C**) and the results

were in agreement with the decreased LAP expression in skin cells from Gli2ΔC2 mice.

It has been previously shown that T_{reg} populations fail to perform suppression on T cells when the later have a reduced ability to respond to TGF-β signals in mouse models of colitis and autoimmune encephalomyelitis (Fahlen et al., 2005, Liu et al., 2003, Zhang et al., 2006). Given the reduced LAP expression, dampened TGF-β, and compromised cell-mediated suppression capacity of Gli2ΔC2 T_{regs}, I decided to examine TGF-β signalling in skin T cell populations, by staining against pSmad2/3. As shown in the representative FACS plots and graph in Figure **7-9 A**, there was a decrease in pSmad2/3 expression in $CD4^+$ T cells from Gli2ΔC2 Oxa-treated mice compared to WT mice and a significantly increased expression of the phosphorylated pathway in Gli2ΔN2 mice compared to the WT littermates. I observed the same significant differences in skin $CD8^+$ T cells when I compared the transgenic mice to their WT littermates (Figure **7-9 B**).

Taken together the data demonstrated an active role for Hh signalling in maintaining active functional T_{reg} populations in the skin and in upregulation of TGF-β, thus indicating one mechanism by which Gli2 activity in $CD4^+$ T cells can control skin-specific immune responses and inflammation.

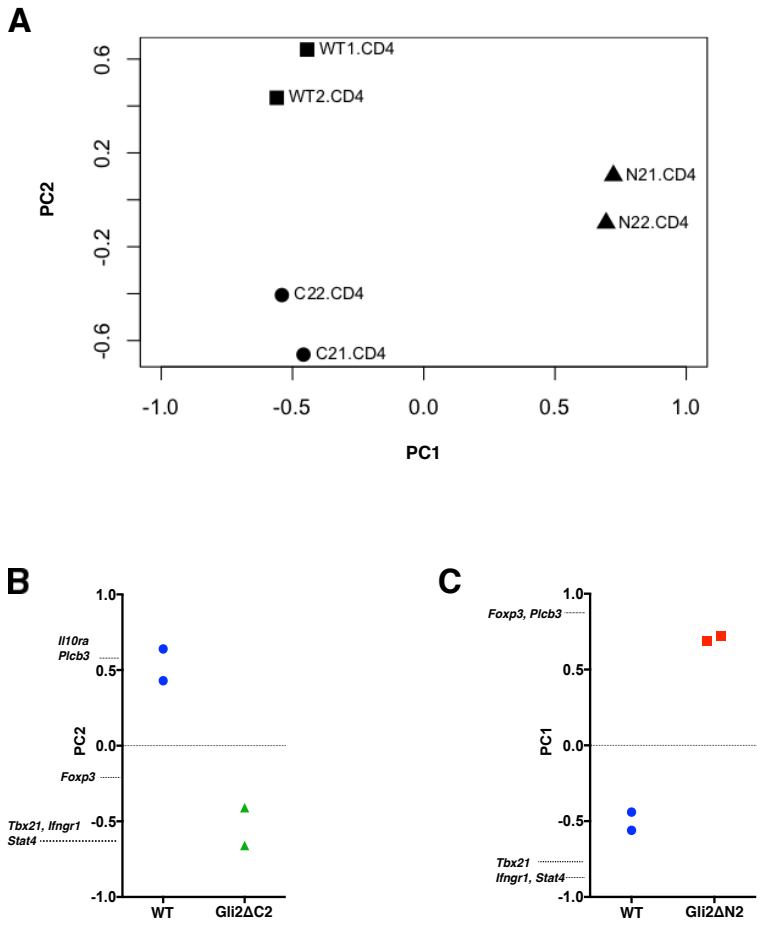


Figure 7-1: PCA analysis of skin CD4⁺ T cells segregates samples according to genotype and reveals differentially expressed genes related to inflammation and immune regulation

(A) PCA analysis of datasets from skin CD4⁺ T cells from Oxa-treated Gli2ΔN2, WT and Gli2ΔC2 samples.

Annotated PC axes with differentially expressed genes. The **(B)** PC2 axis shows relationships between WT (blue circles) and Gli2ΔC2 (green triangles) samples, while **(C)** PC1 axis shows relationships between WT (blue circles) and Gli2ΔN2 (red squares) samples.

Data were generated from two independent experimental groups each with pooled CD4⁺ T cells from skin of Oxa-treated WT (n=3), Gli2ΔN2 (n=3) and Gli2ΔC2 (n=3) mice.

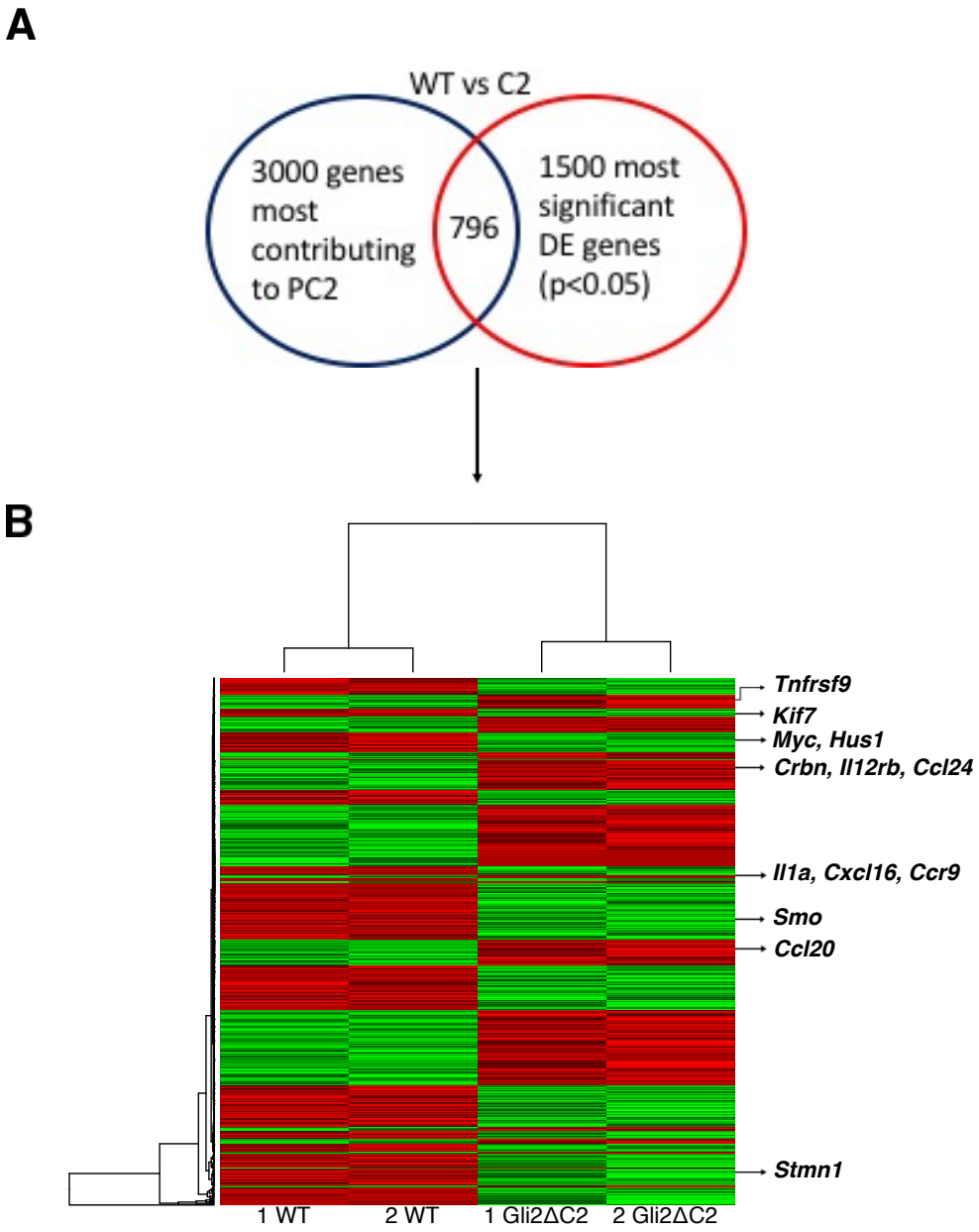


Figure 7-2: Inhibition of Gli2-mediated transcription promotes upregulation of genes related to inflammation and migration and represses the upregulation of Hh signalling component genes in skin $CD4^+$ T cells isolated from Oxa-treated mice

(A) Venn diagram shows the intersection between the 3000 genes that contributed most to PC2 (highest and lowest scores) and the 1500 most significant DEG by Ebayes between WT and Gli2ΔC2 sample datasets.

(B) Intersected genes from the Venn diagram were clustered in a heatmap and genes related to inflammation, migration and Hh signalling pathway were annotated. Green represents lower expression while red shows higher expression levels.

Data were generated from two independent experimental groups each with pooled $CD4^+$ T cells from skin of Oxa-treated WT (n=3) and Gli2ΔC2 (n=3) mice.

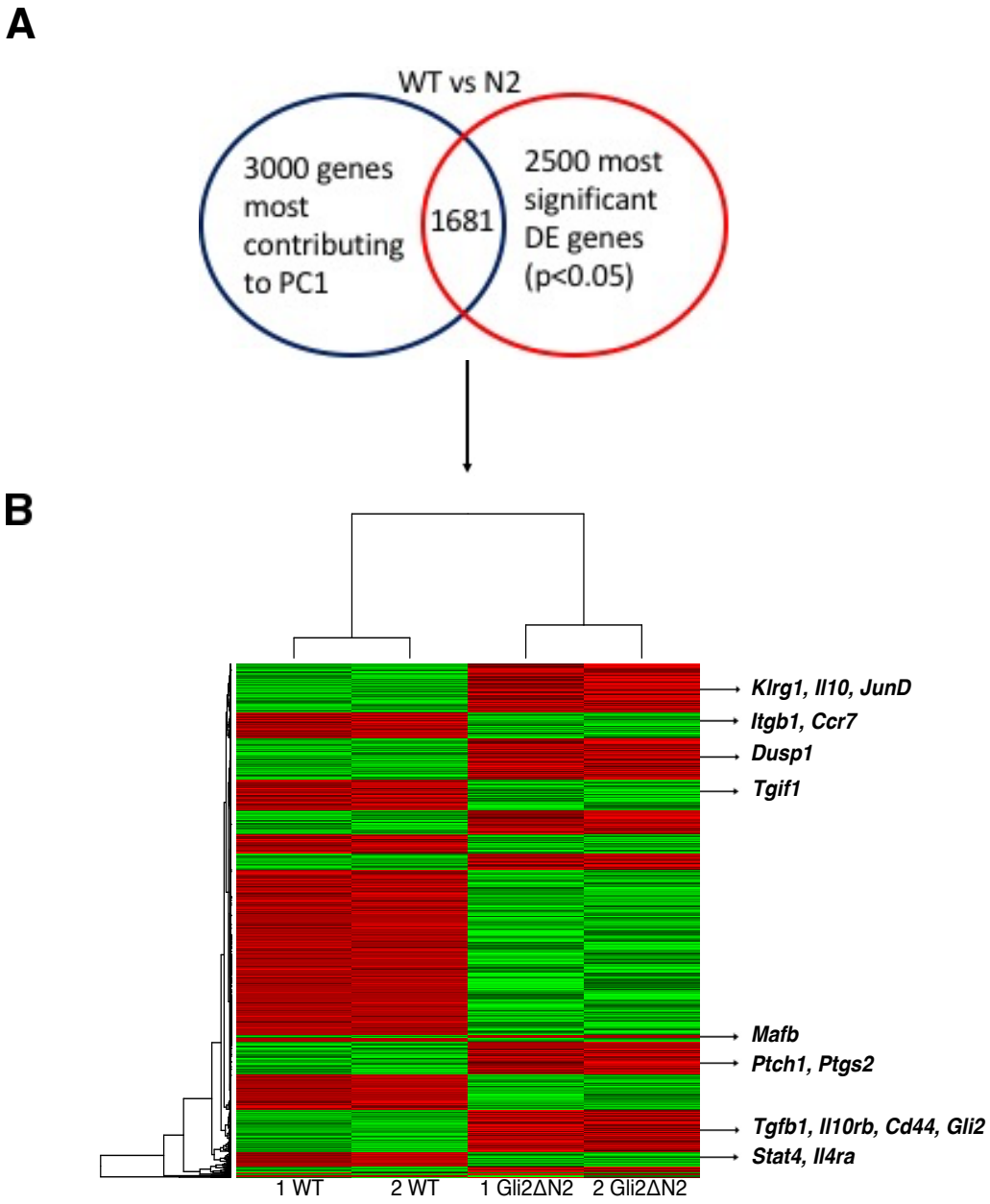


Figure 7-3: Gli2-mediated transcription in T cells suppresses the upregulation of genes related to inflammation, while it favours the expression of genes related to Hh signalling pathway and immune regulation in skin $CD4^+$ T cells isolated from Oxa-treated mice

(A) Venn diagram shows the intersection between the 3000 genes that contributed most to PC1 (highest and lowest scores) and the 2500 most significant DEG by Ebayes between WT and Gli2ΔN2 sample datasets.

(B) Intersected genes from the Venn diagram were clustered in a heatmap and genes related to inflammation, migration, immune regulation and

Hh signalling pathway were annotated. Green represents lower expression while red shows higher expression levels.

Data were generated from two independent experimental groups each with pooled $CD4^+$ T cells from skin of Oxa- treated WT (n=3) and Gli2ΔN2 (n=3) mice.

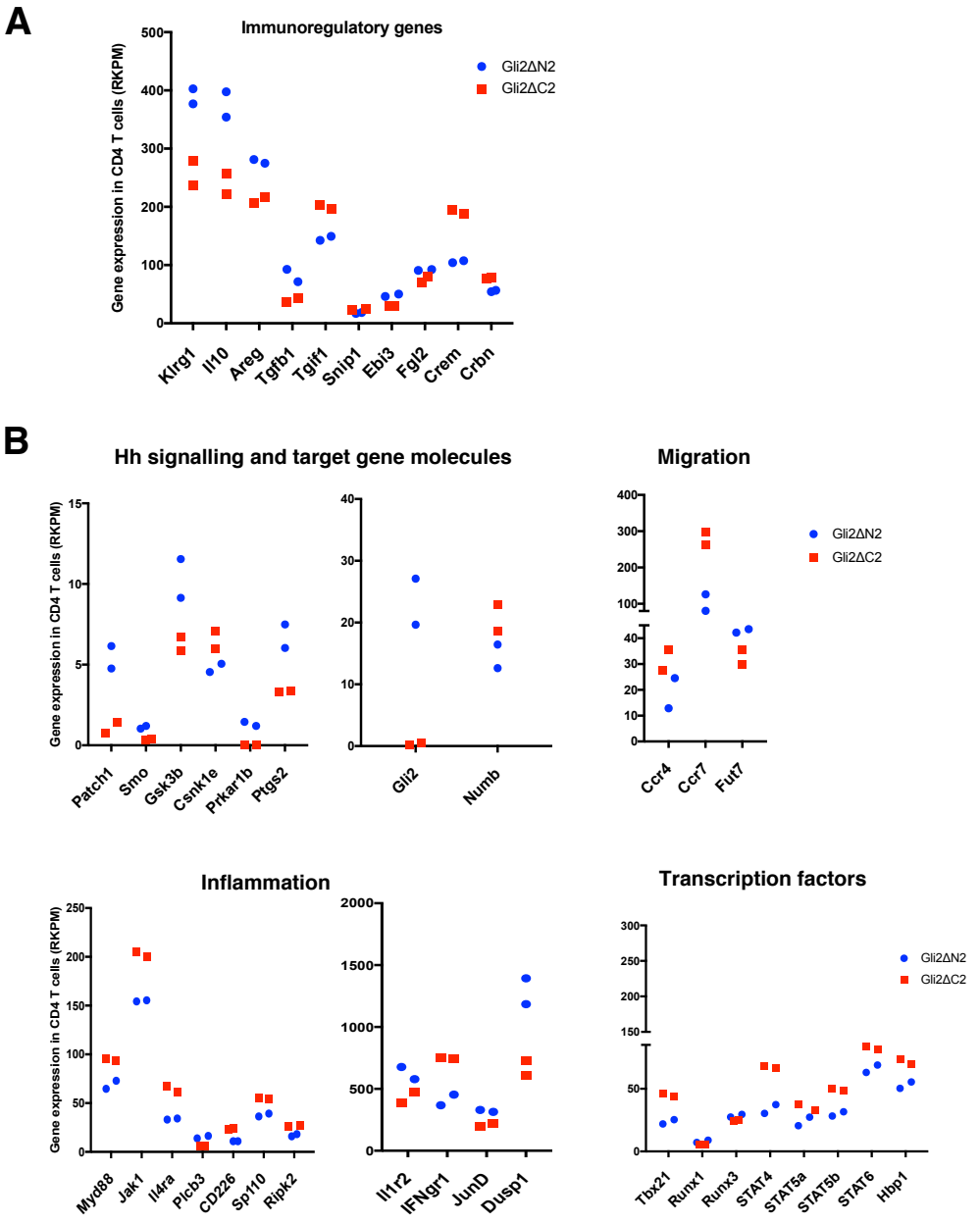
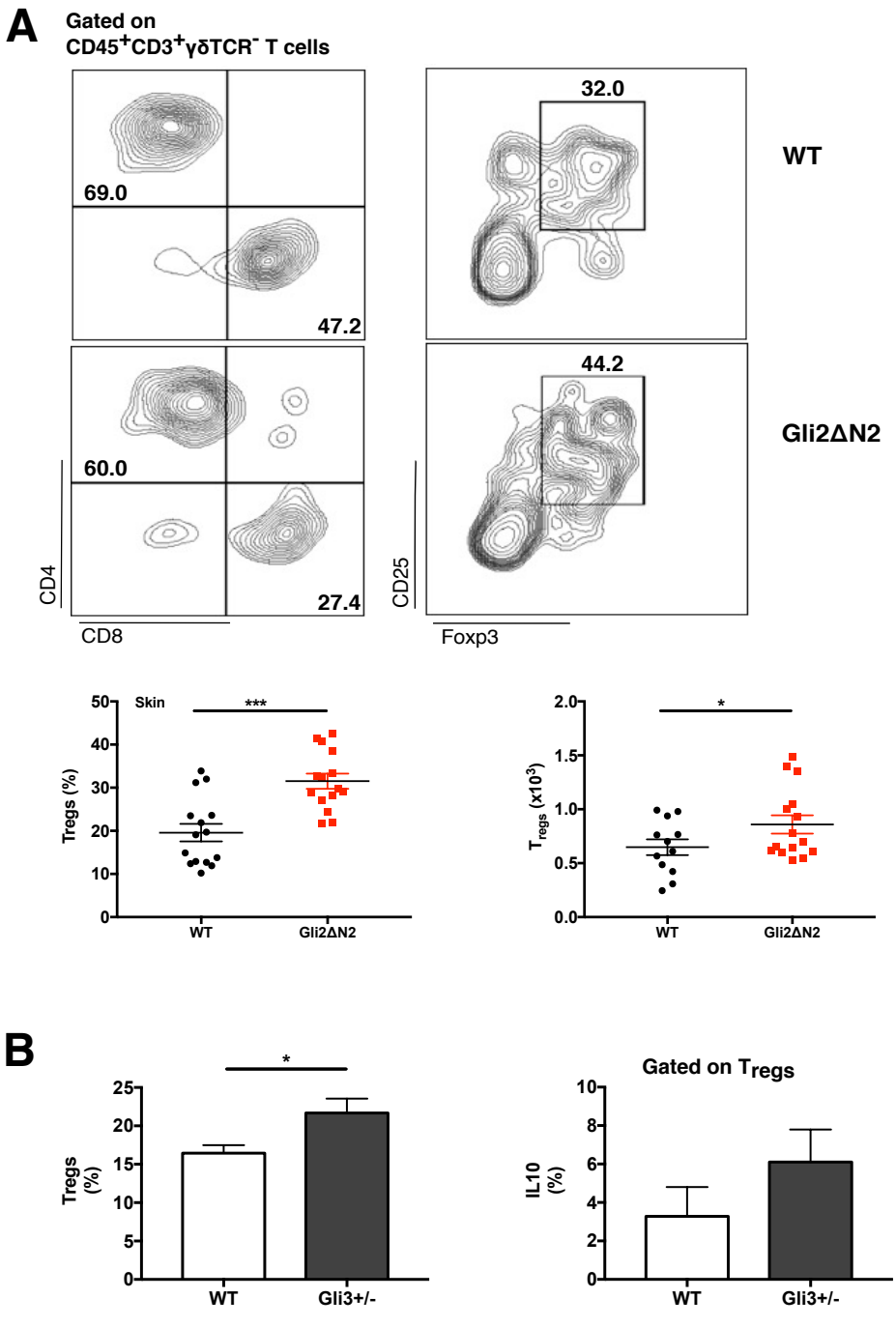


Figure 7-4: Gli2-driven transcription augments immune regulation gene expression and restrains expression of inflammation genes.

Transcript expression (reads per million kilobases) of genes involved in **(A)** immune regulation and **(B)** Hh signalling pathway, inflammation and migration in Gli2ΔN2 (blue circles) and Gli2ΔC2 (red squares) RNA-seq datasets. Data were generated from two independent experimental groups each with pooled $CD4^+$ T cells from skin of Oxa-treated Gli2ΔN2 (n=3) and Gli2ΔC2 (n=3) mice.


A
Gated on
CD45+CD3+γδTCR- T cells
69.0
47.2
60.0
27.4
CD4
CD8
32.0
44.2
CD25
Foxp3
WT
Gli2ΔN2
Skin
***
Tregs (%)
WT
Gli2ΔN2
*
Tregs (x10^3)
WT
Gli2ΔN2
B
*
Tregs (%)
WT
Gli3+/-
Gated on Tregs
IL10 (%)
WT
Gli3+/-

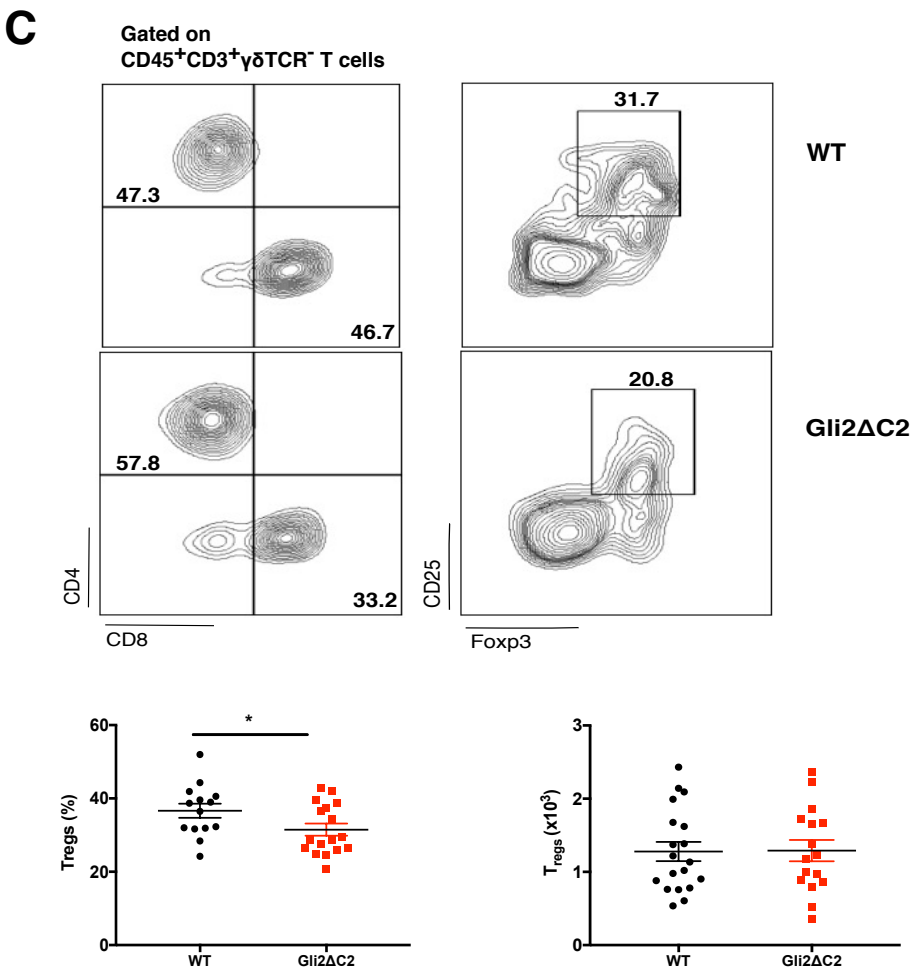


Figure 7-5: Gli2-mediated transcription in T cells increases skin T_{reg} population

Representative contour plots showing CD4 against CD8 and CD25 against Foxp3 staining to identify T_{reg} population isolated from skin of **(A)** Oxa-treated Gli2ΔN2 and WT littermates and **(C)** Oxa-treated Gli2ΔC2 and Oxa-treated WT littermates. Scatter plots in **(A)** and **(C)** show percentages and proportions of T_{regs} isolated from skin (as percentage of the $CD4^+$ T cell populations), where each symbol represents an individual value: WT (black circles), Gli2ΔN2 or Gli2ΔC2 (red squares). The group mean is represented with a bar. Data were generated from two independent experiments with at least 6 mice per group.

(B) Bar charts show percentages of skin T_{reg} population (Left) and IL-10 expressing T_{regs} (Right) from Oxa-treated WT (White bars) and Oxa- treated $Gli3^{+/-}$ (Grey bars). Data were generated from one experiment with 7 mice per group. Plots show mean ± SEM.

*p<0.05 and ***p<0.001

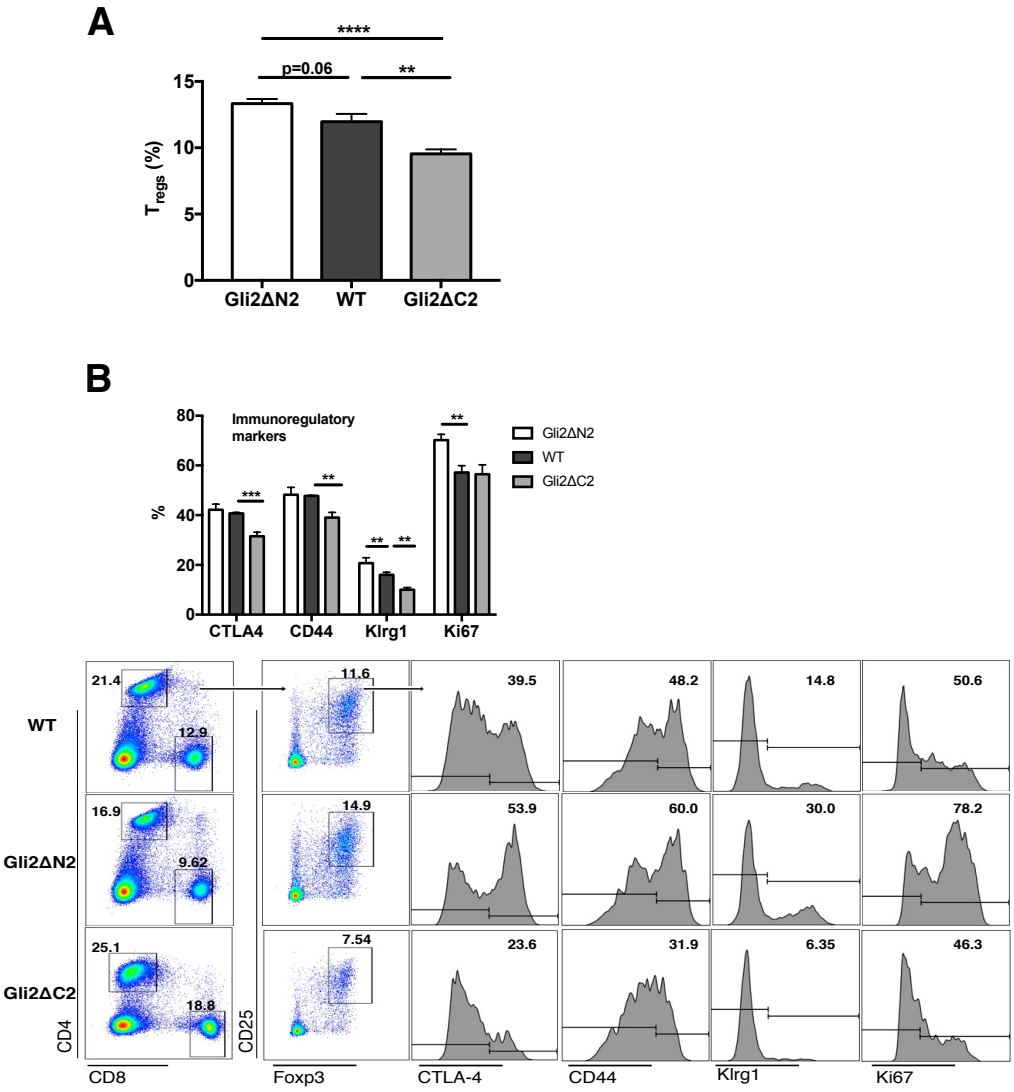


Figure 7-6: Inhibition of Gli2-transcription decreases peripheral T_{reg} numbers and compromises T_{reg} functionality

(A) Bar charts show percentages of T_{reg} populations in Oxa-treated mice from dLNS of Gli2ΔN2 (White bars), WT (dark-grey bars) and Gli2ΔC2 (Light-grey bars).

(B) Bar charts show percentages of CD44-, CTLA-4-, Klrg1- and Ki67-positive $CD4^+CD25^+Foxp3^+$ T_{reg} populations from dLNs in Oxa-treated Gli2ΔN2, WT and Gli2ΔC2 groups determined by FACS-analysis. FACS plots show representative staining from dLNs for the three experimental groups of CD4 against CD8 and CD25 against

intranuclear Foxp3 gated on $CD4^+$ T cells. Histograms show intracellular CTL4-A, cell surface CD44 and Klrg1, and intranuclear Ki67 staining gated on $CD4^+CD25^+Foxp3^+$ T_{reg} cells.

Plots show mean ± SEM **p<0.01, ***p<0.001 and ****p<0.0001.

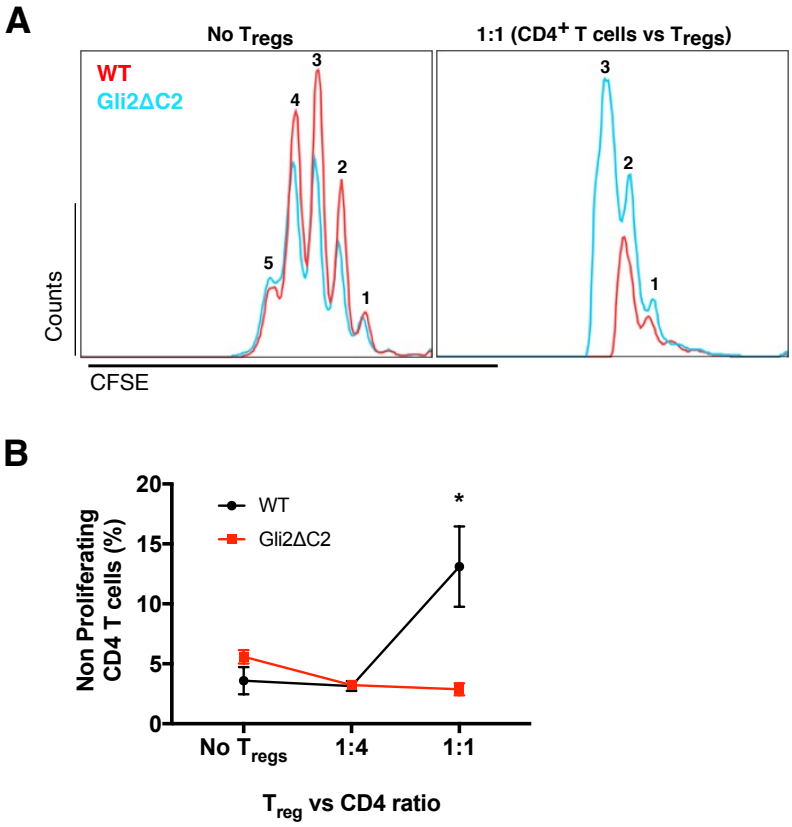


Figure 7-7: Inhibition of Gli2-transcription reduces the *in vitro* immunosuppressive function of T_{regs}

$CD4^+CD25^-$ T cells were co-cultured with $CD4^+CD25^+$ T_{reg} cells from Gli2ΔC2 and WT spleens of Oxa-treated mice at indicated ratios. The T_{reg} effect on $CD4^+$ T cell proliferation was assayed with CFSE staining.

(A) Representative histograms show $CD4^+$ T cell divisions without T_{regs} (Left histogram) and divisions of cells co-cultured with (1:1) CD4:T_{reg} ratio (Right histogram) from Oxa-treated WT (red line) and Gli2ΔC2 (blue line) mice.

(B) The graph shows percentages of non-proliferating $CD4^+$ T cells without T_{regs}, with 1:1 and 1:4 T_{reg}:CD4 ratios. Black line represents Oxa-treated WT and red line Oxa-treated Gli2ΔC2 mice. *$p<0.05$.

Data were generated from one experiment with 5 mice per group.

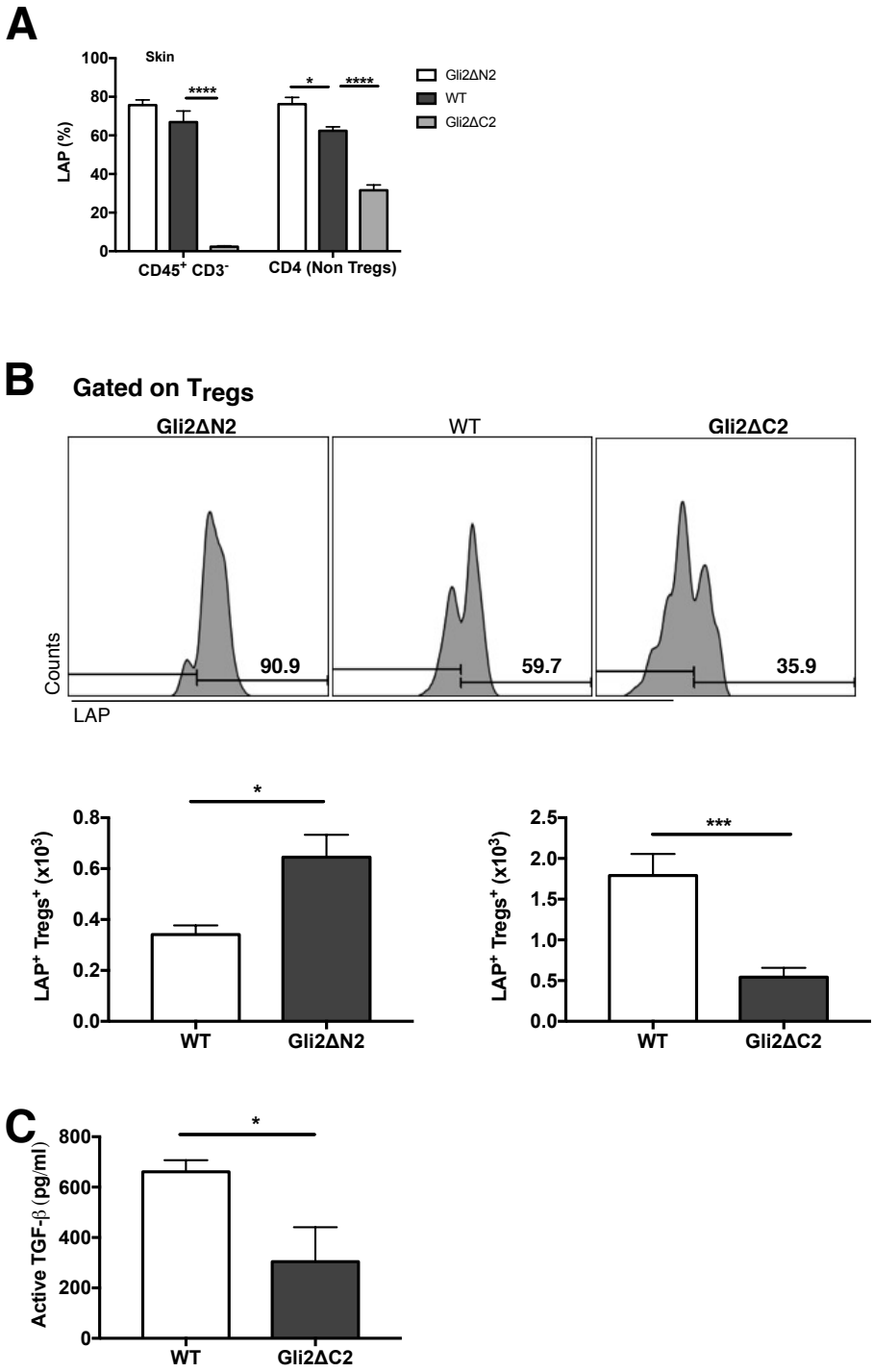


Figure 7-8: Gli2-driven transcription induces TGF-β expression on skin T_{reg} populations upon Oxa-treatment

(A) Percentages of LAP-positive cells (TGF-β expression), gated on $CD45^+CD3^-$ and non-T_{reg} $CD4^+$ T cells from skin of Oxa-treated

Gli2ΔN2 (White bar), WT (Dark-grey bar) and Gli2ΔC2 (Light-grey bar) mice.

(B) Representative histograms of anti-LAP staining on skin T_{reg} populations from Oxa-treated Gli2ΔN2 (Left histogram), WT (Middle histogram) and Gli2ΔC2 (Right histogram). Bar charts show numbers of LAP-expressing skin T_{regs} from WT (White bar) compared to Gli2ΔN2 (Grey bar) littermates (Left) and WT (White bar) compared to Gli2ΔC2 (Grey bar) littermates (Right).

(C) Bar chart shows active TGF-β secretion from supernatants collected from the experiments described in Figure **7-7** from WT (White bar) and Gli2ΔC2 (Grey bar) littermates.

Plots show mean ± SEM. *p<0.05, ***p<0.001 and ****p<0.0001.

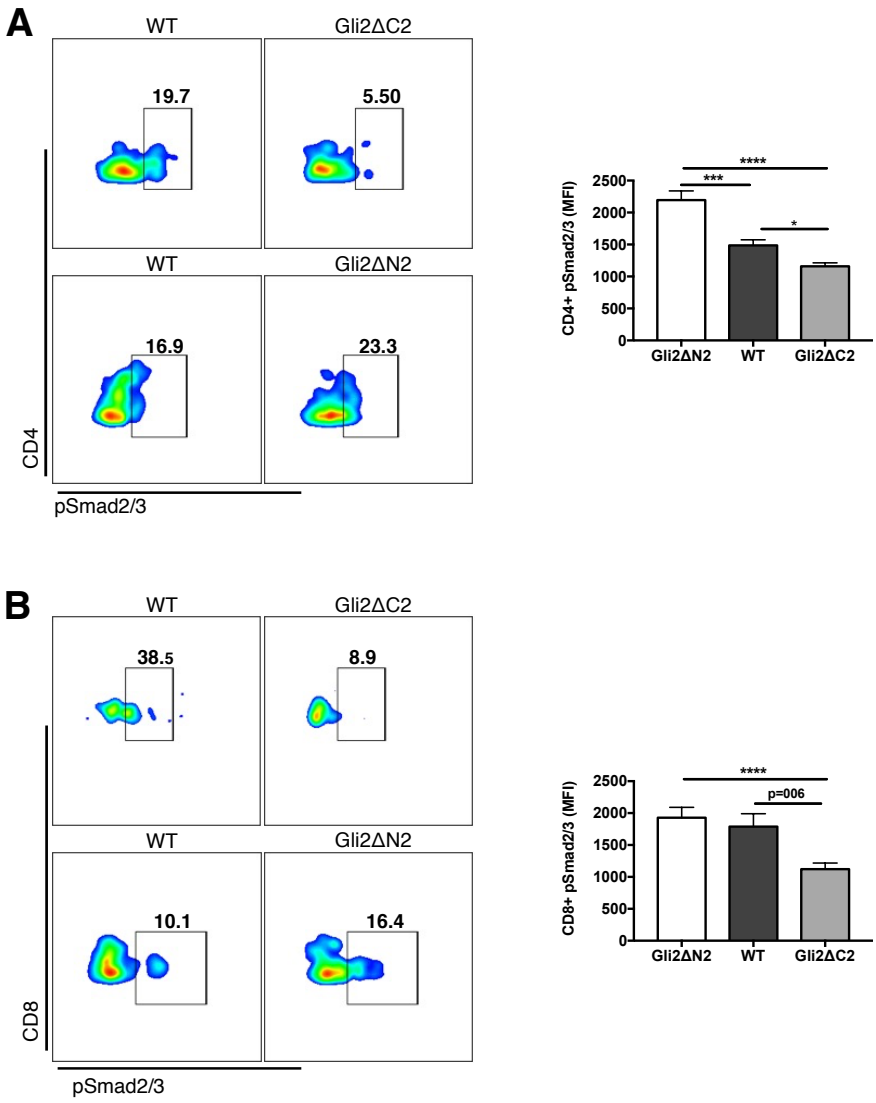


Figure 7-9: Gli2-driven Hh-signalling induces the activation of TGF-β pathway in skin T cell populations

Representative FACS plots showing pSmad2/3 expression in skin **(A)** $CD4^+$ and **(B)** $CD8^+$ T cells from Oxa-treated WT and Gli2ΔC2 littermates (Top panels) and from Oxa-treated WT and Gli2ΔN2 littermates (Bottom panels). Mean MFIs of pSmad2/3 expression in skin CD4+ and skin CD8+ T cells from Gli2ΔN2 (white bar), WT (dark-gey bar) and Gli2ΔC2 (light-grey bar) groups determined by FACS analysis. Data were generated by 6-10 mice per group.

*p<0.05, ***p<0.001 and ****p<0.0001.

7.4 Discussion

In this chapter I have shown that Gli2 controls transcription of inflammation and immune regulation-related genes. By performing RNA-seq on skin $CD4^+$ T cells from the Oxa-treated Gli2ΔN2, WT and Gli2ΔC2 mice, I uncovered pathways that are implicated in skin inflammation and are driven by either inhibition or overexpression of the Gli2 transcription factor in T cells. Among the genes that were highlighted and upregulated in skin $CD4^+$ T cells upon inhibition of Gli2, were the genes related with Th1 (*Stat4*, *Ifngr1*, *Tbx21*) and IFN type I responses (*Jak1*, *Irf1*, *Irf9*, *Irf6*, *Gimap4*, *Gbp2*, *Rtp4*) (Ivashkiv and Donlin, 2014, Kane et al., 2016). IFN I responses are associated with viral infections, where they have both detrimental and positive effects. Research findings have shown that IFN type I expressed by plasmacytoid dendritic cells is responsible for the development of psoriasis and mice lacking an inhibitor of the IFN type I pathway suffered a skin inflammation with characteristics of psoriasis (Hida et al., 2000, Nestle et al., 2005). Additionally, the pathway is implicated in interface dermatitis, a skin inflammation condition which arises as a result of skin autoimmune disorders (Wenzel and Tuting, 2008). The data suggest that the increased expression of these genes upon Gli2-inhibition in T cells, could be involved in the exacerbated skin inflammation in Gli2ΔC2 mice.

Analysis between Gli2ΔN2 and WT datasets, revealed upregulated genes related to immune regulation (*Klrg1*, *Il10*, *Il10rb*, *Tgfb1*, *Ptgs2*) and downregulation of genes related to inflammation (*Stat4*, *Il4ra*). Pro-inflammatory genes such as *Il4* and *Il6* are also upregulated in the datasets of skin $CD4^+$ T cells from Gli2ΔN2 mice compared to WT mice. However,

that could be due to the fact that both genes are direct target genes of Gli2 transcription factor, as it has been previously shown for *Il4* in murine $CD4^+$ T cells and for *Il6* in stromal and B cells (Furmanski et al., 2013, Jackson et al., 2015). The RNA-seq findings are consistent with the results described in the previous chapter, where Gli2ΔN2 mice had attenuated skin inflammation on induction of atopic dermatitis compared to WT mice, whereas Gli2ΔC2 had signs of exacerbated skin inflammation.

Consistent with the reduced inflammation in Gli2ΔN2 mice, skin T_{regs} were significantly increased compared to WT. In addition, the increased expression of *Fut7* was found in skin $CD4^+$ T cells of Oxa-treated Gli2ΔN2 mice (as shown in Figure **7-4 B**), which was previously shown to be necessary for T_{reg} migration to the skin (Dudda et al., 2008). In contrast, less T_{reg} cells were observed in Gli2ΔC2 mice, in line with exacerbated inflammation. Moreover, T_{regs} from Gli2ΔC2 samples, showed an attenuated immune regulatory function. This finding is very interesting as skin of atopic dermatitis patients is characterised by T_{regs} infiltration, which however have lost their function (Hijnen et al., 2009, Ito et al., 2009, Reefer et al., 2008, Zhang et al., 2016). Here I propose that the decreased numbers of T_{regs} together with their compromised function might be due to inhibition of Gli2-mediated Hh signalling pathway. It would be interesting to examine in the future in more depth skin T_{reg} populations and measure their ability to express IL-10 under skin inflammatory conditions, as this is one main tool of immunosuppressive action. Also, *in vitro* suppression assays with Gli2ΔN2 and WT T_{regs}, could be done in order to confirm that under Gli2-driven Hh pathway upregulation, the immunosuppressive activity of

T_{regs} is increased. In the future, adoptive transfer experiments of Gli2ΔN2, WT and Gli2ΔNC2 T_{reg} and $CD4^+$ T cell populations, in RagKO Oxa-treated mice could be a useful tool to examine the *in vivo* capacity of these populations.

It is noteworthy that skin T_{reg} populations from Gli2ΔN2 mice had increased LAP expression, while upon Gli2 inhibition, T_{regs} of Oxa-treated mice, showed reduction of LAP and decreased active TGF-β expression. As mentioned before, TGF-β is responsible for maintenance of immune tolerance. Its loss via Gli2-inhibition in T cells, was associated with the development of more severe dermatitis and skin inflammation. Several studies investigated Hh signalling pathway and TGF-β crosstalk, showing that TGF-β can drive Shh and Gli2 activation in several tissues including skin (Dennler et al., 2007, Liang et al., 2017, Maitah et al., 2011). Additionally, in human $CD4^+$ T cells, Gli2 directly binds and promotes *TGFB1* activation (Furler and Uittenbogaart, 2012). TGF-β pathway is critically implicated in the impairment of T cell responses and TGF-β has been previously shown to alleviate symptoms of atopic dermatitis in mice (Han et al., 2015, Khaheshi et al., 2011). It remains to be investigated whether TGF-β is a direct target of Gli2 transcription factor in murine skin $CD4^+$ T cells and whether it acts with Foxp3 or in a Foxp3-independent mechanism to ameliorate dermatitis symptoms.

In this chapter I have presented RNA-seq data regarding skin $CD4^+$ T cells. This research focuses on the role of $CD4^+$ T cells, while the role of $CD8^+$ T cells in the disease has not been investigated in depth. In the previous chapter I have shown that $CD8^+$ T cell populations are also affected by Gli2-

driven inhibition or overexpression of the Hh pathway. Analysis of the skin $CD8^+$ T cell datasets from the Oxa-treated mice could reveal so far unknown pathways that are implicated in $CD8^+$ T cell responses, as there are no genome wide studies focusing only on this population in the context of dermatitis.

The summary of mechanism of Hh on induction of atopic dermatitis and skin inflammation in mice is proposed in the Conclusions Chapter.

Chapter 8

Chapter 8: Conclusions and future directions

Skin homeostasis is maintained by a tightly controlled microenvironment of keratinocytes, dermal and immune cells. In this study I demonstrate that the morphogen Shh plays an important role in regulating the skin T cell immune responses in the context of chronic atopic dermatitis. I showed that Shh expression in the skin is upregulated on induction of AD and that increased Hh signalling acts as a protective mechanism against disease pathology and skin inflammation. Gli3-mutation increased *Shh* expression in the skin and dampened the inflammatory response on induction of atopic dermatitis, whereas systemic pharmacological blockade of Hh signal transduction reduced induction of *Shh* expression in skin and exacerbated the inflammatory profile.

Induction of atopic dermatitis in the Gli2ΔN2 and Gli2ΔC2 transgenic models demonstrated the importance of Shh signalling to skin T cells in protecting against disease induction and severity, and skin inflammation. I investigated the mechanism by which Gli2-dependent Shh signalling to $CD4^+$ T cells prevents atopic dermatitis and skin inflammation. In my study, Gli2-mediated transcription in response to Shh upregulation increased the skin T_{reg} population and promoted T_{reg} function and TGF-β signalling, thereby decreasing pro-inflammatory responses in skin. These data were further supported by RNA-seq analysis showing that Hh-mediated transcription in skin $CD4^+$ T cells on induction of dermatitis increased expression of immunoregulatory genes (including *Ptgs2*, *Il10*, *Klrg1*, *Areg* and *Fgl2*) and decreased expression of inflammatory and migratory genes

(including *Tnfrsf9*, *Il1a*, *Tgif1*, *Ccr7*, *Ccr9*, *Ccl20* and *Ccl24*). A schematic representation of our proposed model is shown in Figure **8-1**. The observation that increased Hh pathway activation restores the immunosuppressive phenotype of skin T_{regs} has implications for treatments of AD, because non-functional T_{reg} populations are strongly correlated with AD in human patients (Grindebacke et al., 2004, Hijnen et al., 2009, Ito et al., 2009, Verhagen et al., 2006).

The Oxa protocol mouse model of skin inflammation and chronic atopic dermatitis has different kinetics and inflammatory components. However, they all share important features (Jin et al., 2009a). In my experiments I have shown that two weeks of Oxa application led to a chronic phase of skin inflammation, with elevated Th1, Th2 and Th17 and $CD8^+$ T cell responses, specifically characterised by elevated IgE secretion in blood and a strong IFN-γ signature in skin of treated mice. Also, RNA-seq analysis revealed elevated expression of genes implicated in type I and type II interferon pathways expressed by skin $CD4^+$ T cells of Gli2ΔC2 mice (*Ifngr1*, *Tbx21*, *Irf1*, *Irf9*, *Irf3*, *Phf11*, *Gimap4*, *Gbp2*, *Isg15*, *Gch1*, *Gbp3*, *Stat4*), establishing the elevated Th1 responses which are observed in skin of these mice compared to WT and Gli2ΔN2 counterparts.

Independent studies have established the involvement of developmental pathways such as Notch and Wnt signalling in skin inflammation. Loss of Notch signalling results in disruption of epidermal skin barrier and inflammatory skin disease (Blaydon et al., 2011, Dumortier et al., 2010, Murthy et al., 2012). In contrast, Wnt pathway drives inflammatory responses in the skin (Perez-Moreno et al., 2006) and is induced in

psoriatic skin of patients where it is responsible for keratinocyte inflammation (Gudjonsson et al., 2010, Wang et al., 2017b). Genes encoding pathway components of both Wnt (*Tcf4*, *Wnt4*) and Notch pathways (*Dtx1*, *Hes*, *Dtx3*, *Lfng*) were significantly differentially expressed between genotypes in our RNA-seq datasets, suggesting crosstalk between Hh signalling and these other developmental pathways in skin T cells.

To date, studies of the Hh signalling pathway in mouse disease models are controversial. Hh signalling is implicated in a plethora of diseases such as sclerosis, biliary, kidney and lung fibrosis where it promotes fibroblast expansion (Kugler et al., 2015). In lung, Shh is upregulated on induction of allergic asthma but increased Hh pathway activation in T cells exacerbates the disease via IL-4 production and by promoting Th2 differentiation (Furmanski et al., 2013, Standing et al., 2017), while other findings reported that Ptch1 deletion did not alter asthma allergic responses (Michel et al., 2013). This is in contrast to the protective role of Shh upregulation on induction of the mouse models of atopic dermatitis where Hh pathway activation in T cells protects against disease pathology by promoting immune regulation, and highlights the context dependency of Shh's functions and the tissue specificity of immune regulation by $CD4^+$ T cells. The anti-inflammatory action of Hh signalling in skin inflammation is similar to other findings where Shh is elevated upon injury and dampens down inflammation in tissues such as skeletal muscles, brain, pancreas and intestine (Alvarez et al., 2011, Caradu et al., 2018, Singh et al., 2016, Singh et al., 2017, van Dop et al., 2010, Zacharias et al., 2010, Zhou et al., 2012).

Furthermore, the changes that have been observed in Hh signalling have been reported to be similar to wound healing in a range of tissues including skin (Brownell et al., 2011, Huang et al., 2014, Le et al., 2008, Luo et al., 2009, Palladino et al., 2011, Straface et al., 2009, Xiao et al., 2013). My findings, however, demonstrate the beneficial consequences of Shh upregulation in dermatitis and additionally show that the anti-inflammatory action of Shh is orchestrated by T cells. Also, by using our Gli2ΔN2 and Gli2ΔC2 transgenic mice, I could focus on T-cell intrinsic mechanisms, excluding any T cell-independent effects of Shh.

Aberrant expression of Hh proteins or dysregulated Hh pathway activation is involved in many epithelial-derived tumours, including BCC (Takebe et al., 2015, Teglund and Toftgard, 2010). I showed that inhibition of the Hh pathway both by pharmacological blockade and by genetic inhibition of endogenous Hh-mediated transcription in T cells increased pro-inflammatory T cell responses and IFN-γ production in skin $CD4^+$ and $CD8^+$ T cells. This is consistent with a recent study in which pharmacological Hh pathway inhibition, resulted in BCC tumour regression and concomitant increased IFN-γ^+CD8^+ infiltration (Otsuka et al., 2015). As Shh signals to induce regulatory functions in skin T cells, my research indicates that Hh secretion might be a mechanism of immune evasion by skin tumours. Inhibition of Shh signalling in skin cancers will have the additional benefit of releasing T cells to generate an active immune response against the tumour.

It is important to note that pathways in addition to TGF-β signalling for T_{reg} function are involved in the dampening of skin inflammation by Gli2-

mediated transcription in T cells. The RNA-seq data have uncovered increased levels of *Ccl24* and *Ccl8* in skin $CD4^+$ T cells from Oxa-treated Gli2ΔC2 mice, and these chemokines recruit eosinophils to the skin (Islam et al., 2011, Menzies-Gow et al., 2002). In addition, expression of genes for inflammatory signalling pathways implicated in skin inflammation such as TNFR (*Tnfrsf25*), JAK-STAT (*Jak1*) and NF-κB (*Trl4*, *Myd88*, *Cd69*, *Cd48*, *Irf1*) (Kang et al., 2017, Kumari et al., 2013, Palanivel et al., 2014) were increased upon inhibition of Gli2-mediated transcription in skin T cells on induction of dermatitis. Negative regulators of Th2 (*Plcb3*) (Ando et al., 2014), are induced upon constitutive Gli2 expression on T cells, whereas *Jund* reported to repress skin inflammation (Di Meglio et al., 2014), is downregulated after Gli2 conditional inhibition further explaining the ameliorated skin condition in Gli2ΔN2 transgenic mice compared to WT and Gli2ΔC2 counterparts.

Currently, therapeutic research in AD mainly focuses on targeting Th2 cytokines (Gandhi et al., 2016). The observation that upregulated Shh in skin of mice with inflammation suppresses pro-inflammatory cytokine production could have implications for the treatment of dermatitis, as heightened levels of IFN-γ, IL-13 and IL-17 are linked with chronic AD in human patients (Gittler et al., 2012) It remains to be clarified if Hh signalling is elevated in human AD lesional and non-lesional skin and if Hh morphogens have a similar mechanism of action in humans as in mice. In conclusion I define the beneficial effects of Hh signalling in a mouse model with features of chronic AD, and show a mouse Gli2-TGF-β axis which suppresses pro-inflammatory cytokines and restores functional T_{reg}

populations that can be important for development of new strategies to treat atopic dermatitis.

Moreover, in the future it would be of interest to investigate the role of Hh signalling pathway in other skin inflammatory diseases, such as psoriasis. Psoriasis is a chronic skin inflammatory condition characterised by elevated Th17 responses in the skin, leading to keratinocyte hyperproliferation which causes the formation of plaques in epidermis (Blauvelt, 2008, Di Cesare et al., 2009). Major sources of IL-17 production are $CD4^+$ T cells, $CD8^+$ T cells and γδ T cells (Bettelli et al., 2007, Duan et al., 2010, Mangan et al., 2006, Srenathan et al., 2016, Sutton et al., 2009). Recent studies have shown that γδ T cells are a source of IL-17 production in the dermis of psoriatic mice and patients (Cai et al., 2011, Pantelyushin et al., 2012, Shibata et al., 2015). In this project I have already shown that conventional ($CD4^+$ and $CD8^+$) T cells, increased production of IL-17 upon inhibition of Gli2-mediated Hh transcription. Therefore, it would be interesting to examine if Gli2-driven Hh transcription has a protective role also against psoriasis and additionally focus on Hh pathway effects on γδ T cells in the context of the disease.

Finally, the much recent research focuses on the role of a small family of cells called innate lymphoid cells (ILCs) in many diseases including atopic dermatitis (Kim, 2015, Salimi and Ogg, 2014). There are three types of ILCs: ILC1, ILC2 and ILC3, grouped according to the cell lineage markers that they produce: IFN-γ for ILC1, IL-4, IL-5 and IL-13 in mice for ILC2s and IL-17 for ILC3s (Artis and Spits, 2015, Salimi and Ogg, 2014). The role of ILC2s in atopic march diseases such as asthma and atopic dermatitis is

established, and in skin they are critical of the surveillance of cutaneous homeostasis and abnormal elevation leads to dermatitis symptoms (Imai et al., 2013, Kim et al., 2014, Roediger et al., 2013, Salimi et al., 2013).

In preliminary experiments I used the GBS-GFP mice, discussed in Chapter 4, and I have shown that upon the induction of Oxa model of dermatitis, the Hh pathway was significantly activated in skin ILC2s. Taking into consideration that ILCs express the Lck promoter (Moro et al., 2010, Yang et al., 2013) and that they are present in RagKO mice, our Gli2ΔN2 and Gli2ΔC2 mice crossed with RagKO mice, would be appropriate mouse models to examine the role of inhibition or overexpression of Gli2-mediated Hh transcription on ILCs in the context of atopic dermatitis.

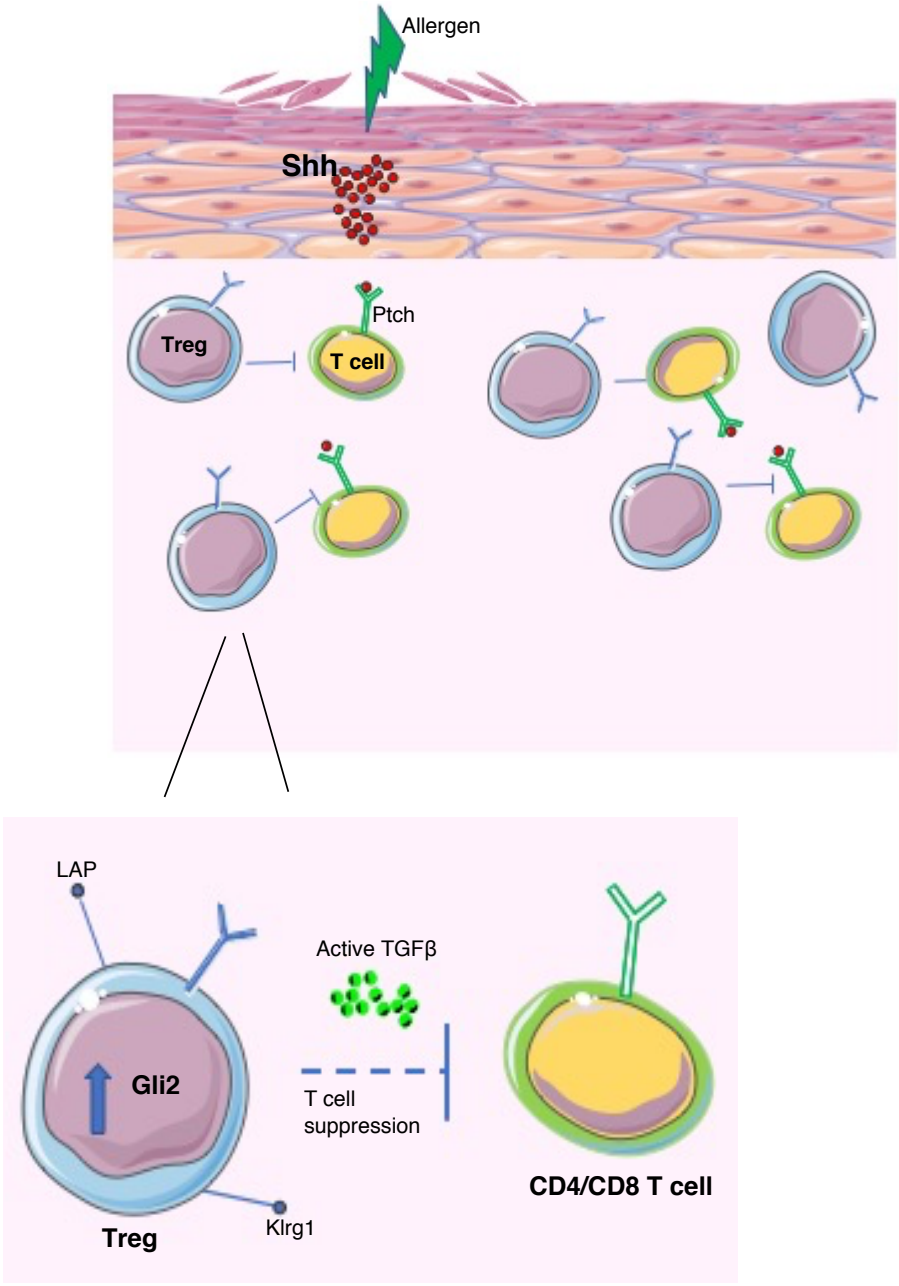


Figure 8-1: Mechanism of Hh signalling action in atopic dermatitis

The figure represents skin under atopic dermatitis conditions. When skin is disturbed, Shh is upregulated by epithelial cells and it binds to Ptch receptor on skin $CD4^+$ and $CD8^+$ T cells. Effector regulatory T cells suppress the harmful T cell responses via Gli2-mediated LAP upregulation and active TGF-β.

Chapter 9

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ABBAS, A. K., MURPHY, K. M. & SHER, A. 1996. Functional diversity of helper T lymphocytes. *Nature,* 383**,** 787-93.

ADOLPHE, C., HETHERINGTON, R., ELLIS, T. & WAINWRIGHT, B. 2006. Patched1 functions as a gatekeeper by promoting cell cycle progression. *Cancer Res,* 66**,** 2081-8.

ADOLPHE, C., NARANG, M., ELLIS, T., WICKING, C., KAUR, P. & WAINWRIGHT, B. 2004. An in vivo comparative study of sonic, desert and Indian hedgehog reveals that hedgehog pathway activity regulates epidermal stem cell homeostasis. *Development,* 131**,** 5009-19.

AEBISCHER, D., WILLRODT, A. H. & HALIN, C. 2014. Oxazolone-induced contact hypersensitivity reduces lymphatic drainage but enhances the induction of adaptive immunity. *PLoS One,* 9**,** e99297.

AGREN, M., KOGERMAN, P., KLEMAN, M. I., WESSLING, M. & TOFTGARD, R. 2004. Expression of the PTCH1 tumor suppressor gene is regulated by alternative promoters and a single functional Gli-binding site. *Gene,* 330**,** 101-14.

AHMAD-NEJAD, P., MRABET-DAHBI, S., BREUER, K., KLOTZ, M., WERFEL, T., HERZ, U., HEEG, K., NEUMAIER, M. & RENZ, H. 2004. The toll-like receptor 2 R753Q polymorphism defines a subgroup of patients with atopic dermatitis having severe phenotype. *J Allergy Clin Immunol,* 113**,** 565-7.

AHN, S. & JOYNER, A. L. 2004. Dynamic changes in the response of cells to positive hedgehog signaling during mouse limb patterning. *Cell,* 118**,** 505-16.

AHO, S., HARDING, C. R., LEE, J. M., MELDRUM, H. & BOSKO, C. A. 2012. Regulatory role for the profilaggrin N-terminal domain in epidermal homeostasis. *J Invest Dermatol,* 132**,** 2376-2385.

AIOI, A., TONOGAITO, H., SUTO, H., HAMADA, K., RA, C. R., OGAWA, H., MAIBACH, H. & MATSUDA, H. 2001. Impairment of skin barrier function in NC/Nga Tnd mice as a possible model for atopic dermatitis. *Br J Dermatol,* 144**,** 12-8.

AKDIS, C. A., AKDIS, M., BIEBER, T., BINDSLEV-JENSEN, C., BOGUNIEWICZ, M., EIGENMANN, P., HAMID, Q., KAPP, A., LEUNG, D. Y., LIPOZENCIC, J., LUGER, T. A., MURARO, A., NOVAK, N., PLATTS-MILLS, T. A., ROSENWASSER, L., SCHEYNIUS, A., SIMONS, F. E., SPERGEL, J., TURJANMAA, K., WAHN, U., WEIDINGER, S., WERFEL, T., ZUBERBIER, T., EUROPEAN ACADEMY OF, A., CLINICAL IMMUNOLOGY/AMERICAN ACADEMY OF ALLERGY, A. & IMMUNOLOGY 2006. Diagnosis and treatment of atopic dermatitis in children and adults: European Academy of Allergology and Clinical Immunology/American Academy of Allergy, Asthma and Immunology/PRACTALL Consensus Report. *J Allergy Clin Immunol,* 118**,** 152-69.

AKIYAMA, M. 2010. FLG mutations in ichthyosis vulgaris and atopic eczema: spectrum of mutations and population genetics. *Br J Dermatol,* 162**,** 472-7.

ALCEDO, J., AYZENZON, M., VON OHLEN, T., NOLL, M. & HOOPER, J. E. 1996. The Drosophila smoothened gene encodes a seven-pass membrane protein, a putative receptor for the hedgehog signal. *Cell,* 86**,** 221-32.

ALEXAKI, V. I., JAVELAUD, D., VAN KEMPEN, L. C., MOHAMMAD, K. S., DENNLER, S., LUCIANI, F., HOEK, K. S., JUAREZ, P., GOYDOS, J. S., FOURNIER, P. J., SIBON, C., BERTOLOTTO, C., VERRECCHIA, F., SAULE, S., DELMAS, V., BALLOTTI, R., LARUE, L., SAIAG, P., GUISE, T. A. & MAUVIEL, A. 2010. GLI2-mediated melanoma invasion and metastasis. *J Natl Cancer Inst,* 102**,** 1148-59.

ALLEN, B. L., SONG, J. Y., IZZI, L., ALTHAUS, I. W., KANG, J. S., CHARRON, F., KRAUSS, R. S. & MCMAHON, A. P. 2011. Overlapping roles and collective requirement for the coreceptors GAS1, CDO, and BOC in SHH pathway function. *Dev Cell,* 20**,** 775-87.

ALVAREZ, J. I., DODELET-DEVILLERS, A., KEBIR, H., IFERGAN, I., FABRE, P. J., TEROUZ, S., SABBAGH, M., WOSIK, K., BOURBONNIERE, L., BERNARD, M., VAN HORSSEN, J., DE VRIES, H. E., CHARRON, F. & PRAT, A. 2011. The Hedgehog Pathway Promotes Blood-Brain Barrier Integrity and CNS Immune Quiescence. *Science,* 334**,** 1727-1731.

AMAKYE, D., JAGANI, Z. & DORSCH, M. 2013. Unraveling the therapeutic potential of the Hedgehog pathway in cancer. *Nat Med,* 19**,** 1410-22.

ANDO, T., XIAO, W., GAO, P., NAMIRANIAN, S., MATSUMOTO, K., TOMIMORI, Y., HONG, H., YAMASHITA, H., KIMURA, M., KASHIWAKURA, J., HATA, T. R., IZUHARA, K., GURISH, M. F., ROERS, A., RAFAELS, N. M., BARNES, K. C., JAMORA, C., KAWAKAMI, Y. & KAWAKAMI, T. 2014. Critical role for mast cell Stat5 activity in skin inflammation. *Cell Rep,* 6**,** 366-76.

ANDREW, D. P., CHANG, M. S., MCNINCH, J., WATHEN, S. T., RIHANEK, M., TSENG, J., SPELLBERG, J. P. & ELIAS, C. G., 3RD 1998. STCP-1 (MDC) CC chemokine acts specifically on chronically activated Th2 lymphocytes and is produced by monocytes on stimulation with Th2 cytokines IL-4 and IL-13. *J Immunol,* 161**,** 5027-38.

ANDREW, D. P., RUFFING, N., KIM, C. H., MIAO, W., HEATH, H., LI, Y., MURPHY, K., CAMPBELL, J. J., BUTCHER, E. C. & WU, L. 2001. C-C chemokine receptor 4 expression defines a major subset of circulating nonintestinal memory T cells of both Th1 and Th2 potential. *J Immunol,* 166**,** 103-11.

ANNES, J. P., MUNGER, J. S. & RIFKIN, D. B. 2003. Making sense of latent TGFbeta activation. *J Cell Sci,* 116**,** 217-24.

ARTIS, D. & SPITS, H. 2015. The biology of innate lymphoid cells. *Nature,* 517**,** 293-301.

ASAI, J., TAKENAKA, H., KUSANO, K. F., II, M., LUEDEMANN, C., CURRY, C., EATON, E., IWAKURA, A., TSUTSUMI, Y., HAMADA, H., KISHIMOTO, S., THORNE, T., KISHORE, R. & LOSORDO, D. W. 2006. Topical sonic hedgehog gene therapy accelerates wound healing in diabetes by enhancing endothelial progenitor cell-mediated microvascular remodeling. *Circulation,* 113**,** 2413-24.

AUTENGRUBER, A., GEREKE, M., HANSEN, G., HENNIG, C. & BRUDER, D. 2012. Impact of enzymatic tissue disintegration on the level of surface molecule

expression and immune cell function. *Eur J Microbiol Immunol (Bp),* 2**,** 112-20.

AZA-BLANC, P., LIN, H. Y., RUIZ I ALTABA, A. & KORNBERG, T. B. 2000. Expression of the vertebrate Gli proteins in Drosophila reveals a distribution of activator and repressor activities. *Development,* 127**,** 4293-301.

BAI, C. B., AUERBACH, W., LEE, J. S., STEPHEN, D. & JOYNER, A. L. 2002. Gli2, but not Gli1, is required for initial Shh signaling and ectopic activation of the Shh pathway. *Development,* 129**,** 4753-61.

BAI, C. B. & JOYNER, A. L. 2001. Gli1 can rescue the in vivo function of Gli2. *Development,* 128**,** 5161-72.

BAI, C. B., STEPHEN, D. & JOYNER, A. L. 2004. All mouse ventral spinal cord patterning by hedgehog is Gli dependent and involves an activator function of Gli3. *Dev Cell,* 6**,** 103-15.

BALASKAS, N., RIBEIRO, A., PANOVSKA, J., DESSAUD, E., SASAI, N., PAGE, K. M., BRISCOE, J. & RIBES, V. 2012. Gene regulatory logic for reading the Sonic Hedgehog signaling gradient in the vertebrate neural tube. *Cell,* 148**,** 273-84.

BARBARULO, A., LAU, C. I., MENGRELIS, K., ROSS, S., SOLANKI, A., SALDANA, J. I. & CROMPTON, T. 2016. Hedgehog Signalling in the Embryonic Mouse Thymus. *J Dev Biol,* 4**,** 22.

BARKER, J. N., PALMER, C. N., ZHAO, Y., LIAO, H., HULL, P. R., LEE, S. P., ALLEN, M. H., MEGGITT, S. J., REYNOLDS, N. J., TREMBATH, R. C. & MCLEAN, W. H. 2007. Null mutations in the filaggrin gene (FLG) determine major susceptibility to early-onset atopic dermatitis that persists into adulthood. *J Invest Dermatol,* 127**,** 564-7.

BARNES, K. C. 2010. An update on the genetics of atopic dermatitis: scratching the surface in 2009. *J Allergy Clin Immunol,* 125**,** 16-29 e1-11; quiz 30-1.

BARTON, D., HOGENESCH, H. & WEIH, F. 2000. Mice lacking the transcription factor RelB develop T cell-dependent skin lesions similar to human atopic dermatitis. *Eur J Immunol,* 30**,** 2323-32.

BEACHY, P. A., COOPER, M. K., YOUNG, K. E., VON KESSLER, D. P., PARK, W. J., HALL, T. M., LEAHY, D. J. & PORTER, J. A. 1997. Multiple roles of cholesterol in hedgehog protein biogenesis and signaling. *Cold Spring Harb Symp Quant Biol,* 62**,** 191-204.

BEACHY, P. A., KARHADKAR, S. S. & BERMAN, D. M. 2004. Tissue repair and stem cell renewal in carcinogenesis. *Nature,* 432**,** 324-331.

BEATTIE, P. E. & LEWIS-JONES, M. S. 2006. A comparative study of impairment of quality of life in children with skin disease and children with other chronic childhood diseases. *Br J Dermatol,* 155**,** 145-51.

BEDOUI, S., WHITNEY, P. G., WAITHMAN, J., EIDSMO, L., WAKIM, L., CAMINSCHI, I., ALLAN, R. S., WOJTASIAK, M., SHORTMAN, K., CARBONE, F. R., BROOKS, A. G. & HEATH, W. R. 2009. Cross-presentation of viral and self antigens by skin-derived CD103+ dendritic cells. *Nat Immunol,* 10**,** 488-95.

BEHNIAFARD, N., GHARAGOZLOU, M., FARHADI, E., KHALEDI, M., SOTOUDEH, S., DARABI, B., FATHI, S. M., GHOLIZADEH MOGHADDAM, Z., MAHMOUDI, M., AGHAMOHAMMADI, A., AMIRZARGAR, A. A. & REZAEI, N. 2012. TNF-

alpha single nucleotide polymorphisms in atopic dermatitis. *Eur Cytokine Netw,* 23**,** 163-5.

BENCK, C. J., MARTINOV, T., FIFE, B. T. & CHATTERJEA, D. 2016. Isolation of Infiltrating Leukocytes from Mouse Skin Using Enzymatic Digest and Gradient Separation. *J Vis Exp***,** e53638.

BENNETT, C. L., CHRISTIE, J., RAMSDELL, F., BRUNKOW, M. E., FERGUSON, P. J., WHITESELL, L., KELLY, T. E., SAULSBURY, F. T., CHANCE, P. F. & OCHS, H. D. 2001. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet,* 27**,** 20-1.

BERG, E. L., YOSHINO, T., ROTT, L. S., ROBINSON, M. K., WARNOCK, R. A., KISHIMOTO, T. K., PICKER, L. J. & BUTCHER, E. C. 1991. The cutaneous lymphocyte antigen is a skin lymphocyte homing receptor for the vascular lectin endothelial cell-leukocyte adhesion molecule 1. *J Exp Med,* 174**,** 1461-6.

BETTELLI, E., KORN, T. & KUCHROO, V. K. 2007. Th17: the third member of the effector T cell trilogy. *Curr Opin Immunol,* 19**,** 652-7.

BHARDWAJ, G., MURDOCH, B., WU, D., BAKER, D. P., WILLIAMS, K. P., CHADWICK, K., LING, L. E., KARANU, F. N. & BHATIA, M. 2001. Sonic hedgehog induces the proliferation of primitive human hematopoietic cells via BMP regulation. *Nat Immunol,* 2**,** 172-80.

BIEBER, T. 2008. Mechanisms of disease: Atopic dermatitis. *New England Journal of Medicine,* 358**,** 1483-1494.

BIEBER, T. 2010. Atopic dermatitis. *Ann Dermatol,* 22**,** 125-37.

BIEDERMANN, T., ROCKEN, M. & CARBALLIDO, J. M. 2004. TH1 and TH2 lymphocyte development and regulation of TH cell-mediated immune responses of the skin. *J Investig Dermatol Symp Proc,* 9**,** 5-14.

BIEDERMANN, T., SCHWARZLER, C., LAMETSCHWANDTNER, G., THOMA, G., CARBALLIDO-PERRIG, N., KUND, J., DE VRIES, J. E., ROT, A. & CARBALLIDO, J. M. 2002. Targeting CLA/E-selectin interactions prevents CCR4-mediated recruitment of human Th2 memory cells to human skin in vivo. *Eur J Immunol,* 32**,** 3171-80.

BITGOOD, M. J., SHEN, L. & MCMAHON, A. P. 1996. Sertoli cell signaling by Desert hedgehog regulates the male germline. *Curr Biol,* 6**,** 298-304.

BLANPAIN, C. & FUCHS, E. 2014. Stem cell plasticity. Plasticity of epithelial stem cells in tissue regeneration. *Science,* 344**,** 1242281.

BLAUVELT, A. 2008. T-helper 17 cells in psoriatic plaques and additional genetic links between IL-23 and psoriasis. *J Invest Dermatol,* 128**,** 1064-7.

BLAYDON, D. C., BIANCHERI, P., DI, W. L., PLAGNOL, V., CABRAL, R. M., BROOKE, M. A., VAN HEEL, D. A., RUSCHENDORF, F., TOYNBEE, M., WALNE, A., O'TOOLE, E. A., MARTIN, J. E., LINDLEY, K., VULLIAMY, T., ABRAMS, D. J., MACDONALD, T. T., HARPER, J. I. & KELSELL, D. P. 2011. Inflammatory skin and bowel disease linked to ADAM17 deletion. *N Engl J Med,* 365**,** 1502-8.

BOGUNIEWICZ, M., ABRAMOVITS, W., PALLER, A., WHITAKER-WORTH, D. L., PRENDERGAST, M., CHENG, J. W., WANG, P. & TONG, K. B. 2007. A multiple-domain framework of clinical, economic, and patient-reported

outcomes for evaluating benefits of intervention in atopic dermatitis. *J Drugs Dermatol,* 6**,** 416-23.

BONIFACE, K., BERNARD, F. X., GARCIA, M., GURNEY, A. L., LECRON, J. C. & MOREL, F. 2005. IL-22 inhibits epidermal differentiation and induces proinflammatory gene expression and migration of human keratinocytes. *J Immunol,* 174**,** 3695-702.

BONIFACE, K., DIVEU, C., MOREL, F., PEDRETTI, N., FROGER, J., RAVON, E., GARCIA, M., VENEREAU, E., PREISSER, L., GUIGNOUARD, E., GUILLET, G., DAGREGORIO, G., PENE, J., MOLES, J. P., YSSEL, H., CHEVALIER, S., BERNARD, F. X., GASCAN, H. & LECRON, J. C. 2007. Oncostatin M secreted by skin infiltrating T lymphocytes is a potent keratinocyte activator involved in skin inflammation. *J Immunol,* 178**,** 4615-22.

BOS, J. D., ZONNEVELD, I., DAS, P. K., KRIEG, S. R., VAN DER LOOS, C. M. & KAPSENBERG, M. L. 1987. The skin immune system (SIS): distribution and immunophenotype of lymphocyte subpopulations in normal human skin. *J Invest Dermatol,* 88**,** 569-73.

BOTTING, R. A., BERTRAM, K. M., BAHARLOU, H., SANDGREN, K. J., FLETCHER, J., RHODES, J. W., RANA, H., PLASTO, T. M., WANG, X. M., LIM, J. J. K., BARNOUTI, L., KOHOUT, M. P., PAPADOPOULOS, T., MERTEN, S., OLBOURNE, N., CUNNINGHAM, A. L., HANIFFA, M. & HARMAN, A. N. 2017. Phenotypic and functional consequences of different isolation protocols on skin mononuclear phagocytes. *J Leukoc Biol,* 101**,** 1393-1403.

BRISCOE, J., CHEN, Y., JESSELL, T. M. & STRUHL, G. 2001. A hedgehog-insensitive form of patched provides evidence for direct long-range morphogen activity of sonic hedgehog in the neural tube. *Mol Cell,* 7**,** 1279-91.

BRISCOE, J. & THEROND, P. P. 2013. The mechanisms of Hedgehog signalling and its roles in development and disease. *Nature Reviews Molecular Cell Biology,* 14**,** 416-429.

BROMLEY, S. K., YAN, S., TOMURA, M., KANAGAWA, O. & LUSTER, A. D. 2013. Recirculating memory T cells are a unique subset of CD4+ T cells with a distinct phenotype and migratory pattern. *J Immunol,* 190**,** 970-6.

BROWN, S. J., KROBOTH, K., SANDILANDS, A., CAMPBELL, L. E., POHLER, E., KEZIC, S., CORDELL, H. J., MCLEAN, W. H. & IRVINE, A. D. 2012. Intragenic copy number variation within filaggrin contributes to the risk of atopic dermatitis with a dose-dependent effect. *J Invest Dermatol,* 132**,** 98-104.

BROWN, S. J. & MCLEAN, W. H. 2009. Eczema genetics: current state of knowledge and future goals. *J Invest Dermatol,* 129**,** 543-52.

BROWN, S. J., RELTON, C. L., LIAO, H., ZHAO, Y., SANDILANDS, A., MCLEAN, W. H., CORDELL, H. J. & REYNOLDS, N. J. 2009. Filaggrin haploinsufficiency is highly penetrant and is associated with increased severity of eczema: further delineation of the skin phenotype in a prospective epidemiological study of 792 school children. *Br J Dermatol,* 161**,** 884-9.

BROWNELL, I., GUEVARA, E., BAI, C. B., LOOMIS, C. A. & JOYNER, A. L. 2011. Nerve-derived sonic hedgehog defines a niche for hair follicle stem cells capable of becoming epidermal stem cells. *Cell Stem Cell,* 8**,** 552-65.

BUITRAGO, A., GYLFE, E., HENRIKSSON, C. & PERTOFT, H. 1977. Rapid isolation of pancreatic islets from collagenase digested pancreas by sedimentation through Percol at unit gravity. *Biochem Biophys Res Commun,* 79**,** 823-8.

BURGLIN, T. R. 2008. Evolution of hedgehog and hedgehog-related genes, their origin from Hog proteins in ancestral eukaryotes and discovery of a novel Hint motif. *BMC Genomics,* 9**,** 127.

BURKE, R., NELLEN, D., BELLOTTO, M., HAFEN, E., SENTI, K. A., DICKSON, B. J. & BASLER, K. 1999. Dispatched, a novel sterol-sensing domain protein dedicated to the release of cholesterol-modified hedgehog from signaling cells. *Cell,* 99**,** 803-15.

CAI, Y., SHEN, X., DING, C., QI, C., LI, K., LI, X., JALA, V. R., ZHANG, H. G., WANG, T., ZHENG, J. & YAN, J. 2011. Pivotal role of dermal IL-17-producing gammadelta T cells in skin inflammation. *Immunity,* 35**,** 596-610.

CALLAHAN, C. A., OFSTAD, T., HORNG, L., WANG, J. K., ZHEN, H. H., COULOMBE, P. A. & ORO, A. E. 2004. MIM/BEG4, a Sonic hedgehog-responsive gene that potentiates Gli-dependent transcription. *Genes Dev,* 18**,** 2724-9.

CALLEJO, A., CULI, J. & GUERRERO, I. 2008. Patched, the receptor of Hedgehog, is a lipoprotein receptor. *Proc Natl Acad Sci U S A,* 105**,** 912-7.

CAMPBELL, J. J., HARALDSEN, G., PAN, J., ROTTMAN, J., QIN, S., PONATH, P., ANDREW, D. P., WARNKE, R., RUFFING, N., KASSAM, N., WU, L. & BUTCHER, E. C. 1999. The chemokine receptor CCR4 in vascular recognition by cutaneous but not intestinal memory T cells. *Nature,* 400**,** 776-80.

CAPRONI, M., TORCHIA, D., ANTIGA, E., VOLPI, W., DEL BIANCO, E. & FABBRI, P. 2006. The effects of tacrolimus ointment on regulatory T lymphocytes in atopic dermatitis. *J Clin Immunol,* 26**,** 370-5.

CARADU, C., GUY, A., JAMES, C., REYNAUD, A., GADEAU, A. P. & RENAULT, M. A. 2018. Endogenous Sonic Hedgehog limits inflammation and angiogenesis in the ischaemic skeletal muscle of mice. *Cardiovasc Res,* 114**,** 759-770.

CARDONA, I. D., GOLEVA, E., OU, L. S. & LEUNG, D. Y. M. 2006. Staphylococcal enterotoxin B inhibits regulatory T cells by inducing glucocorticoid-induced TNF receptor-related protein ligand on monocytes. *Journal of Allergy and Clinical Immunology,* 117**,** 688-695.

CASPARY, T., GARCIA-GARCIA, M. J., HUANGFU, D., EGGENSCHWILER, J. T., WYLER, M. R., RAKEMAN, A. S., ALCORN, H. L. & ANDERSON, K. V. 2002. Mouse Dispatched homolog1 is required for long-range, but not juxtacrine, Hh signaling. *Curr Biol,* 12**,** 1628-32.

CHAN, L. S., ROBINSON, N. & XU, L. 2001. Expression of interleukin-4 in the epidermis of transgenic mice results in a pruritic inflammatory skin disease: an experimental animal model to study atopic dermatitis. *J Invest Dermatol,* 117**,** 977-83.

CHANG, D. T., LOPEZ, A., VON KESSLER, D. P., CHIANG, C., SIMANDL, B. K., ZHAO, R., SELDIN, M. F., FALLON, J. F. & BEACHY, P. A. 1994. Products, genetic linkage and limb patterning activity of a murine hedgehog gene. *Development,* 120**,** 3339-53.

CHARBONNIER, L. M., WANG, S., GEORGIEV, P., SEFIK, E. & CHATILA, T. A. 2015. Control of peripheral tolerance by regulatory T cell-intrinsic Notch signaling. *Nature Immunology,* 16**,** 1162-1173.

CHEN, J. K., TAIPALE, J., COOPER, M. K. & BEACHY, P. A. 2002a. Inhibition of Hedgehog signaling by direct binding of cyclopamine to Smoothened. *Genes Dev,* 16**,** 2743-8.

CHEN, J. K., TAIPALE, J., YOUNG, K. E., MAITI, T. & BEACHY, P. A. 2002b. Small molecule modulation of Smoothened activity. *Proc Natl Acad Sci U S A,* 99**,** 14071-6.

CHEN, M. L., YAN, B. S., BANDO, Y., KUCHROO, V. K. & WEINER, H. L. 2008. Latency-associated peptide identifies a novel CD4+CD25+ regulatory T cell subset with TGFbeta-mediated function and enhanced suppression of experimental autoimmune encephalomyelitis. *J Immunol,* 180**,** 7327-37.

CHEN, Y., LI, S., TONG, C., ZHAO, Y., WANG, B., LIU, Y., JIA, J. & JIANG, J. 2010. G protein-coupled receptor kinase 2 promotes high-level Hedgehog signaling by regulating the active state of Smo through kinase-dependent and kinase-independent mechanisms in Drosophila. *Genes Dev,* 24**,** 2054-67.

CHEN, Y. & STRUHL, G. 1996. Dual roles for patched in sequestering and transducing Hedgehog. *Cell,* 87**,** 553-63.

CHENG, G., YUAN, X., TSAI, M. S., PODACK, E. R., YU, A. & MALEK, T. R. 2012. IL-2 receptor signaling is essential for the development of Klrg1+ terminally differentiated T regulatory cells. *J Immunol,* 189**,** 1780-91.

CHIANG, C., LITINGTUNG, Y., LEE, E., YOUNG, K. E., CORDEN, J. L., WESTPHAL, H. & BEACHY, P. A. 1996. Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. *Nature,* 383**,** 407-13.

CHIANG, C., SWAN, R. Z., GRACHTCHOUK, M., BOLINGER, M., LITINGTUNG, Y., ROBERTSON, E. K., COOPER, M. K., GAFFIELD, W., WESTPHAL, H., BEACHY, P. A. & DLUGOSZ, A. A. 1999. Essential role for Sonic hedgehog during hair follicle morphogenesis. *Dev Biol,* 205**,** 1-9.

CHODACZEK, G., PAPANNA, V., ZAL, M. A. & ZAL, T. 2012. Body-barrier surveillance by epidermal gammadelta TCRs. *Nat Immunol,* 13**,** 272-82.

CHUANG, P. T., KAWCAK, T. & MCMAHON, A. P. 2003. Feedback control of mammalian Hedgehog signaling by the Hedgehog-binding protein, Hip1, modulates Fgf signaling during branching morphogenesis of the lung. *Genes Dev,* 17**,** 342-7.

CHUNG, M. K., KIM, H. J., LEE, Y. S., HAN, M. E., YOON, S., BAEK, S. Y., KIM, B. S., KIM, J. B. & OH, S. O. 2010. Hedgehog signaling regulates proliferation of prostate cancer cells via stathmin1. *Clin Exp Med,* 10**,** 51-7.

CLARK, R. A., CHONG, B., MIRCHANDANI, N., BRINSTER, N. K., YAMANAKA, K., DOWGIERT, R. K. & KUPPER, T. S. 2006. The vast majority of CLA+ T cells are resident in normal skin. *J Immunol,* 176**,** 4431-9.

CLARK, R. A., WATANABE, R., TEAGUE, J. E., SCHLAPBACH, C., TAWA, M. C., ADAMS, N., DOROSARIO, A. A., CHANEY, K. S., CUTLER, C. S., LEBOEUF, N. R., CARTER, J. B., FISHER, D. C. & KUPPER, T. S. 2012. Skin effector memory T cells do not recirculate and provide immune protection in alemtuzumab-treated CTCL patients. *Sci Transl Med,* 4**,** 117ra7.

COBOURNE, M. T., HARDCASTLE, Z. & SHARPE, P. T. 2001. Sonic hedgehog regulates epithelial proliferation and cell survival in the developing tooth germ. *J Dent Res,* 80**,** 1974-9.

CORCORAN, R. B. & SCOTT, M. P. 2006. Oxysterols stimulate Sonic hedgehog signal transduction and proliferation of medulloblastoma cells. *Proc Natl Acad Sci U S A,* 103**,** 8408-13.

CORNELISSEN, C., MARQUARDT, Y., CZAJA, K., WENZEL, J., FRANK, J., LUSCHER-FIRZLAFF, J., LUSCHER, B. & BARON, J. M. 2012. IL-31 regulates differentiation and filaggrin expression in human organotypic skin models. *J Allergy Clin Immunol,* 129**,** 426-33, 433 e1-8.

CORTES, J. R., SANCHEZ-DIAZ, R., BOVOLENTA, E. R., BARREIRO, O., LASARTE, S., MATESANZ-MARIN, A., TORIBIO, M. L., SANCHEZ-MADRID, F. & MARTIN, P. 2014. Maintenance of immune tolerance by Foxp3+ regulatory T cells requires CD69 expression. *J Autoimmun,* 55**,** 51-62.

CRIDLAND, S. O., KEYS, J. R., PAPATHANASIOU, P. & PERKINS, A. C. 2009. Indian hedgehog supports definitive erythropoiesis. *Blood Cells Mol Dis,* 43**,** 149-55.

CROMPTON, T., OUTRAM, S. V. & HAGER-THEODORIDES, A. L. 2007. Sonic hedgehog signalling in T-cell development and activation. *Nat Rev Immunol,* 7**,** 726-35.

CRON, R. Q., GAJEWSKI, T. F., SHARROW, S. O., FITCH, F. W., MATIS, L. A. & BLUESTONE, J. A. 1989. Phenotypic and functional analysis of murine CD3+,CD4-,CD8- TCR-gamma delta-expressing peripheral T cells. *J Immunol,* 142**,** 3754-62.

CZARNOWICKI, T., SANTAMARIA-BABI, L. F. & GUTTMAN-YASSKY, E. 2017. Circulating CLA(+) T cells in atopic dermatitis and their possible role as peripheral biomarkers. *Allergy,* 72**,** 366-372.

DAI, P., AKIMARU, H., TANAKA, Y., MAEKAWA, T., NAKAFUKU, M. & ISHII, S. 1999. Sonic Hedgehog-induced activation of the Gli1 promoter is mediated by GLI3. *J Biol Chem,* 274**,** 8143-52.

DANSO, M. O., VAN DRONGELEN, V., MULDER, A., VAN ESCH, J., SCOTT, H., VAN SMEDEN, J., EL GHALBZOURI, A. & BOUWSTRA, J. A. 2014. TNF-alpha and Th2 cytokines induce atopic dermatitis-like features on epidermal differentiation proteins and stratum corneum lipids in human skin equivalents. *J Invest Dermatol,* 134**,** 1941-1950.

DARDALHON, V., AWASTHI, A., KWON, H., GALILEOS, G., GAO, W., SOBEL, R. A., MITSDOERFFER, M., STROM, T. B., ELYAMAN, W., HO, I. C., KHOURY, S., OUKKA, M. & KUCHROO, V. K. 2008a. IL-4 inhibits TGF-beta-induced Foxp3+ T cells and, together with TGF-beta, generates IL-9+ IL-10+ Foxp3(-) effector T cells. *Nat Immunol,* 9**,** 1347-55.

DARDALHON, V., KORN, T., KUCHROO, V. K. & ANDERSON, A. C. 2008b. Role of Th1 and Th17 cells in organ-specific autoimmunity. *J Autoimmun,* 31**,** 252-6.

DE BENEDETTO, A., RAFAELS, N. M., MCGIRT, L. Y., IVANOV, A. I., GEORAS, S. N., CHEADLE, C., BERGER, A. E., ZHANG, K., VIDYASAGAR, S., YOSHIDA, T., BOGUNIEWICZ, M., HATA, T., SCHNEIDER, L. C., HANIFIN, J. M., GALLO, R. L., NOVAK, N., WEIDINGER, S., BEATY, T. H., LEUNG, D. Y., BARNES, K. C. &

BECK, L. A. 2011. Tight junction defects in patients with atopic dermatitis. *J Allergy Clin Immunol,* 127**,** 773-86 e1-7.

DE LA ROCHE, M., RITTER, A. T., ANGUS, K. L., DINSMORE, C., EARNSHAW, C. H., REITER, J. F. & GRIFFITHS, G. M. 2013. Hedgehog signaling controls T cell killing at the immunological synapse. *Science,* 342**,** 1247-50.

DEB PAL, A. & BANERJEE, S. 2015. Epstein-Barr virus latent membrane protein 2A mediated activation of Sonic Hedgehog pathway induces HLA class Ia downregulation in gastric cancer cells. *Virology,* 484**,** 22-32.

DENNLER, S., ANDRE, J., ALEXAKI, I., LI, A., MAGNALDO, T., TEN DIJKE, P., WANG, X. J., VERRECCHIA, F. & MAUVIEL, A. 2007. Induction of sonic hedgehog mediators by transforming growth factor-beta: Smad3-dependent activation of Gli2 and Gli1 expression in vitro and in vivo. *Cancer Research,* 67**,** 6981-6986.

DEPIANTO, D., KERNS, M. L., DLUGOSZ, A. A. & COULOMBE, P. A. 2010. Keratin 17 promotes epithelial proliferation and tumor growth by polarizing the immune response in skin. *Nat Genet,* 42**,** 910-4.

DESSAUD, E., MCMAHON, A. P. & BRISCOE, J. 2008. Pattern formation in the vertebrate neural tube: a sonic hedgehog morphogen-regulated transcriptional network. *Development,* 135**,** 2489-503.

DESSAUD, E., YANG, L. L., HILL, K., COX, B., ULLOA, F., RIBEIRO, A., MYNETT, A., NOVITCH, B. G. & BRISCOE, J. 2007. Interpretation of the sonic hedgehog morphogen gradient by a temporal adaptation mechanism. *Nature,* 450**,** 717-20.

DI CESARE, A., DI MEGLIO, P. & NESTLE, F. O. 2009. The IL-23/Th17 axis in the immunopathogenesis of psoriasis. *J Invest Dermatol,* 129**,** 1339-50.

DI MEGLIO, P., DUARTE, J. H., AHLFORS, H., OWENS, N. D., LI, Y., VILLANOVA, F., TOSI, I., HIROTA, K., NESTLE, F. O., MROWIETZ, U., GILCHRIST, M. J. & STOCKINGER, B. 2014. Activation of the aryl hydrocarbon receptor dampens the severity of inflammatory skin conditions. *Immunity,* 40**,** 989-1001.

DI MEGLIO, P., PERERA, G. K. & NESTLE, F. O. 2011. The Multitasking Organ: Recent Insights into Skin Immune Function. *Immunity,* 35**,** 857-869.

DIEU-NOSJEAN, M. C., MASSACRIER, C., HOMEY, B., VANBERVLIET, B., PIN, J. J., VICARI, A., LEBECQUE, S., DEZUTTER-DAMBUYANT, C., SCHMITT, D., ZLOTNIK, A. & CAUX, C. 2000. Macrophage inflammatory protein 3alpha is expressed at inflamed epithelial surfaces and is the most potent chemokine known in attracting Langerhans cell precursors. *J Exp Med,* 192**,** 705-18.

DILLON, S. R., SPRECHER, C., HAMMOND, A., BILSBOROUGH, J., ROSENFELD-FRANKLIN, M., PRESNELL, S. R., HAUGEN, H. S., MAURER, M., HARDER, B., JOHNSTON, J., BORT, S., MUDRI, S., KUIJPER, J. L., BUKOWSKI, T., SHEA, P., DONG, D. L., DASOVICH, M., GRANT, F. J., LOCKWOOD, L., LEVIN, S. D., LECIEL, C., WAGGIE, K., DAY, H., TOPOUZIS, S., KRAMER, J., KUESTNER, R., CHEN, Z., FOSTER, D., PARRISH-NOVAK, J. & GROSS, J. A. 2004. Interleukin 31, a cytokine produced by activated T cells, induces dermatitis in mice. *Nat Immunol,* 5**,** 752-60.

DOLD, S., WJST, M., VON MUTIUS, E., REITMEIR, P. & STIEPEL, E. 1992. Genetic risk for asthma, allergic rhinitis, and atopic dermatitis. *Arch Dis Child,* 67**,** 1018-22.

DRAKOPOULOU, E., OUTRAM, S. V., ROWBOTHAM, N. J., ROSS, S. E., FURMANSKI, A. L., SALDANA, J. I., HAGER-THEODORIDES, A. L. & CROMPTON, T. 2010. Non-redundant role for the transcription factor Gli1 at multiple stages of thymocyte development. *Cell Cycle,* 9**,** 4144-52.

DUAN, J., CHUNG, H., TROY, E. & KASPER, D. L. 2010. Microbial colonization drives expansion of IL-1 receptor 1-expressing and IL-17-producing gamma/delta T cells. *Cell Host Microbe,* 7**,** 140-50.

DUDDA, J. C., PERDUE, N., BACHTANIAN, E. & CAMPBELL, D. J. 2008. Foxp3+ regulatory T cells maintain immune homeostasis in the skin. *J Exp Med,* 205**,** 1559-65.

DUMAN-SCHEEL, M., WENG, L., XIN, S. & DU, W. 2002. Hedgehog regulates cell growth and proliferation by inducing Cyclin D and Cyclin E. *Nature,* 417**,** 299-304.

DUMORTIER, A., DURHAM, A. D., DI PIAZZA, M., VAUCLAIR, S., KOCH, U., FERRAND, G., FERRERO, I., DEMEHRI, S., SONG, L. L., FARR, A. G., LEONARD, W. J., KOPAN, R., MIELE, L., HOHL, D., FINKE, D. & RADTKE, F. 2010. Atopic dermatitis-like disease and associated lethal myeloproliferative disorder arise from loss of Notch signaling in the murine skin. *PLoS One,* 5**,** e9258.

DWYER, J. R., SEVER, N., CARLSON, M., NELSON, S. F., BEACHY, P. A. & PARHAMI, F. 2007. Oxysterols are novel activators of the hedgehog signaling pathway in pluripotent mesenchymal cells. *J Biol Chem,* 282**,** 8959-68.

DYER, M. A., FARRINGTON, S. M., MOHN, D., MUNDAY, J. R. & BARON, M. H. 2001. Indian hedgehog activates hematopoiesis and vasculogenesis and can respecify prospective neurectodermal cell fate in the mouse embryo. *Development,* 128**,** 1717-30.

ECHELARD, Y., EPSTEIN, D. J., ST-JACQUES, B., SHEN, L., MOHLER, J., MCMAHON, J. A. & MCMAHON, A. P. 1993. Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. *Cell,* 75**,** 1417-30.

EKANAYAKE-MUDIYANSELAGE, S., ASCHAUER, H., SCHMOOK, F. P., JENSEN, J. M., MEINGASSNER, J. G. & PROKSCH, E. 1998. Expression of epidermal keratins and the cornified envelope protein involucrin is influenced by permeability barrier disruption. *J Invest Dermatol,* 111**,** 517-23.

EL ANDALOUSSI, A., GRAVES, S., MENG, F., MANDAL, M., MASHAYEKHI, M. & AIFANTIS, I. 2006. Hedgehog signaling controls thymocyte progenitor homeostasis and differentiation in the thymus. *Nat Immunol,* 7**,** 418-26.

ELLIS, T., SMYTH, I., RILEY, E., GRAHAM, S., ELLIOT, K., NARANG, M., KAY, G. F., WICKING, C. & WAINWRIGHT, B. 2003. Patched 1 conditional null allele in mice. *Genesis,* 36**,** 158-61.

EPSTEIN, E. H. 2008. Basal cell carcinomas: attack of the hedgehog. *Nat Rev Cancer,* 8**,** 743-54.

EPSTEIN, E. H., JR. & MUNDERLOH, N. H. 1978. Human skin collagen. Presence of type I and type III at all levels of the dermis. *J Biol Chem,* 253**,** 1336-7.

ESPARZA-GORDILLO, J., WEIDINGER, S., FOLSTER-HOLST, R., BAUERFEIND, A., RUSCHENDORF, F., PATONE, G., ROHDE, K., MARENHOLZ, I., SCHULZ, F., KERSCHER, T., HUBNER, N., WAHN, U., SCHREIBER, S., FRANKE, A., VOGLER, R., HEATH, S., BAURECHT, H., NOVAK, N., RODRIGUEZ, E., ILLIG, T., LEE-KIRSCH, M. A., CIECHANOWICZ, A., KUREK, M., PISKACKOVA, T., MACEK, M., LEE, Y. A. & RUETHER, A. 2009. A common variant on chromosome 11q13 is associated with atopic dermatitis. *Nat Genet,* 41**,** 596-601.

EUGSTER, C., PANAKOVA, D., MAHMOUD, A. & EATON, S. 2007. Lipoprotein-heparan sulfate interactions in the Hh pathway. *Dev Cell,* 13**,** 57-71.

EWALD, D. A., NODA, S., OLIVA, M., LITMAN, T., NAKAJIMA, S., LI, X., XU, H., WORKMAN, C. T., SCHEIPERS, P., SVITACHEVA, N., LABUDA, T., KRUEGER, J. G., SUAREZ-FARINAS, M., KABASHIMA, K. & GUTTMAN-YASSKY, E. 2017. Major differences between human atopic dermatitis and murine models, as determined by using global transcriptomic profiling. *J Allergy Clin Immunol,* 139**,** 562-571.

FAHLEN, L., READ, S., GORELIK, L., HURST, S. D., COFFMAN, R. L., FLAVELL, R. A. & POWRIE, F. 2005. T cells that cannot respond to TGF-beta escape control by CD4(+)CD25(+) regulatory T cells. *J Exp Med,* 201**,** 737-46.

FAIAO-FLORES, F., ALVES-FERNANDES, D. K., PENNACCHI, P. C., SANDRI, S., VICENTE, A. L., SCAPULATEMPO-NETO, C., VAZQUEZ, V. L., REIS, R. M., CHAUHAN, J., GODING, C. R., SMALLEY, K. S. & MARIA-ENGLER, S. S. 2017. Targeting the hedgehog transcription factors GLI1 and GLI2 restores sensitivity to vemurafenib-resistant human melanoma cells. *Oncogene,* 36**,** 1849-1861.

FALLON, P. G., SASAKI, T., SANDILANDS, A., CAMPBELL, L. E., SAUNDERS, S. P., MANGAN, N. E., CALLANAN, J. J., KAWASAKI, H., SHIOHAMA, A., KUBO, A., SUNDBERG, J. P., PRESLAND, R. B., FLECKMAN, P., SHIMIZU, N., KUDOH, J., IRVINE, A. D., AMAGAI, M. & MCLEAN, W. H. 2009. A homozygous frameshift mutation in the mouse Flg gene facilitates enhanced percutaneous allergen priming. *Nat Genet,* 41**,** 602-8.

FARRAR, J. D., ASNAGLI, H. & MURPHY, K. M. 2002. T helper subset development: roles of instruction, selection, and transcription. *J Clin Invest,* 109**,** 431-5.

FELDMEYER, L., WERNER, S., FRENCH, L. E. & BEER, H. D. 2010. Interleukin-1, inflammasomes and the skin. *Eur J Cell Biol,* 89**,** 638-44.

FEUERER, M., HILL, J. A., KRETSCHMER, K., VON BOEHMER, H., MATHIS, D. & BENOIST, C. 2010. Genomic definition of multiple ex vivo regulatory T cell subphenotypes. *Proc Natl Acad Sci U S A,* 107**,** 5919-24.

FONTENOT, J. D., GAVIN, M. A. & RUDENSKY, A. Y. 2003. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol,* 4**,** 330-6.

FORSTER, R., DAVALOS-MISSLITZ, A. C. & ROT, A. 2008. CCR7 and its ligands: balancing immunity and tolerance. *Nat Rev Immunol,* 8**,** 362-71.

FOWLER, A. J., SHEU, M. Y., SCHMUTH, M., KAO, J., FLUHR, J. W., RHEIN, L., COLLINS, J. L., WILLSON, T. M., MANGELSDORF, D. J., ELIAS, P. M. & FEINGOLD, K. R. 2003. Liver X receptor activators display anti-inflammatory activity in irritant and allergic contact dermatitis models:

liver-X-receptor-specific inhibition of inflammation and primary cytokine production. *J Invest Dermatol,* 120**,** 246-55.

FRANK-KAMENETSKY, M., ZHANG, X. M., BOTTEGA, S., GUICHERIT, O., WICHTERLE, H., DUDEK, H., BUMCROT, D., WANG, F. Y., JONES, S., SHULOK, J., RUBIN, L. L. & PORTER, J. A. 2002. Small-molecule modulators of Hedgehog signaling: identification and characterization of Smoothened agonists and antagonists. *J Biol,* 1**,** 10.

FRIMPONG-BOATENG, K., VAN ROOIJEN, N. & GEIBEN-LYNN, R. 2010. Regulatory T cells suppress natural killer cells during plasmid DNA vaccination in mice, blunting the CD8+ T cell immune response by the cytokine TGFbeta. *PLoS One,* 5**,** e12281.

FUCCILLO, M., JOYNER, A. L. & FISHELL, G. 2006. Morphogen to mitogen: the multiple roles of hedgehog signalling in vertebrate neural development. *Nat Rev Neurosci,* 7**,** 772-83.

FUCHS, E. 2007. Scratching the surface of skin development. *Nature,* 445**,** 834-42.

FUCHS, E. 2008. Skin stem cells: rising to the surface. *J Cell Biol,* 180**,** 273-84.

FURLER, R. L. & UITTENBOGAART, C. H. 2012. GLI2 regulates TGF-beta1 in human CD4+ T cells: implications in cancer and HIV pathogenesis. *PLoS One,* 7**,** e40874.

FURMANSKI, A. L., BARBARULO, A., SOLANKI, A., LAU, C. I., SAHNI, H., SALDANA, J. I., D'ACQUISTO, F. & CROMPTON, T. 2015. The transcriptional activator Gli2 modulates T-cell receptor signalling through attenuation of AP-1 and NFkappaB activity. *J Cell Sci,* 128**,** 2085-95.

FURMANSKI, A. L., SALDANA, J. I., ONO, M., SAHNI, H., PASCHALIDIS, N., D'ACQUISTO, F. & CROMPTON, T. 2013. Tissue-derived hedgehog proteins modulate Th differentiation and disease. *J Immunol,* 190**,** 2641-9.

FURMANSKI, A. L., SALDANA, J. I., ROWBOTHAM, N. J., ROSS, S. E. & CROMPTON, T. 2012. Role of Hedgehog signalling at the transition from double-positive to single-positive thymocyte. *Eur J Immunol,* 42**,** 489-99.

GAILANI, M. R., STAHLE-BACKDAHL, M., LEFFELL, D. J., GLYNN, M., ZAPHIROPOULOS, P. G., PRESSMAN, C., UNDEN, A. B., DEAN, M., BRASH, D. E., BALE, A. E. & TOFTGARD, R. 1996. The role of the human homologue of Drosophila patched in sporadic basal cell carcinomas. *Nat Genet,* 14**,** 78-81.

GANDHI, N. A., BENNETT, B. L., GRAHAM, N. M., PIROZZI, G., STAHL, N. & YANCOPOULOS, G. D. 2016. Targeting key proximal drivers of type 2 inflammation in disease. *Nat Rev Drug Discov,* 15**,** 35-50.

GAO, J., GRAVES, S., KOCH, U., LIU, S., JANKOVIC, V., BUONAMICI, S., EL ANDALOUSSI, A., NIMER, S. D., KEE, B. L., TAICHMAN, R., RADTKE, F. & AIFANTIS, I. 2009a. Hedgehog signaling is dispensable for adult hematopoietic stem cell function. *Cell Stem Cell,* 4**,** 548-58.

GAO, P. S., RAFAELS, N. M., HAND, T., MURRAY, T., BOGUNIEWICZ, M., HATA, T., SCHNEIDER, L., HANIFIN, J. M., GALLO, R. L., GAO, L., BEATY, T. H., BECK, L. A., BARNES, K. C. & LEUNG, D. Y. 2009b. Filaggrin mutations that confer risk of atopic dermatitis confer greater risk for eczema herpeticum. *J Allergy Clin Immunol,* 124**,** 507-13, 513 e1-7.

GAO, P. S., RAFAELS, N. M., MU, D., HAND, T., MURRAY, T., BOGUNIEWICZ, M., HATA, T., SCHNEIDER, L., HANIFIN, J. M., GALLO, R. L., GAO, L., BEATY, T. H., BECK, L. A., LEUNG, D. Y. & BARNES, K. C. 2010. Genetic variants in thymic stromal lymphopoietin are associated with atopic dermatitis and eczema herpeticum. *J Allergy Clin Immunol,* 125**,** 1403-1407 e4.

GEBHARDT, T., WAKIM, L. M., EIDSMO, L., READING, P. C., HEATH, W. R. & CARBONE, F. R. 2009. Memory T cells in nonlymphoid tissue that provide enhanced local immunity during infection with herpes simplex virus. *Nat Immunol,* 10**,** 524-30.

GEBHARDT, T., WHITNEY, P. G., ZAID, A., MACKAY, L. K., BROOKS, A. G., HEATH, W. R., CARBONE, F. R. & MUELLER, S. N. 2011. Different patterns of peripheral migration by memory CD4+ and CD8+ T cells. *Nature,* 477**,** 216-9.

GITTLER, J. K., SHEMER, A., SUAREZ-FARINAS, M., FUENTES-DUCULAN, J., GULEWICZ, K. J., WANG, C. Q., MITSUI, H., CARDINALE, I., DE GUZMAN STRONG, C., KRUEGER, J. G. & GUTTMAN-YASSKY, E. 2012. Progressive activation of T(H)2/T(H)22 cytokines and selective epidermal proteins characterizes acute and chronic atopic dermatitis. *J Allergy Clin Immunol,* 130**,** 1344-54.

GODDEERIS, M. M., RHO, S., PETIET, A., DAVENPORT, C. L., JOHNSON, G. A., MEYERS, E. N. & KLINGENSMITH, J. 2008. Intracardiac septation requires hedgehog-dependent cellular contributions from outside the heart. *Development,* 135**,** 1887-95.

GRACHTCHOUK, M., MO, R., YU, S., ZHANG, X., SASAKI, H., HUI, C. C. & DLUGOSZ, A. A. 2000. Basal cell carcinomas in mice overexpressing Gli2 in skin. *Nat Genet,* 24**,** 216-7.

GRACHTCHOUK, M., PERO, J., YANG, S. H., ERMILOV, A. N., MICHAEL, L. E., WANG, A., WILBERT, D., PATEL, R. M., FERRIS, J., DIENER, J., ALLEN, M., LIM, S., SYU, L. J., VERHAEGEN, M. & DLUGOSZ, A. A. 2011. Basal cell carcinomas in mice arise from hair follicle stem cells and multiple epithelial progenitor populations. *J Clin Invest,* 121**,** 1768-81.

GRANATO, M., ZOMPETTA, C., VESCARELLI, E., RIZZELLO, C., CARDI, A., VALIA, S., ANTONELLI, G., MARCHESE, C., TORRISI, M. R., FAGGIONI, A. & CIRONE, M. 2016. HCV derived from sera of HCV-infected patients induces pro-fibrotic effects in human primary fibroblasts by activating GLI2. *Sci Rep,* 6**,** 30649.

GRAY, E. E., RAMIREZ-VALLE, F., XU, Y., WU, S., WU, Z., KARJALAINEN, K. E. & CYSTER, J. G. 2013. Deficiency in IL-17-committed Vgamma4(+) gammadelta T cells in a spontaneous Sox13-mutant CD45.1(+) congenic mouse substrain provides protection from dermatitis. *Nat Immunol,* 14**,** 584-92.

GREWE, M., WALTHER, S., GYUFKO, K., CZECH, W., SCHOPF, E. & KRUTMANN, J. 1995. Analysis of the cytokine pattern expressed in situ in inhalant allergen patch test reactions of atopic dermatitis patients. *J Invest Dermatol,* 105**,** 407-10.

GRICE, E. A. & SEGRE, J. A. 2011. The skin microbiome. *Nat Rev Microbiol,* 9**,** 244-53.

GRICHNIK, J. M. 2008. Melanoma, nevogenesis, and stem cell biology. *J Invest Dermatol,* 128**,** 2365-80.

GRIGORYEV, D. N., HOWELL, M. D., WATKINS, T. N., CHEN, Y. C., CHEADLE, C., BOGUNIEWICZ, M., BARNES, K. C. & LEUNG, D. Y. 2010. Vaccinia virus-specific molecular signature in atopic dermatitis skin. *J Allergy Clin Immunol,* 125**,** 153-159 e28.

GRINDEBACKE, H., WING, K., ANDERSSON, A. C., SURI-PAYER, E., RAK, S. & RUDIN, A. 2004. Defective suppression of Th2 cytokines by CD4CD25 regulatory T cells in birch allergics during birch pollen season. *Clin Exp Allergy,* 34**,** 1364-72.

GRUBER, R., ELIAS, P. M., CRUMRINE, D., LIN, T. K., BRANDNER, J. M., HACHEM, J. P., PRESLAND, R. B., FLECKMAN, P., JANECKE, A. R., SANDILANDS, A., MCLEAN, W. H., FRITSCH, P. O., MILDNER, M., TSCHACHLER, E. & SCHMUTH, M. 2011. Filaggrin genotype in ichthyosis vulgaris predicts abnormalities in epidermal structure and function. *Am J Pathol,* 178**,** 2252-63.

GUDJONSSON, J. E., JOHNSTON, A., DYSON, M., VALDIMARSSON, H. & ELDER, J. T. 2007. Mouse models of psoriasis. *J Invest Dermatol,* 127**,** 1292-308.

GUDJONSSON, J. E., JOHNSTON, A., STOLL, S. W., RIBLETT, M. B., XING, X., KOCHKODAN, J. J., DING, J., NAIR, R. P., APHALE, A., VOORHEES, J. J. & ELDER, J. T. 2010. Evidence for altered Wnt signaling in psoriatic skin. *J Invest Dermatol,* 130**,** 1849-59.

GUILLIAMS, M., CROZAT, K., HENRI, S., TAMOUTOUNOUR, S., GRENOT, P., DEVILARD, E., DE BOVIS, B., ALEXOPOULOU, L., DALOD, M. & MALISSEN, B. 2010. Skin-draining lymph nodes contain dermis-derived CD103(-) dendritic cells that constitutively produce retinoic acid and induce Foxp3(+) regulatory T cells. *Blood,* 115**,** 1958-68.

GUPTA, S., TAKEBE, N. & LORUSSO, P. 2010. Targeting the Hedgehog pathway in cancer. *Ther Adv Med Oncol,* 2**,** 237-50.

GUTTMAN-YASSKY, E., LOWES, M. A., FUENTES-DUCULAN, J., ZABA, L. C., CARDINALE, I., NOGRALES, K. E., KHATCHERIAN, A., NOVITSKAYA, I., CARUCCI, J. A., BERGMAN, R. & KRUEGER, J. G. 2008. Low Expression of the IL-23/Th17 Pathway in Atopic Dermatitis Compared to Psoriasis. *Journal of Immunology,* 181**,** 7420-7427.

GUTTMAN-YASSKY, E., SUAREZ-FARINAS, M., CHIRICOZZI, A., NOGRALES, K. E., SHEMER, A., FUENTES-DUCULAN, J., CARDINALE, I., LIN, P., BERGMAN, R., BOWCOCK, A. M. & KRUEGER, J. G. 2009. Broad defects in epidermal cornification in atopic dermatitis identified through genomic analysis. *J Allergy Clin Immunol,* 124**,** 1235-1244 e58.

HAGER-THEODORIDES, A. L., DESSENS, J. T., OUTRAM, S. V. & CROMPTON, T. 2005. The transcription factor Gli3 regulates differentiation of fetal CD4(-)CD8(-) double-negative thymocytes. *Blood,* 106**,** 1296-1304.

HAGER-THEODORIDES, A. L., FURMANSKI, A. L., ROSS, S. E., OUTRAM, S. V., ROWBOTHAM, N. J. & CROMPTON, T. 2009. The Gli3 transcription factor expressed in the thymus stroma controls thymocyte negative selection via Hedgehog-dependent and -independent mechanisms. *J Immunol,* 183**,** 3023-32.

HAN, S. C., KOO, D. H., KANG, N. J., YOON, W. J., KANG, G. J., KANG, H. K. & YOO, E. S. 2015. Docosahexaenoic Acid Alleviates Atopic Dermatitis by Generating Tregs and IL-10/TGF-beta-Modified Macrophages via a TGF-beta-Dependent Mechanism. *J Invest Dermatol,* 135**,** 1556-1564.

HAN, Y. G., KWOK, B. H. & KERNAN, M. J. 2003. Intraflagellar transport is required in Drosophila to differentiate sensory cilia but not sperm. *Curr Biol,* 13**,** 1679-86.

HAO, L., JOHNSEN, R., LAUTER, G., BAILLIE, D. & BURGLIN, T. R. 2006. Comprehensive analysis of gene expression patterns of hedgehog-related genes. *BMC Genomics,* 7**,** 280.

HARFE, B. D., SCHERZ, P. J., NISSIM, S., TIAN, H., MCMAHON, A. P. & TABIN, C. J. 2004. Evidence for an expansion-based temporal Shh gradient in specifying vertebrate digit identities. *Cell,* 118**,** 517-28.

HASHMI, S. & MARINKOVICH, M. P. 2011. Molecular organization of the basement membrane zone. *Clin Dermatol,* 29**,** 398-411.

HATTORI, K., NISHIKAWA, M., WATCHARANURAK, K., IKOMA, A., KABASHIMA, K., TOYOTA, H., TAKAHASHI, Y., TAKAHASHI, R., WATANABE, Y. & TAKAKURA, Y. 2010. Sustained exogenous expression of therapeutic levels of IFN-gamma ameliorates atopic dermatitis in NC/Nga mice via Th1 polarization. *J Immunol,* 184**,** 2729-35.

HAVRAN, W. L., CHIEN, Y. H. & ALLISON, J. P. 1991. Recognition of self antigens by skin-derived T cells with invariant gamma delta antigen receptors. *Science,* 252**,** 1430-2.

HAYDAY, A. C. 2009. Gammadelta T cells and the lymphoid stress-surveillance response. *Immunity,* 31**,** 184-96.

HE, M., SUBRAMANIAN, R., BANGS, F., OMELCHENKO, T., LIEM, K. F., JR., KAPOOR, T. M. & ANDERSON, K. V. 2014. The kinesin-4 protein Kif7 regulates mammalian Hedgehog signalling by organizing the cilium tip compartment. *Nat Cell Biol,* 16**,** 663-72.

HE, R., OYOSHI, M. K., JIN, H. & GEHA, R. S. 2007. Epicutaneous antigen exposure induces a Th17 response that drives airway inflammation after inhalation challenge. *Proc Natl Acad Sci U S A,* 104**,** 15817-22.

HEATH, W. R. & CARBONE, F. R. 2013. The skin-resident and migratory immune system in steady state and memory: innate lymphocytes, dendritic cells and T cells. *Nat Immunol,* 14**,** 978-85.

HELDIN, C. H., MIYAZONO, K. & TEN DIJKE, P. 1997. TGF-beta signalling from cell membrane to nucleus through SMAD proteins. *Nature,* 390**,** 465-71.

HELLSTROM, M., HELLSTROM, S., ENGSTROM-LAURENT, A. & BERTHEIM, U. 2014. The structure of the basement membrane zone differs between keloids, hypertrophic scars and normal skin: a possible background to an impaired function. *J Plast Reconstr Aesthet Surg,* 67**,** 1564-72.

HENDERSON, J., NORTHSTONE, K., LEE, S. P., LIAO, H., ZHAO, Y., PEMBREY, M., MUKHOPADHYAY, S., SMITH, G. D., PALMER, C. N., MCLEAN, W. H. & IRVINE, A. D. 2008. The burden of disease associated with filaggrin mutations: a population-based, longitudinal birth cohort study. *J Allergy Clin Immunol,* 121**,** 872-7 e9.

HENNINO, A., VOCANSON, M., TOUSSAINT, Y., RODET, K., BENETIERE, J., SCHMITT, A. M., ARIES, M. F., BERARD, F., ROZIERES, A. & NICOLAS, J. F. 2007. Skin-infiltrating CD8(+) T cells initiate atopic dermatitis lesions. *Journal of Immunology,* 178**,** 5571-5577.

HERSHKO, A. Y., SUZUKI, R., CHARLES, N., ALVAREZ-ERRICO, D., SARGENT, J. L., LAURENCE, A. & RIVERA, J. 2011. Mast cell interleukin-2 production contributes to suppression of chronic allergic dermatitis. *Immunity,* 35**,** 562-71.

HERZOG, F., WINTER, H. & SCHWEIZER, J. 1994. The large type II 70-kDa keratin of mouse epidermis is the ortholog of human keratin K2e. *J Invest Dermatol,* 102**,** 165-70.

HIDA, S., OGASAWARA, K., SATO, K., ABE, M., TAKAYANAGI, H., YOKOCHI, T., SATO, T., HIROSE, S., SHIRAI, T., TAKI, S. & TANIGUCHI, T. 2000. CD8(+) T cell-mediated skin disease in mice lacking IRF-2, the transcriptional attenuator of interferon-alpha/beta signaling. *Immunity,* 13**,** 643-55.

HIJNEN, D., HAECK, I., VAN KRAATS, A. A., NIJHUIS, E., DE BRUIN-WELLER, M. S., BRUIJNZEEL-KOOMEN, C. A. & KNOL, E. F. 2009. Cyclosporin A reduces CD4(+)CD25(+) regulatory T-cell numbers in patients with atopic dermatitis. *J Allergy Clin Immunol,* 124**,** 856-8.

HIJNEN, D., KNOL, E. F., GENT, Y. Y., GIOVANNONE, B., BEIJN, S. J. P., KUPPER, T. S., BRUIJNZEEL-KOOMEN, C. A. F. M. & CLARK, R. A. 2013. CD8(+) T Cells in the Lesional Skin of Atopic Dermatitis and Psoriasis Patients Are an Important Source of IFN-gamma, IL-13, IL-17, and IL-22. *Journal of Investigative Dermatology,* 133**,** 973-979.

HILL, R. E., HEANEY, S. J. & LETTICE, L. A. 2003. Sonic hedgehog: restricted expression and limb dysmorphologies. *J Anat,* 202**,** 13-20.

HOFFJAN, S. & EPPLEN, J. T. 2005. The genetics of atopic dermatitis: recent findings and future options. *J Mol Med (Berl),* 83**,** 682-92.

HOFMANN, I., STOVER, E. H., CULLEN, D. E., MAO, J., MORGAN, K. J., LEE, B. H., KHARAS, M. G., MILLER, P. G., CORNEJO, M. G., OKABE, R., ARMSTRONG, S. A., GHILARDI, N., GOULD, S., DE SAUVAGE, F. J., MCMAHON, A. P. & GILLILAND, D. G. 2009. Hedgehog signaling is dispensable for adult murine hematopoietic stem cell function and hematopoiesis. *Cell Stem Cell,* 4**,** 559-67.

HOLLA, S., STEPHEN-VICTOR, E., PRAKHAR, P., SHARMA, M., SAHA, C., UDUPA, V., KAVERI, S. V., BAYRY, J. & BALAJI, K. N. 2016. Mycobacteria-responsive sonic hedgehog signaling mediates programmed death-ligand 1- and prostaglandin E2-induced regulatory T cell expansion. *Sci Rep,* 6**,** 24193.

HOMEY, B., ALENIUS, H., MULLER, A., SOTO, H., BOWMAN, E. P., YUAN, W., MCEVOY, L., LAUERMA, A. I., ASSMANN, T., BUNEMANN, E., LEHTO, M., WOLFF, H., YEN, D., MARXHAUSEN, H., TO, W., SEDGWICK, J., RUZICKA, T., LEHMANN, P. & ZLOTNIK, A. 2002. CCL27-CCR10 interactions regulate T cell-mediated skin inflammation. *Nat Med,* 8**,** 157-65.

HONDA, T., EGAWA, G., GRABBE, S. & KABASHIMA, K. 2013. Update of immune events in the murine contact hypersensitivity model: toward the understanding of allergic contact dermatitis. *J Invest Dermatol,* 133**,** 303-15.

HORI, S., NOMURA, T. & SAKAGUCHI, S. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science,* 299**,** 1057-61.

HOWELL, M. D., KIM, B. E., GAO, P., GRANT, A. V., BOGUNIEWICZ, M., DEBENEDETTO, A., SCHNEIDER, L., BECK, L. A., BARNES, K. C. & LEUNG, D. Y. 2009. Cytokine modulation of atopic dermatitis filaggrin skin expression. *J Allergy Clin Immunol,* 124**,** R7-R12.

HSU, Y. C., LI, L. & FUCHS, E. 2014. Transit-amplifying cells orchestrate stem cell activity and tissue regeneration. *Cell,* 157**,** 935-49.

HU, M. C., MO, R., BHELLA, S., WILSON, C. W., CHUANG, P. T., HUI, C. C. & ROSENBLUM, N. D. 2006. GLI3-dependent transcriptional repression of Gli1, Gli2 and kidney patterning genes disrupts renal morphogenesis. *Development,* 133**,** 569-78.

HUANG, C., TANG, M., YEHLING, E. & ZHANG, X. 2014. Overexpressing sonic hedgehog peptide restores periosteal bone formation in a murine bone allograft transplantation model. *Mol Ther,* 22**,** 430-439.

HUANG, C. H., KUO, I. C., XU, H., LEE, Y. S. & CHUA, K. Y. 2003. Mite allergen induces allergic dermatitis with concomitant neurogenic inflammation in mouse. *J Invest Dermatol,* 121**,** 289-93.

HUEHN, J., SIEGMUND, K., LEHMANN, J. C., SIEWERT, C., HAUBOLD, U., FEUERER, M., DEBES, G. F., LAUBER, J., FREY, O., PRZYBYLSKI, G. K., NIESNER, U., DE LA ROSA, M., SCHMIDT, C. A., BRAUER, R., BUER, J., SCHEFFOLD, A. & HAMANN, A. 2004. Developmental stage, phenotype, and migration distinguish naive- and effector/memory-like CD4+ regulatory T cells. *J Exp Med,* 199**,** 303-13.

HUI, C. C. & ANGERS, S. 2011. Gli proteins in development and disease. *Annu Rev Cell Dev Biol,* 27**,** 513-37.

HUI, C. C. & JOYNER, A. L. 1993. A mouse model of greig cephalopolysyndactyly syndrome: the extra-toesJ mutation contains an intragenic deletion of the Gli3 gene. *Nat Genet,* 3**,** 241-6.

HUSE, M., MUIR, T. W., XU, L., CHEN, Y. G., KURIYAN, J. & MASSAGUE, J. 2001. The TGF beta receptor activation process: an inhibitor- to substrate-binding switch. *Mol Cell,* 8**,** 671-82.

HUTCHIN, M. E., KARIAPPER, M. S., GRACHTCHOUK, M., WANG, A., WEI, L., CUMMINGS, D., LIU, J., MICHAEL, L. E., GLICK, A. & DLUGOSZ, A. A. 2005. Sustained Hedgehog signaling is required for basal cell carcinoma proliferation and survival: conditional skin tumorigenesis recapitulates the hair growth cycle. *Genes Dev,* 19**,** 214-23.

IGYARTO, B. Z., HALEY, K., ORTNER, D., BOBR, A., GERAMI-NEJAD, M., EDELSON, B. T., ZURAWSKI, S. M., MALISSEN, B., ZURAWSKI, G., BERMAN, J. & KAPLAN, D. H. 2011. Skin-resident murine dendritic cell subsets promote distinct and opposing antigen-specific T helper cell responses. *Immunity,* 35**,** 260-72.

IGYARTO, B. Z. & KAPLAN, D. H. 2013. Antigen presentation by Langerhans cells. *Curr Opin Immunol,* 25**,** 115-9.

IIKURA, M., SUTO, H., KAJIWARA, N., OBOKI, K., OHNO, T., OKAYAMA, Y., SAITO, H., GALLI, S. J. & NAKAE, S. 2007. IL-33 can promote survival, adhesion and cytokine production in human mast cells. *Lab Invest,* 87**,** 971-8.

IKRAM, M. S., NEILL, G. W., REGL, G., EICHBERGER, T., FRISCHAUF, A. M., ABERGER, F., QUINN, A. & PHILPOTT, M. 2004. GLI2 is expressed in normal human epidermis and BCC and induces GLI1 expression by binding to its promoter. *J Invest Dermatol,* 122**,** 1503-9.

IMAI, Y., YASUDA, K., SAKAGUCHI, Y., HANEDA, T., MIZUTANI, H., YOSHIMOTO, T., NAKANISHI, K. & YAMANISHI, K. 2013. Skin-specific expression of IL-33 activates group 2 innate lymphoid cells and elicits atopic dermatitis-like inflammation in mice. *Proc Natl Acad Sci U S A,* 110**,** 13921-6.

INGHAM, P. W. & MCMAHON, A. P. 2001. Hedgehog signaling in animal development: paradigms and principles. *Genes Dev,* 15**,** 3059-87.

INGHAM, P. W. & MCMAHON, A. P. 2009. Hedgehog signalling: Kif7 is not that fishy after all. *Curr Biol,* 19**,** R729-31.

INGHAM, P. W., NAKANO, Y. & SEGER, C. 2011. Mechanisms and functions of Hedgehog signalling across the metazoa. *Nat Rev Genet,* 12**,** 393-406.

INGHAM, P. W. & PLACZEK, M. 2006. Orchestrating ontogenesis: variations on a theme by sonic hedgehog. *Nat Rev Genet,* 7**,** 841-50.

INGHAM, P. W., TAYLOR, A. M. & NAKANO, Y. 1991. Role of the Drosophila patched gene in positional signalling. *Nature,* 353**,** 184-7.

IRVINE, A. D. 2007. Fleshing out filaggrin phenotypes. *Journal of Investigative Dermatology,* 127**,** 504-507.

IRVINE, A. D., MCLEAN, W. H. & LEUNG, D. Y. 2011. Filaggrin mutations associated with skin and allergic diseases. *N Engl J Med,* 365**,** 1315-27.

ISHIDA-YAMAMOTO, A., TAKAHASHI, H., PRESLAND, R. B., DALE, B. A. & IIZUKA, H. 1998. Translocation of profilaggrin N-terminal domain into keratinocyte nuclei with fragmented DNA in normal human skin and loricrin keratoderma. *Lab Invest,* 78**,** 1245-53.

ISLAM, S. A., CHANG, D. S., COLVIN, R. A., BYRNE, M. H., MCCULLY, M. L., MOSER, B., LIRA, S. A., CHARO, I. F. & LUSTER, A. D. 2011. Mouse CCL8, a CCR8 agonist, promotes atopic dermatitis by recruiting IL-5(+) T(H)2 cells. *Nature Immunology,* 12**,** 167-U86.

ITO, M., YANG, Z., ANDL, T., CUI, C., KIM, N., MILLAR, S. E. & COTSARELIS, G. 2007. Wnt-dependent de novo hair follicle regeneration in adult mouse skin after wounding. *Nature,* 447**,** 316-20.

ITO, Y., ADACHI, Y., MAKINO, T., HIGASHIYAMA, H., FUCHIZAWA, T., SHIMIZU, T. & MIYAWAKI, T. 2009. Expansion of FOXP3-positive CD4+CD25+ T cells associated with disease activity in atopic dermatitis. *Ann Allergy Asthma Immunol,* 103**,** 160-5.

IVASHKIV, L. B. & DONLIN, L. T. 2014. Regulation of type I interferon responses. *Nat Rev Immunol,* 14**,** 36-49.

IZZI, L., LEVESQUE, M., MORIN, S., LANIEL, D., WILKES, B. C., MILLE, F., KRAUSS, R. S., MCMAHON, A. P., ALLEN, B. L. & CHARRON, F. 2011. Boc and Gas1 each form distinct Shh receptor complexes with Ptch1 and are required for Shh-mediated cell proliferation. *Dev Cell,* 20**,** 788-801.

JACKSON, D. A., SMITH, T. D., AMARSAIKHAN, N., HAN, W., NEIL, M. S., BOI, S. K., VRABEL, A. M., TOLOSA, E. J., ALMADA, L. L., FERNANDEZ-ZAPICO, M. E. & ELSAWA, S. F. 2015. Modulation of the IL-6 Receptor alpha Underlies GLI2-

Mediated Regulation of Ig Secretion in Waldenstrom Macroglobulinemia Cells. *J Immunol,* 195**,** 2908-16.

JACOB, J. & BRISCOE, J. 2003. Gli proteins and the control of spinal-cord patterning. *EMBO Rep,* 4**,** 761-5.

JAKS, V., KASPER, M. & TOFTGARD, R. 2010. The hair follicle-a stem cell zoo. *Exp Cell Res,* 316**,** 1422-8.

JAMIESON, C., CORTES, J. E., OEHLER, V., BACCARANI, M., KANTARJIAN, H. M., PAPAYANNIDIS, C., RICE, K. N., ZHANG, X. X., SHAIK, N., COURTNEY, R., LEVIN, W. J. & MARTINELLI, G. 2011. Phase 1 Dose-Escalation Study of PF-04449913, An Oral Hedgehog (Hh) Inhibitor, in Patients with Select Hematologic Malignancies. *Blood,* 118**,** 195-196.

JAVELAUD, D., ALEXAKI, V. I., DENNLER, S., MOHAMMAD, K. S., GUISE, T. A. & MAUVIEL, A. 2011. TGF-beta/SMAD/GLI2 signaling axis in cancer progression and metastasis. *Cancer Res,* 71**,** 5606-10.

JAVELAUD, D., PIERRAT, M. J. & MAUVIEL, A. 2012. Crosstalk between TGF-beta and hedgehog signaling in cancer. *FEBS Lett,* 586**,** 2016-25.

JIA, J., ZHANG, L., ZHANG, Q., TONG, C., WANG, B., HOU, F., AMANAI, K. & JIANG, J. 2005. Phosphorylation by double-time/CKIepsilon and CKIalpha targets cubitus interruptus for Slimb/beta-TRCP-mediated proteolytic processing. *Dev Cell,* 9**,** 819-30.

JIANG, J. & HUI, C. C. 2008. Hedgehog signaling in development and cancer. *Dev Cell,* 15**,** 801-12.

JIANG, X., CLARK, R. A., LIU, L., WAGERS, A. J., FUHLBRIGGE, R. C. & KUPPER, T. S. 2012. Skin infection generates non-migratory memory CD8+ T(RM) cells providing global skin immunity. *Nature,* 483**,** 227-31.

JIN, H., HE, R., OYOSHI, M. & GEHA, R. S. 2009a. Animal models of atopic dermatitis. *J Invest Dermatol,* 129**,** 31-40.

JIN, H., KUMAR, L., MATHIAS, C., ZURAKOWSKI, D., OETTGEN, H., GORELIK, L. & GEHA, R. 2009b. Toll-like receptor 2 is important for the T(H)1 response to cutaneous sensitization. *J Allergy Clin Immunol,* 123**,** 875-82 e1.

JOHANSSON, O., LIANG, Y., HEILBORN, J. D. & MARCUSSON, J. A. 1998. Langerhans cells in prurigo nodularis investigated by HLA-DR and S-100 immunofluorescence double staining. *J Dermatol Sci,* 17**,** 24-32.

JOHNSON, R. L., GRENIER, J. K. & SCOTT, M. P. 1995. patched overexpression alters wing disc size and pattern: transcriptional and post-transcriptional effects on hedgehog targets. *Development,* 121**,** 4161-70.

JUNG, Y., MCCALL, S. J., LI, Y. X. & DIEHL, A. M. 2007. Bile ductules and stromal cells express hedgehog ligands and/or hedgehog target genes in primary biliary cirrhosis. *Hepatology,* 45**,** 1091-6.

KABASHIMA, K. 2013. New concept of the pathogenesis of atopic dermatitis: interplay among the barrier, allergy, and pruritus as a trinity. *J Dermatol Sci,* 70**,** 3-11.

KAMATA, Y., TANIGUCHI, A., YAMAMOTO, M., NOMURA, J., ISHIHARA, K., TAKAHARA, H., HIBINO, T. & TAKEDA, A. 2009. Neutral cysteine protease bleomycin hydrolase is essential for the breakdown of deiminated filaggrin into amino acids. *J Biol Chem,* 284**,** 12829-36.

KANE, M., ZANG, T. M., RIHN, S. J., ZHANG, F., KUECK, T., ALIM, M., SCHOGGINS, J., RICE, C. M., WILSON, S. J. & BIENIASZ, P. D. 2016. Identification of Interferon-Stimulated Genes with Antiretroviral Activity. *Cell Host Microbe,* 20**,** 392-405.

KANG, D. H., HAN, M. E., SONG, M. H., LEE, Y. S., KIM, E. H., KIM, H. J., KIM, G. H., KIM, D. H., YOON, S., BAEK, S. Y., KIM, B. S., KIM, J. B. & OH, S. O. 2009. The role of hedgehog signaling during gastric regeneration. *J Gastroenterol,* 44**,** 372-9.

KANG, J., DING, Y., LI, B., LIU, H., YANG, X. & CHEN, M. 2017. TRPA1 mediated aggravation of allergic contact dermatitis induced by DINP and regulated by NF-kappaB activation. *Sci Rep,* 7**,** 43586.

KANG, S., GRAHAM, J. M., JR., OLNEY, A. H. & BIESECKER, L. G. 1997. GLI3 frameshift mutations cause autosomal dominant Pallister-Hall syndrome. *Nat Genet,* 15**,** 266-8.

KAPOOR, R., MENON, C., HOFFSTAD, O., BILKER, W., LECLERC, P. & MARGOLIS, D. J. 2008. The prevalence of atopic triad in children with physician-confirmed atopic dermatitis. *J Am Acad Dermatol,* 58**,** 68-73.

KARLSSON, L., BONDJERS, C. & BETSHOLTZ, C. 1999. Roles for PDGF-A and sonic hedgehog in development of mesenchymal components of the hair follicle. *Development,* 126**,** 2611-21.

KASHIWAGI, M., HOSOI, J., LAI, J. F., BRISSETTE, J., ZIEGLER, S. F., MORGAN, B. A. & GEORGOPOULOS, K. 2017. Direct control of regulatory I cells by keratinocytes. *Nature Immunology,* 18**,** 334-343.

KAWAKAMI, T., ANDO, T., KIMURA, M., WILSON, B. S. & KAWAKAMI, Y. 2009. Mast cells in atopic dermatitis. *Curr Opin Immunol,* 21**,** 666-78.

KESKI-OJA, J., KOLI, K. & VON MELCHNER, H. 2004. TGF-beta activation by traction? *Trends Cell Biol,* 14**,** 657-9.

KHAHESHI, I., KESHAVARZ, S., IMANI FOOLADI, A. A., EBRAHIMI, M., YAZDANI, S., PANAHI, Y., SHOHRATI, M. & NOURANI, M. R. 2011. Loss of expression of TGF-betas and their receptors in chronic skin lesions induced by sulfur mustard as compared with chronic contact dermatitis patients. *BMC Dermatol,* 11**,** 2.

KIEHL, P., FALKENBERG, K., VOGELBRUCH, M. & KAPP, A. 2001. Tissue eosinophilia in acute and chronic atopic dermatitis: a morphometric approach using quantitative image analysis of immunostaining. *Br J Dermatol,* 145**,** 720-9.

KIM, B. S. 2015. Innate lymphoid cells in the skin. *J Invest Dermatol,* 135**,** 673-678.

KIM, B. S., WANG, K., SIRACUSA, M. C., SAENZ, S. A., BRESTOFF, J. R., MONTICELLI, L. A., NOTI, M., TAIT WOJNO, E. D., FUNG, T. C., KUBO, M. & ARTIS, D. 2014. Basophils promote innate lymphoid cell responses in inflamed skin. *J Immunol,* 193**,** 3717-25.

KOGA, C., KABASHIMA, K., SHIRAISHI, N., KOBAYASHI, M. & TOKURA, Y. 2008. Possible pathogenic role of Th17 cells for atopic dermatitis. *J Invest Dermatol,* 128**,** 2625-30.

KONDO, S. & SAUDER, D. N. 1997. Tumor necrosis factor (TNF) receptor type 1 (p55) is a main mediator for TNF-alpha-induced skin inflammation. *Eur J Immunol,* 27**,** 1713-8.

KONISHI, H., TSUTSUI, H., MURAKAMI, T., YUMIKURA-FUTATSUGI, S., YAMANAKA, K., TANAKA, M., IWAKURA, Y., SUZUKI, N., TAKEDA, K., AKIRA, S., NAKANISHI, K. & MIZUTANI, H. 2002. IL-18 contributes to the spontaneous development of atopic dermatitis-like inflammatory skin lesion independently of IgE/stat6 under specific pathogen-free conditions. *Proc Natl Acad Sci U S A,* 99**,** 11340-5.

KONKEL, J. E., JIN, W., ABBATIELLO, B., GRAINGER, J. R. & CHEN, W. 2014. Thymocyte apoptosis drives the intrathymic generation of regulatory T cells. *Proc Natl Acad Sci U S A,* 111**,** E465-73.

KONKEL, J. E., ZHANG, D. F., ZANVIT, P., CHIA, C., ZANGARLE-MURRAY, T., JIN, W. W., WANG, S. L. & CHEN, W. J. 2017. Transforming Growth Factor-beta Signaling in Regulatory T Cells Controls T Helper-17 Cells and Tissue-Specific Immune Responses. *Immunity,* 46**,** 660-674.

KOSAKA, H., YOSHIMOTO, T., YOSHIMOTO, T., FUJIMOTO, J. & NAKANISHI, K. 2008. Interferon-gamma is a therapeutic target molecule for prevention of postoperative adhesion formation. *Nat Med,* 14**,** 437-41.

KOVACS, J. J., WHALEN, E. J., LIU, R., XIAO, K., KIM, J., CHEN, M., WANG, J., CHEN, W. & LEFKOWITZ, R. J. 2008. Beta-arrestin-mediated localization of smoothened to the primary cilium. *Science,* 320**,** 1777-81.

KRAUSE, A., XU, Y., JOH, J., HUBNER, R., GESS, A., ILIC, T. & WORGALL, S. 2010. Overexpression of sonic Hedgehog in the lung mimics the effect of lung injury and compensatory lung growth on pulmonary Sca-1 and CD34 positive cells. *Mol Ther,* 18**,** 404-12.

KRAUSS, S., CONCORDET, J. P. & INGHAM, P. W. 1993. A functionally conserved homolog of the Drosophila segment polarity gene hh is expressed in tissues with polarizing activity in zebrafish embryos. *Cell,* 75**,** 1431-44.

KUGLER, M. C., JOYNER, A. L., LOOMIS, C. A. & MUNGER, J. S. 2015. Sonic hedgehog signaling in the lung. From development to disease. *Am J Respir Cell Mol Biol,* 52**,** 1-13.

KUKURBA, K. R. & MONTGOMERY, S. B. 2015. RNA Sequencing and Analysis. *Cold Spring Harb Protoc,* 2015**,** 951-69.

KUMARI, S., BONNET, M. C., ULVMAR, M. H., WOLK, K., KARAGIANNI, N., WITTE, E., UTHOFF-HACHENBERG, C., RENAULD, J. C., KOLLIAS, G., TOFTGARD, R., SABAT, R., PASPARAKIS, M. & HAASE, I. 2013. Tumor necrosis factor receptor signaling in keratinocytes triggers interleukin-24-dependent psoriasis-like skin inflammation in mice. *Immunity,* 39**,** 899-911.

KUO, I. H., YOSHIDA, T., DE BENEDETTO, A. & BECK, L. A. 2013. The cutaneous innate immune response in patients with atopic dermatitis. *J Allergy Clin Immunol,* 131**,** 266-78.

KYTTALA, M., TALLILA, J., SALONEN, R., KOPRA, O., KOHLSCHMIDT, N., PAAVOLA-SAKKI, P., PELTONEN, L. & KESTILA, M. 2006. MKS1, encoding a component of the flagellar apparatus basal body proteome, is mutated in Meckel syndrome. *Nat Genet,* 38**,** 155-7.

LAKE, J. P., PIERCE, C. W. & KENNEDY, J. D. 1991. CD8+ alpha/beta or gamma/delta T cell receptor-bearing T cells from athymic nude mice are cytolytically active in vivo. *J Immunol,* 147**,** 1121-6.

LAN, X., WEN, H., CHENG, K., PLAGOV, A., MARASHI SHOSHTARI, S. S., MALHOTRA, A. & SINGHAL, P. C. 2017. Hedgehog pathway plays a vital role in HIV-induced epithelial-mesenchymal transition of podocyte. *Exp Cell Res,* 352**,** 193-201.

LANGE, J., HEINZMANN, A., ZEHLE, C. & KOPP, M. 2005. CT genotype of promotor polymorphism C159T in the CD14 gene is associated with lower prevalence of atopic dermatitis and lower IL-13 production. *Pediatr Allergy Immunol,* 16**,** 456-7.

LAOUINI, D., ELKHAL, A., YALCINDAG, A., KAWAMOTO, S., OETTGEN, H. & GEHA, R. S. 2005. COX-2 inhibition enhances the TH2 immune response to epicutaneous sensitization. *J Allergy Clin Immunol,* 116**,** 390-6.

LAU, C. I., BARBARULO, A., SOLANKI, A., SALDANA, J. I. & CROMPTON, T. 2017. The kinesin motor protein Kif7 is required for T-cell development and normal MHC expression on thymic epithelial cells (TEC) in the thymus. *Oncotarget,* 8**,** 24163-24176.

LAU, C. I., OUTRAM, S. V., SALDANA, J. I., FURMANSKI, A. L., DESSENS, J. T. & CROMPTON, T. 2012. Regulation of murine normal and stress-induced erythropoiesis by Desert Hedgehog. *Blood,* 119**,** 4741-51.

LAWRENCE, D. A. 2001. Latent-TGF-beta: an overview. *Mol Cell Biochem,* 219**,** 163-70.

LAZAREVIC, V. & GLIMCHER, L. H. 2011. T-bet in disease. *Nat Immunol,* 12**,** 597-606.

LE, H., KLEINERMAN, R., LERMAN, O. Z., BROWN, D., GALIANO, R., GURTNER, G. C., WARREN, S. M., LEVINE, J. P. & SAADEH, P. B. 2008. Hedgehog signaling is essential for normal wound healing. *Wound Repair Regen,* 16**,** 768-73.

LEBRE, M. C., VAN DER AAR, A. M., VAN BAARSEN, L., VAN CAPEL, T. M., SCHUITEMAKER, J. H., KAPSENBERG, M. L. & DE JONG, E. C. 2007. Human keratinocytes express functional Toll-like receptor 3, 4, 5, and 9. *J Invest Dermatol,* 127**,** 331-41.

LEE, R. T., ZHAO, Z. & INGHAM, P. W. 2016. Hedgehog signalling. *Development,* 143**,** 367-72.

LEE, W. & LEE, G. R. 2018. Transcriptional regulation and development of regulatory T cells. *Exp Mol Med,* 50**,** e456.

LEONARD, J. M., YE, H., WETMORE, C. & KARNITZ, L. M. 2008. Sonic Hedgehog signaling impairs ionizing radiation-induced checkpoint activation and induces genomic instability. *J Cell Biol,* 183**,** 385-91.

LEUNG, D. Y., BOGUNIEWICZ, M., HOWELL, M. D., NOMURA, I. & HAMID, Q. A. 2004. New insights into atopic dermatitis. *J Clin Invest,* 113**,** 651-7.

LEWANDOWSKI, J. P., DU, F., ZHANG, S., POWELL, M. B., FALKENSTEIN, K. N., JI, H. & VOKES, S. A. 2015. Spatiotemporal regulation of GLI target genes in the mammalian limb bud. *Dev Biol,* 406**,** 92-103.

LEWIS, P. M., DUNN, M. P., MCMAHON, J. A., LOGAN, M., MARTIN, J. F., ST-JACQUES, B. & MCMAHON, A. P. 2001. Cholesterol modification of sonic hedgehog is required for long-range signaling activity and effective modulation of signaling by Ptc1. *Cell,* 105**,** 599-612.

LI, C., CHI, S. & XIE, J. 2011. Hedgehog signaling in skin cancers. *Cell Signal,* 23**,** 1235-43.

LI, M. O. & FLAVELL, R. A. 2008. TGF-beta: a master of all T cell trades. *Cell,* 134**,** 392-404.

LI, Z. J., NIEUWENHUIS, E., NIEN, W., ZHANG, X., ZHANG, J., PUVIINDRAN, V., WAINWRIGHT, B. J., KIM, P. C. & HUI, C. C. 2012. Kif7 regulates Gli2 through Sufu-dependent and -independent functions during skin development and tumorigenesis. *Development,* 139**,** 4152-61.

LIANG, R. F., SUMOVA, B., CORDAZZO, C., MALLANO, T., ZHANG, Y., WOHLFAHRT, T., DEES, C., RAMMING, A., KRASOWSKA, D., MICHALSKA-JAKUBUS, M., DISTLER, O., SCHETT, G., SENOLT, L. & DISTLER, J. H. W. 2017. The transcription factor GLI2 as a downstream mediator of transforming growth factor-beta-induced fibroblast activation in SSc. *Annals of the Rheumatic Diseases,* 76.

LIEW, F. Y., PITMAN, N. I. & MCINNES, I. B. 2010. Disease-associated functions of IL-33: the new kid in the IL-1 family. *Nat Rev Immunol,* 10**,** 103-10.

LIU, A., WANG, B. & NISWANDER, L. A. 2005. Mouse intraflagellar transport proteins regulate both the activator and repressor functions of Gli transcription factors. *Development,* 132**,** 3103-11.

LIU, H., HU, B., XU, D. & LIEW, F. Y. 2003. CD4+CD25+ regulatory T cells cure murine colitis: the role of IL-10, TGF-beta, and CTLA4. *J Immunol,* 171**,** 5012-7.

LIU, H., JIAN, Q., XUE, K., MA, C., XIE, F., WANG, R., LIAO, W., LIU, Y., CHI, S. & LI, C. 2014. The MEK/ERK signalling cascade is required for sonic hedgehog signalling pathway-mediated enhancement of proliferation and inhibition of apoptosis in normal keratinocytes. *Exp Dermatol,* 23**,** 896-901.

LIU, L., KUGLER, M. C., LOOMIS, C. A., SAMDANI, R., ZHAO, Z., CHEN, G. J., BRANDT, J. P., BROWNELL, I., JOYNER, A. L., ROM, W. N. & MUNGER, J. S. 2013. Hedgehog signaling in neonatal and adult lung. *Am J Respir Cell Mol Biol,* 48**,** 703-10.

LIU, Y., ZHANG, P., LI, J., KULKARNI, A. B., PERRUCHE, S. & CHEN, W. 2008. A critical function for TGF-beta signaling in the development of natural CD4+CD25+Foxp3+ regulatory T cells. *Nat Immunol,* 9**,** 632-40.

LIU, Y. J., SOUMELIS, V., WATANABE, N., ITO, T., WANG, Y. H., MALEFYT RDE, W., OMORI, M., ZHOU, B. & ZIEGLER, S. F. 2007. TSLP: an epithelial cell cytokine that regulates T cell differentiation by conditioning dendritic cell maturation. *Annu Rev Immunol,* 25**,** 193-219.

LONSDALE-ECCLES, J. D., TELLER, D. C. & DALE, B. A. 1982. Characterization of a phosphorylated form of the intermediate filament-aggregating protein filaggrin. *Biochemistry,* 21**,** 5940-8.

LOPEZ-BRAVO, M. & ARDAVIN, C. 2008. In vivo induction of immune responses to pathogens by conventional dendritic cells. *Immunity,* 29**,** 343-51.

LU, Y., LI, J., CHENG, J. & LUBAHN, D. B. 2015. Genes targeted by the Hedgehog-signaling pathway can be regulated by Estrogen related receptor beta. *BMC Mol Biol,* 16**,** 19.

LUM, L., YAO, S., MOZER, B., ROVESCALLI, A., VON KESSLER, D., NIRENBERG, M. & BEACHY, P. A. 2003. Identification of Hedgehog pathway components by RNAi in Drosophila cultured cells. *Science,* 299**,** 2039-45.

LUO, J. D., HU, T. P., WANG, L., CHEN, M. S., LIU, S. M. & CHEN, A. F. 2009. Sonic hedgehog improves delayed wound healing via enhancing cutaneous nitric oxide function in diabetes. *Am J Physiol Endocrinol Metab,* 297**,** E525-31.

LUTZ, M. B., ASSMANN, C. U., GIROLOMONI, G. & RICCIARDI-CASTAGNOLI, P. 1996. Different cytokines regulate antigen uptake and presentation of a precursor dendritic cell line. *Eur J Immunol,* 26**,** 586-94.

MACLEOD, A. S. & HAVRAN, W. L. 2011. Functions of skin-resident gammadelta T cells. *Cell Mol Life Sci,* 68**,** 2399-408.

MADISON, B. B., BRAUNSTEIN, K., KUIZON, E., PORTMAN, K., QIAO, X. T. & GUMUCIO, D. L. 2005. Epithelial hedgehog signals pattern the intestinal crypt-villus axis. *Development,* 132**,** 279-89.

MAITAH, M. Y., ALI, S., AHMAD, A., GADGEEL, S. & SARKAR, F. H. 2011. Upregulation of sonic hedgehog contributes to TGF-beta 1-induced epithelial to mesenchymal transition in NSCLC cells. *Cancer Research,* 71.

MALHOTRA, N., LEYVA-CASTILLO, J. M., JADHAV, U., BARREIRO, O., KAM, C., O'NEILL, N. K., MEYLAN, F., CHAMBON, P., VON ANDRIAN, U. H., SIEGEL, R. M., WANG, E. C., SHIVDASANI, R. & GEHA, R. S. 2018. RORalpha-expressing T regulatory cells restrain allergic skin inflammation. *Sci Immunol,* 3.

MALLOL, J., CRANE, J., VON MUTIUS, E., ODHIAMBO, J., KEIL, U., STEWART, A. & GROUP, I. P. T. S. 2013. The International Study of Asthma and Allergies in Childhood (ISAAC) Phase Three: a global synthesis. *Allergol Immunopathol (Madr),* 41**,** 73-85.

MAN, M. Q., HATANO, Y., LEE, S. H., MAN, M., CHANG, S., FEINGOLD, K. R., LEUNG, D. Y., HOLLERAN, W., UCHIDA, Y. & ELIAS, P. M. 2008. Characterization of a hapten-induced, murine model with multiple features of atopic dermatitis: structural, immunologic, and biochemical changes following single versus multiple oxazolone challenges. *J Invest Dermatol,* 128**,** 79-86.

MANCINI, A. J., KAULBACK, K. & CHAMLIN, S. L. 2008. The socioeconomic impact of atopic dermatitis in the United States: a systematic review. *Pediatr Dermatol,* 25**,** 1-6.

MANGAN, P. R., HARRINGTON, L. E., O'QUINN, D. B., HELMS, W. S., BULLARD, D. C., ELSON, C. O., HATTON, R. D., WAHL, S. M., SCHOEB, T. R. & WEAVER, C. T. 2006. Transforming growth factor-beta induces development of the T(H)17 lineage. *Nature,* 441**,** 231-4.

MANTOVANI, A., BISWAS, S. K., GALDIERO, M. R., SICA, A. & LOCATI, M. 2013. Macrophage plasticity and polarization in tissue repair and remodelling. *J Pathol,* 229**,** 176-85.

MARENHOLZ, I., NICKEL, R., RUSCHENDORF, F., SCHULZ, F., ESPARZA-GORDILLO, J., KERSCHER, T., GRUBER, C., LAU, S., WORM, M., KEIL, T., KUREK, M., ZALUGA, E., WAHN, U. & LEE, Y. A. 2006. Filaggrin loss-of-function mutations predispose to phenotypes involved in the atopic march. *J Allergy Clin Immunol,* 118**,** 866-71.

MARKOVA, N. G., MAREKOV, L. N., CHIPEV, C. C., GAN, S. Q., IDLER, W. W. & STEINERT, P. M. 1993. Profilaggrin is a major epidermal calcium-binding protein. *Mol Cell Biol,* 13**,** 613-25.

MARTINON, F., MAYOR, A. & TSCHOPP, J. 2009. The inflammasomes: guardians of the body. *Annu Rev Immunol,* 27**,** 229-65.

MASUYA, H., SAGAI, T., WAKANA, S., MORIWAKI, K. & SHIROISHI, T. 1995. A duplicated zone of polarizing activity in polydactylous mouse mutants. *Genes Dev,* 9**,** 1645-53.

MATSUDA, H., WATANABE, N., GEBA, G. P., SPERL, J., TSUDZUKI, M., HIROI, J., MATSUMOTO, M., USHIO, H., SAITO, S., ASKENASE, P. W. & RA, C. 1997. Development of atopic dermatitis-like skin lesion with IgE hyperproduction in NC/Nga mice. *Int Immunol,* 9**,** 461-6.

MAURYA, A. K., BEN, J., ZHAO, Z., LEE, R. T., NIAH, W., NG, A. S., IYU, A., YU, W., ELWORTHY, S., VAN EEDEN, F. J. & INGHAM, P. W. 2013. Positive and negative regulation of Gli activity by Kif7 in the zebrafish embryo. *PLoS Genet,* 9**,** e1003955.

MCALEER, M. A. & IRVINE, A. D. 2013. The multifunctional role of filaggrin in allergic skin disease. *Journal of Allergy and Clinical Immunology,* 131**,** 280-291.

MCCALLA-MARTIN, A. C., CHEN, X., LINDER, K. E., ESTRADA, J. L. & PIEDRAHITA, J. A. 2010. Varying phenotypes in swine versus murine transgenic models constitutively expressing the same human Sonic hedgehog transcriptional activator, K5-HGLI2 Delta N. *Transgenic Res,* 19**,** 869-87.

MCKELVEY, K., JACKSON, C. J. & XUE, M. 2014. Activated protein C: A regulator of human skin epidermal keratinocyte function. *World J Biol Chem,* 5**,** 169-79.

MCMAHON, A. P., INGHAM, P. W. & TABIN, C. J. 2003. Developmental roles and clinical significance of hedgehog signaling. *Curr Top Dev Biol,* 53**,** 1-114.

MEEPHANSAN, J., TSUDA, H., KOMINE, M., TOMINAGA, S. & OHTSUKI, M. 2012. Regulation of IL-33 expression by IFN-gamma and tumor necrosis factor-alpha in normal human epidermal keratinocytes. *J Invest Dermatol,* 132**,** 2593-600.

MEIXNER, A., KARRETH, F., KENNER, L. & WAGNER, E. F. 2004. JunD regulates lymphocyte proliferation and T helper cell cytokine expression. *EMBO J,* 23**,** 1325-35.

MEMPEL, T. R., PITTET, M. J., KHAZAIE, K., WENINGER, W., WEISSLEDER, R., VON BOEHMER, H. & VON ANDRIAN, U. H. 2006. Regulatory T cells reversibly suppress cytotoxic T cell function independent of effector differentiation. *Immunity,* 25**,** 129-41.

MENZIES-GOW, A., YING, S., SABROE, I., STUBBS, V. L., SOLER, D., WILLIAMS, T. J. & KAY, A. B. 2002. Eotaxin (CCL11) and eotaxin-2 (CCL24) induce recruitment of eosinophils, basophils, neutrophils, and macrophages as well as features of early- and late-phase allergic reactions following cutaneous injection in human atopic and nonatopic volunteers. *Journal of Immunology,* 169**,** 2712-2718.

MERCHANT, A. A. & MATSUI, W. 2009. Smoothening the controversial role of hedgehog in hematopoiesis. *Cell Stem Cell,* 4**,** 470-1.

MERCHANT, M., VAJDOS, F. F., ULTSCH, M., MAUN, H. R., WENDT, U., CANNON, J., DESMARAIS, W., LAZARUS, R. A., DE VOS, A. M. & DE SAUVAGE, F. J.

2004. Suppressor of fused regulates Gli activity through a dual binding mechanism. *Mol Cell Biol,* 24**,** 8627-41.

METHOT, N. & BASLER, K. 1999. Hedgehog controls limb development by regulating the activities of distinct transcriptional activator and repressor forms of Cubitus interruptus. *Cell,* 96**,** 819-31.

MICHEL, K. D., UHMANN, A., DRESSEL, R., VAN DEN BRANDT, J., HAHN, H. & REICHARDT, H. M. 2013. The hedgehog receptor patched1 in T cells is dispensable for adaptive immunity in mice. *PLoS One,* 8**,** e61034.

MIKESH, L. M., ARAMADHAKA, L. R., MOSKALUK, C., ZIGRINO, P., MAUCH, C. & FOX, J. W. 2013. Proteomic anatomy of human skin. *J Proteomics,* 84**,** 190-200.

MILENKOVIC, L., SCOTT, M. P. & ROHATGI, R. 2009. Lateral transport of Smoothened from the plasma membrane to the membrane of the cilium. *J Cell Biol,* 187**,** 365-74.

MILL, P., MO, R., FU, H., GRACHTCHOUK, M., KIM, P. C., DLUGOSZ, A. A. & HUI, C. C. 2003. Sonic hedgehog-dependent activation of Gli2 is essential for embryonic hair follicle development. *Genes Dev,* 17**,** 282-94.

MILLER, L. S. & MODLIN, R. L. 2007. Human keratinocyte Toll-like receptors promote distinct immune responses. *J Invest Dermatol,* 127**,** 262-3.

MIN, Y., WI, S. M., KANG, J. A., YANG, T., PARK, C. S., PARK, S. G., CHUNG, S., SHIM, J. H., CHUN, E. & LEE, K. Y. 2016. Cereblon negatively regulates TLR4 signaling through the attenuation of ubiquitination of TRAF6. *Cell Death Dis,* 7**,** e2313.

MINAMI, Y., MINAMI, H., MIYAMOTO, T., YOSHIMOTO, G., KOBAYASHI, Y., MUNAKATA, W., ONISHI, Y., KOBAYASHI, M., IKUTA, M., CHAN, G., WOOLFSON, A., ONO, C., SHAIK, M. N., FUJII, Y., ZHENG, X. & NAOE, T. 2017. Phase I study of glasdegib (PF-04449913), an oral smoothened inhibitor, in Japanese patients with select hematologic malignancies. *Cancer Sci,* 108**,** 1628-1633.

MIYAZONO, K., ICHIJO, H. & HELDIN, C. H. 1993. Transforming growth factor-beta: latent forms, binding proteins and receptors. *Growth Factors,* 8**,** 11-22.

MIZUTANI, H., SCHECHTER, N., LAZARUS, G., BLACK, R. A. & KUPPER, T. S. 1991. Rapid and specific conversion of precursor interleukin 1 beta (IL-1 beta) to an active IL-1 species by human mast cell chymase. *J Exp Med,* 174**,** 821-5.

MO, R., FREER, A. M., ZINYK, D. L., CRACKOWER, M. A., MICHAUD, J., HENG, H. H., CHIK, K. W., SHI, X. M., TSUI, L. C., CHENG, S. H., JOYNER, A. L. & HUI, C. 1997. Specific and redundant functions of Gli2 and Gli3 zinc finger genes in skeletal patterning and development. *Development,* 124**,** 113-23.

MORAR, N., WILLIS-OWEN, S. A., MOFFATT, M. F. & COOKSON, W. O. 2006. The genetics of atopic dermatitis. *J Allergy Clin Immunol,* 118**,** 24-34; quiz 35-6.

MORI, T., KABASHIMA, K., YOSHIKI, R., SUGITA, K., SHIRAISHI, N., ONOUE, A., KURODA, E., KOBAYASHI, M., YAMASHITA, U. & TOKURA, Y. 2008. Cutaneous hypersensitivities to hapten are controlled by IFN-gamma-upregulated keratinocyte Th1 chemokines and IFN-gamma-downregulated langerhans cell Th2 chemokines. *J Invest Dermatol,* 128**,** 1719-27.

MORO, K., YAMADA, T., TANABE, M., TAKEUCHI, T., IKAWA, T., KAWAMOTO, H., FURUSAWA, J., OHTANI, M., FUJII, H. & KOYASU, S. 2010. Innate production of T(H)2 cytokines by adipose tissue-associated c-Kit(+)Sca-1(+) lymphoid cells. *Nature,* 463**,** 540-4.

MOSSER, D. M. & EDWARDS, J. P. 2008. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol,* 8**,** 958-69.

MUENKE, M. & BEACHY, P. A. 2000. Genetics of ventral forebrain development and holoprosencephaly. *Curr Opin Genet Dev,* 10**,** 262-9.

MUNCHHOF, M. J., LI, Q., SHAVNYA, A., BORZILLO, G. V., BOYDEN, T. L., JONES, C. S., LAGRECA, S. D., MARTINEZ-ALSINA, L., PATEL, N., PELLETIER, K., REITER, L. A., ROBBINS, M. D. & TKALCEVIC, G. T. 2012. Discovery of PF-04449913, a Potent and Orally Bioavailable Inhibitor of Smoothened. *ACS Med Chem Lett,* 3**,** 106-11.

MURTHY, A., SHAO, Y. W., NARALA, S. R., MOLYNEUX, S. D., ZUNIGA-PFLUCKER, J. C. & KHOKHA, R. 2012. Notch activation by the metalloproteinase ADAM17 regulates myeloproliferation and atopic barrier immunity by suppressing epithelial cytokine synthesis. *Immunity,* 36**,** 105-19.

NACHTERGAELE, S., MYDOCK, L. K., KRISHNAN, K., RAMMOHAN, J., SCHLESINGER, P. H., COVEY, D. F. & ROHATGI, R. 2012. Oxysterols are allosteric activators of the oncoprotein Smoothened. *Nat Chem Biol,* 8**,** 211-20.

NAGAO, K., GINHOUX, F., LEITNER, W. W., MOTEGI, S., BENNETT, C. L., CLAUSEN, B. E., MERAD, M. & UDEY, M. C. 2009. Murine epidermal Langerhans cells and langerin-expressing dermal dendritic cells are unrelated and exhibit distinct functions. *Proc Natl Acad Sci U S A,* 106**,** 3312-7.

NAGAO, K., KOBAYASHI, T., MORO, K., OHYAMA, M., ADACHI, T., KITASHIMA, D. Y., UEHA, S., HORIUCHI, K., TANIZAKI, H., KABASHIMA, K., KUBO, A., CHO, Y. H., CLAUSEN, B. E., MATSUSHIMA, K., SUEMATSU, M., FURTADO, G. C., LIRA, S. A., FARBER, J. M., UDEY, M. C. & AMAGAI, M. 2012. Stress-induced production of chemokines by hair follicles regulates the trafficking of dendritic cells in skin. *Nat Immunol,* 13**,** 744-52.

NAGARAJAN, P., ROMANO, R. A. & SINHA, S. 2008. Transcriptional control of the differentiation program of interfollicular epidermal keratinocytes. *Crit Rev Eukaryot Gene Expr,* 18**,** 57-79.

NAKAJIMA, S., KITOH, A., EGAWA, G., NATSUAKI, Y., NAKAMIZO, S., MONIAGA, C. S., OTSUKA, A., HONDA, T., HANAKAWA, S., AMANO, W., IWAKURA, Y., NAKAE, S., KUBO, M., MIYACHI, Y. & KABASHIMA, K. 2014. IL-17A as an Inducer for Th2 Immune Responses in Murine Atopic Dermatitis Models. *Journal of Investigative Dermatology,* 134**,** 2122-2130.

NAKAMURA, Y., FRANCHI, L., KAMBE, N., MENG, G., STROBER, W. & NUNEZ, G. 2012. Critical role for mast cells in interleukin-1beta-driven skin inflammation associated with an activating mutation in the nlrp3 protein. *Immunity,* 37**,** 85-95.

NAYLOR, E. C., WATSON, R. E. & SHERRATT, M. J. 2011. Molecular aspects of skin ageing. *Maturitas,* 69**,** 249-56.

NEMOTO-HASEBE, I., AKIYAMA, M., NOMURA, T., SANDILANDS, A., MCLEAN, W. H. & SHIMIZU, H. 2009. Clinical severity correlates with impaired barrier in filaggrin-related eczema. *J Invest Dermatol,* 129**,** 682-9.

NESTLE, F. O., CONRAD, C., TUN-KYI, A., HOMEY, B., GOMBERT, M., BOYMAN, O., BURG, G., LIU, Y. J. & GILLIET, M. 2005. Plasmacytoid predendritic cells initiate psoriasis through interferon-alpha production. *J Exp Med,* 202**,** 135-43.

NESTLE, F. O., DI MEGLIO, P., QIN, J. Z. & NICKOLOFF, B. J. 2009. Skin immune sentinels in health and disease. *Nat Rev Immunol,* 9**,** 679-91.

NG, J. M. & CURRAN, T. 2011. The Hedgehog's tale: developing strategies for targeting cancer. *Nat Rev Cancer,* 11**,** 493-501.

NICKEL, R., BECK, L. A., STELLATO, C. & SCHLEIMER, R. P. 1999. Chemokines and allergic disease. *J Allergy Clin Immunol,* 104**,** 723-42.

NICKOLOFF, B. J. & NAIDU, Y. 1994. Perturbation of epidermal barrier function correlates with initiation of cytokine cascade in human skin. *J Am Acad Dermatol,* 30**,** 535-46.

NILSSON, M., UNDEN, A. B., KRAUSE, D., MALMQWIST, U., RAZA, K., ZAPHIROPOULOS, P. G. & TOFTGARD, R. 2000. Induction of basal cell carcinomas and trichoepitheliomas in mice overexpressing GLI-1. *Proc Natl Acad Sci U S A,* 97**,** 3438-43.

NOGRALES, K. E., ZABA, L. C., SHEMER, A., FUENTES-DUCULAN, J., CARDINALE, I., KIKUCHI, T., RAMON, M., BERGMAN, R., KRUEGER, J. G. & GUTTMAN-YASSKY, E. 2009. IL-22-producing "T22" T cells account for upregulated IL-22 in atopic dermatitis despite reduced IL-17-producing TH17 T cells. *J Allergy Clin Immunol,* 123**,** 1244-52 e2.

NOMURA, T., KABASHIMA, K. & MIYACHI, Y. 2014. The panoply of alphabetaT cells in the skin. *J Dermatol Sci,* 76**,** 3-9.

NOMURA, T., SANDILANDS, A., AKIYAMA, M., LIAO, H., EVANS, A. T., SAKAI, K., OTA, M., SUGIURA, H., YAMAMOTO, K., SATO, H., PALMER, C. N., SMITH, F. J., MCLEAN, W. H. & SHIMIZU, H. 2007. Unique mutations in the filaggrin gene in Japanese patients with ichthyosis vulgaris and atopic dermatitis. *J Allergy Clin Immunol,* 119**,** 434-40.

NOVAIS, F. O., CARVALHO, A. M., CLARK, M. L., CARVALHO, L. P., BEITING, D. P., BRODSKY, I. E., CARVALHO, E. M. & SCOTT, P. 2017. CD8+ T cell cytotoxicity mediates pathology in the skin by inflammasome activation and IL-1beta production. *PLoS Pathog,* 13**,** e1006196.

NOVAK, N., KRUSE, S., POTRECK, J., MAINTZ, L., JENNECK, C., WEIDINGER, S., FIMMERS, R. & BIEBER, T. 2005. Single nucleotide polymorphisms of the IL18 gene are associated with atopic eczema. *J Allergy Clin Immunol,* 115**,** 828-33.

NUSSLEIN-VOLHARD, C. & WIESCHAUS, E. 1980. Mutations affecting segment number and polarity in Drosophila. *Nature,* 287**,** 795-801.

NUTTEN, S. 2015. Atopic dermatitis: global epidemiology and risk factors. *Ann Nutr Metab,* 66 Suppl 1**,** 8-16.

OCHI, H., ABRAHAM, M., ISHIKAWA, H., FRENKEL, D., YANG, K., BASSO, A. S., WU, H., CHEN, M. L., GANDHI, R., MILLER, A., MARON, R. & WEINER, H. L. 2006. Oral CD3-specific antibody suppresses autoimmune encephalomyelitis by inducing CD4+ CD25- LAP+ T cells. *Nat Med,* 12**,** 627-35.

OCHOA, B., SYN, W. K., DELGADO, I., KARACA, G. F., JUNG, Y., WANG, J., ZUBIAGA, A. M., FRESNEDO, O., OMENETTI, A., ZDANOWICZ, M., CHOI, S. S. & DIEHL,

A. M. 2010. Hedgehog signaling is critical for normal liver regeneration after partial hepatectomy in mice. *Hepatology,* 51**,** 1712-23.

OGUCHI, M., KOBAYASI, T. & ASBOE-HANSEN, G. 1985. Secretion of type IV collagen by keratinocytes of human adult. *J Invest Dermatol,* 85**,** 79-81.

OLSZEWSKI, W. L. 2005. The innate reaction of the human skin lymphatic system to foreign and self-antigens. *Lymphat Res Biol,* 3**,** 50-7.

OMENETTI, A., YANG, L., LI, Y. X., MCCALL, S. J., JUNG, Y., SICKLICK, J. K., HUANG, J., CHOI, S., SUZUKI, A. & DIEHL, A. M. 2007. Hedgehog-mediated mesenchymal-epithelial interactions modulate hepatic response to bile duct ligation. *Lab Invest,* 87**,** 499-514.

OMOTO, Y., YAMANAKA, K., TOKIME, K., KITANO, S., KAKEDA, M., AKEDA, T., KUROKAWA, I., GABAZZA, E. C., TSUTSUI, H., KATAYAMA, N., YAMANISHI, K., NAKANISHI, K. & MIZUTANI, H. 2010. Granzyme B is a novel interleukin-18 converting enzyme. *J Dermatol Sci,* 59**,** 129-35.

ONOUE, A., KABASHIMA, K., KOBAYASHI, M., MORI, T. & TOKURA, Y. 2009. Induction of eosinophil- and Th2-attracting epidermal chemokines and cutaneous late-phase reaction in tape-stripped skin. *Exp Dermatol,* 18**,** 1036-43.

ORO, A. E. & HIGGINS, K. 2003. Hair cycle regulation of Hedgehog signal reception. *Dev Biol,* 255**,** 238-48.

ORO, A. E., HIGGINS, K. M., HU, Z., BONIFAS, J. M., EPSTEIN, E. H., JR. & SCOTT, M. P. 1997. Basal cell carcinomas in mice overexpressing sonic hedgehog. *Science,* 276**,** 817-21.

OTSUKA, A., DREIER, J., CHENG, P. F., NAGELI, M., LEHMANN, H., FELDERER, L., FREW, I. J., MATSUSHITA, S., LEVESQUE, M. P. & DUMMER, R. 2015. Hedgehog pathway inhibitors promote adaptive immune responses in basal cell carcinoma. *Clin Cancer Res,* 21**,** 1289-97.

OTTAVIANI, M., CAMERA, E. & PICARDO, M. 2010. Lipid mediators in acne. *Mediators Inflamm,* 2010.

OUTRAM, S. V., HAGER-THEODORIDES, A. L., SHAH, D. K., ROWBOTHAM, N. J., DRAKOPOULOU, E., ROSS, S. E., LANSKE, B., DESSENS, J. T. & CROMPTON, T. 2009. Indian hedgehog (Ihh) both promotes and restricts thymocyte differentiation. *Blood,* 113**,** 2217-28.

OUTRAM, S. V., VARAS, A., PEPICELLI, C. V. & CROMPTON, T. 2000. Hedgehog signaling regulates differentiation from double-negative to double-positive thymocyte. *Immunity,* 13**,** 187-97.

OYOSHI, M. K., HE, R., LI, Y., MONDAL, S., YOON, J., AFSHAR, R., CHEN, M., LEE, D. M., LUO, H. R., LUSTER, A. D., CHO, J. S., MILLER, L. S., LARSON, A., MURPHY, G. F. & GEHA, R. S. 2012. Leukotriene B4-driven neutrophil recruitment to the skin is essential for allergic skin inflammation. *Immunity,* 37**,** 747-58.

OYOSHI, M. K., MURPHY, G. F. & GEHA, R. S. 2009. Filaggrin-deficient mice exhibit TH17-dominated skin inflammation and permissiveness to epicutaneous sensitization with protein antigen. *J Allergy Clin Immunol,* 124**,** 485-93, 493 e1.

PAK, E. & SEGAL, R. A. 2016. Hedgehog Signal Transduction: Key Players, Oncogenic Drivers, and Cancer Therapy. *Dev Cell,* 38**,** 333-44.

PALANIVEL, J. A., MACBETH, A. E., CHETTY, N. C. & LEVELL, N. J. 2014. An insight into JAK-STAT signalling in dermatology. *Clin Exp Dermatol,* 39**,** 513-8.

PALLADINO, M., GATTO, I., NERI, V., STRAINO, S., SILVER, M., TRITARELLI, A., PICCIONI, A., SMITH, R. C., GAETANI, E., LOSORDO, D. W., CREA, F., CAPOGROSSI, M. & POLA, R. 2011. Pleiotropic beneficial effects of sonic hedgehog gene therapy in an experimental model of peripheral limb ischemia. *Mol Ther,* 19**,** 658-66.

PALMER, C. N., IRVINE, A. D., TERRON-KWIATKOWSKI, A., ZHAO, Y., LIAO, H., LEE, S. P., GOUDIE, D. R., SANDILANDS, A., CAMPBELL, L. E., SMITH, F. J., O'REGAN, G. M., WATSON, R. M., CECIL, J. E., BALE, S. J., COMPTON, J. G., DIGIOVANNA, J. J., FLECKMAN, P., LEWIS-JONES, S., ARSECULERATNE, G., SERGEANT, A., MUNRO, C. S., EL HOUATE, B., MCELREAVEY, K., HALKJAER, L. B., BISGAARD, H., MUKHOPADHYAY, S. & MCLEAN, W. H. 2006. Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. *Nat Genet,* 38**,** 441-6.

PAN, J., WANG, Q. & SNELL, W. J. 2005. Cilium-generated signaling and cilia-related disorders. *Lab Invest,* 85**,** 452-63.

PANDURO, M., BENOIST, C. & MATHIS, D. 2016. Tissue Tregs. *Annu Rev Immunol,* 34**,** 609-33.

PANTELYUSHIN, S., HAAK, S., INGOLD, B., KULIG, P., HEPPNER, F. L., NAVARINI, A. A. & BECHER, B. 2012. Rorgammat+ innate lymphocytes and gammadelta T cells initiate psoriasiform plaque formation in mice. *J Clin Invest,* 122**,** 2252-6.

PARK, H. L., BAI, C., PLATT, K. A., MATISE, M. P., BEEGHLY, A., HUI, C. C., NAKASHIMA, M. & JOYNER, A. L. 2000. Mouse Gli1 mutants are viable but have defects in SHH signaling in combination with a Gli2 mutation. *Development,* 127**,** 1593-605.

PARMANTIER, E., LYNN, B., LAWSON, D., TURMAINE, M., NAMINI, S. S., CHAKRABARTI, L., MCMAHON, A. P., JESSEN, K. R. & MIRSKY, R. 1999. Schwann cell-derived Desert hedgehog controls the development of peripheral nerve sheaths. *Neuron,* 23**,** 713-24.

PASPARAKIS, M., HAASE, I. & NESTLE, F. O. 2014. Mechanisms regulating skin immunity and inflammation. *Nature Reviews Immunology,* 14**,** 289-301.

PEARTON, D. J., DALE, B. A. & PRESLAND, R. B. 2002. Functional analysis of the profilaggrin N-terminal peptide: identification of domains that regulate nuclear and cytoplasmic distribution. *J Invest Dermatol,* 119**,** 661-9.

PEREIRA TDE, A., WITEK, R. P., SYN, W. K., CHOI, S. S., BRADRICK, S., KARACA, G. F., AGBOOLA, K. M., JUNG, Y., OMENETTI, A., MOYLAN, C. A., YANG, L., FERNANDEZ-ZAPICO, M. E., JHAVERI, R., SHAH, V. H., PEREIRA, F. E. & DIEHL, A. M. 2010. Viral factors induce Hedgehog pathway activation in humans with viral hepatitis, cirrhosis, and hepatocellular carcinoma. *Lab Invest,* 90**,** 1690-703.

PEREZ-MORENO, M., DAVIS, M. A., WONG, E., PASOLLI, H. A., REYNOLDS, A. B. & FUCHS, E. 2006. p120-catenin mediates inflammatory responses in the skin. *Cell,* 124**,** 631-44.

PETERS, C., WOLF, A., WAGNER, M., KUHLMANN, J. & WALDMANN, H. 2004. The cholesterol membrane anchor of the Hedgehog protein confers stable

membrane association to lipid-modified proteins. *Proc Natl Acad Sci U S A,* 101**,** 8531-6.

PETROVA, R. & JOYNER, A. L. 2014. Roles for Hedgehog signaling in adult organ homeostasis and repair. *Development,* 141**,** 3445-57.

PEUKERT, S. & MILLER-MOSLIN, K. 2010. Small-molecule inhibitors of the hedgehog signaling pathway as cancer therapeutics. *ChemMedChem,* 5**,** 500-12.

PIETSCH, T., WAHA, A., KOCH, A., KRAUS, J., ALBRECHT, S., TONN, J., SORENSEN, N., BERTHOLD, F., HENK, B., SCHMANDT, N., WOLF, H. K., VON DEIMLING, A., WAINWRIGHT, B., CHENEVIX-TRENCH, G., WIESTLER, O. D. & WICKING, C. 1997. Medulloblastomas of the desmoplastic variant carry mutations of the human homologue of Drosophila patched. *Cancer Res,* 57**,** 2085-8.

POGACH, M. S., CAO, Y., MILLIEN, G., RAMIREZ, M. I. & WILLIAMS, M. C. 2007. Key developmental regulators change during hyperoxia-induced injury and recovery in adult mouse lung. *J Cell Biochem,* 100**,** 1415-29.

POLA, R., LING, L. E., SILVER, M., CORBLEY, M. J., KEARNEY, M., BLAKE PEPINSKY, R., SHAPIRO, R., TAYLOR, F. R., BAKER, D. P., ASAHARA, T. & ISNER, J. M. 2001. The morphogen Sonic hedgehog is an indirect angiogenic agent upregulating two families of angiogenic growth factors. *Nat Med,* 7**,** 706-11.

POULIN, L. F., HENRI, S., DE BOVIS, B., DEVILARD, E., KISSENPFENNIG, A. & MALISSEN, B. 2007. The dermis contains langerin+ dendritic cells that develop and function independently of epidermal Langerhans cells. *J Exp Med,* 204**,** 3119-31.

PRESLAND, R. B., HAYDOCK, P. V., FLECKMAN, P., NIRUNSUKSIRI, W. & DALE, B. A. 1992. Characterization of the human epidermal profilaggrin gene. Genomic organization and identification of an S-100-like calcium binding domain at the amino terminus. *J Biol Chem,* 267**,** 23772-81.

PROKSCH, E., BRANDNER, J. M. & JENSEN, J. M. 2008. The skin: an indispensable barrier. *Exp Dermatol,* 17**,** 1063-72.

PROKSCH, E., BRASCH, J. & STERRY, W. 1996. Integrity of the permeability barrier regulates epidermal Langerhans cell density. *Br J Dermatol,* 134**,** 630-8.

RAAP, U., WICHMANN, K., BRUDER, M., STANDER, S., WEDI, B., KAPP, A. & WERFEL, T. 2008. Correlation of IL-31 serum levels with severity of atopic dermatitis. *J Allergy Clin Immunol,* 122**,** 421-3.

RAMSBOTTOM, S. A. & POWNALL, M. E. 2016. Regulation of Hedgehog Signalling Inside and Outside the Cell. *Journal of Developmental Biology,* 4.

RAWLINGS, A. V. & HARDING, C. R. 2004. Moisturization and skin barrier function. *Dermatol Ther,* 17 Suppl 1**,** 43-8.

REEFER, A. J., SATINOVER, S. M., SOLGA, M. D., LANNIGAN, J. A., NGUYEN, J. T., WILSON, B. B. & WOODFOLK, J. A. 2008. Analysis of CD25hiCD4+ "regulatory" T-cell subtypes in atopic dermatitis reveals a novel T(H)2-like population. *J Allergy Clin Immunol,* 121**,** 415-422 e3.

REGL, G., KASPER, M., SCHNIDAR, H., EICHBERGER, T., NEILL, G. W., IKRAM, M. S., QUINN, A. G., PHILPOTT, M. P., FRISCHAUF, A. M. & ABERGER, F. 2004. The zinc-finger transcription factor GLI2 antagonizes contact inhibition and differentiation of human epidermal cells. *Oncogene,* 23**,** 1263-74.

REIFENBERGER, J., WOLTER, M., KNOBBE, C. B., KOHLER, B., SCHONICKE, A., SCHARWACHTER, C., KUMAR, K., BLASCHKE, B., RUZICKA, T. & REIFENBERGER, G. 2005. Somatic mutations in the PTCH, SMOH, SUFUH and TP53 genes in sporadic basal cell carcinomas. *Br J Dermatol,* 152**,** 43-51.

REIFENBERGER, J., WOLTER, M., WEBER, R. G., MEGAHED, M., RUZICKA, T., LICHTER, P. & REIFENBERGER, G. 1998. Missense mutations in SMOH in sporadic basal cell carcinomas of the skin and primitive neuroectodermal tumors of the central nervous system. *Cancer Res,* 58**,** 1798-803.

RIDDLE, R. D., JOHNSON, R. L., LAUFER, E. & TABIN, C. 1993. Sonic hedgehog mediates the polarizing activity of the ZPA. *Cell,* 75**,** 1401-16.

RIOBO, N. A. & MANNING, D. R. 2007. Pathways of signal transduction employed by vertebrate Hedgehogs. *Biochem J,* 403**,** 369-79.

RITCHIE, M. E., PHIPSON, B., WU, D., HU, Y., LAW, C. W., SHI, W. & SMYTH, G. K. 2015. limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res,* 43**,** e47.

ROBERTS, A. B., ANZANO, M. A., LAMB, L. C., SMITH, J. M. & SPORN, M. B. 1981. New class of transforming growth factors potentiated by epidermal growth factor: isolation from non-neoplastic tissues. *Proc Natl Acad Sci U S A,* 78**,** 5339-43.

ROEDIGER, B., KYLE, R., YIP, K. H., SUMARIA, N., GUY, T. V., KIM, B. S., MITCHELL, A. J., TAY, S. S., JAIN, R., FORBES-BLOM, E., CHEN, X., TONG, P. L., BOLTON, H. A., ARTIS, D., PAUL, W. E., FAZEKAS DE ST GROTH, B., GRIMBALDESTON, M. A., LE GROS, G. & WENINGER, W. 2013. Cutaneous immunosurveillance and regulation of inflammation by group 2 innate lymphoid cells. *Nat Immunol,* 14**,** 564-73.

ROELINK, H., AUGSBURGER, A., HEEMSKERK, J., KORZH, V., NORLIN, S., RUIZ I ALTABA, A., TANABE, Y., PLACZEK, M., EDLUND, T., JESSELL, T. M. & ET AL. 1994. Floor plate and motor neuron induction by vhh-1, a vertebrate homolog of hedgehog expressed by the notochord. *Cell,* 76**,** 761-75.

ROOP, D. 1995. Defects in the barrier. *Science,* 267**,** 474-5.

ROWBOTHAM, N. J., FURMANSKI, A. L., HAGER-THEODORIDES, A. L., ROSS, S. E., DRAKOPOULOU, E., KOUFARIS, C., OUTRAM, S. V. & CROMPTON, T. 2008. Repression of hedgehog signal transduction in T-lineage cells increases TCR-induced activation and proliferation. *Cell Cycle,* 7**,** 904-8.

ROWBOTHAM, N. J., HAGER-THEODORIDES, A. L., CEBECAUER, M., SHAH, D. K., DRAKOPOULOU, E., DYSON, J., OUTRAM, S. V. & CROMPTON, T. 2007. Activation of the Hedgehog signaling pathway in T-lineage cells inhibits TCR repertoire selection in the thymus and peripheral T-cell activation. *Blood,* 109**,** 3757-66.

ROWBOTHAM, N. J., HAGER-THEODORIDES, A. L., FURMANSKI, A. L., ROSS, S. E., OUTRAM, S. V., DESSENS, J. T. & CROMPTON, T. 2009. Sonic hedgehog negatively regulates pre-TCR-induced differentiation by a Gli2-dependent mechanism. *Blood,* 113**,** 5144-56.

RUBIN, A. I., CHEN, E. H. & RATNER, D. 2005. Basal-cell carcinoma. *N Engl J Med,* 353**,** 2262-9.

RUBTSOV, Y. P., RASMUSSEN, J. P., CHI, E. Y., FONTENOT, J., CASTELLI, L., YE, X., TREUTING, P., SIEWE, L., ROERS, A., HENDERSON, W. R., JR., MULLER, W. & RUDENSKY, A. Y. 2008. Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. *Immunity,* 28**,** 546-58.

SACEDON, R., DIEZ, B., NUNEZ, V., HERNANDEZ-LOPEZ, C., GUTIERREZ-FRIAS, C., CEJALVO, T., OUTRAM, S. V., CROMPTON, T., ZAPATA, A. G., VICENTE, A. & VARAS, A. 2005. Sonic hedgehog is produced by follicular dendritic cells and protects germinal center B cells from apoptosis. *J Immunol,* 174**,** 1456-61.

SACEDON, R., VARAS, A., HERNANDEZ-LOPEZ, C., GUTIERREZ-DEFRIAS, C., CROMPTON, T., ZAPATA, A. G. & VICENTE, A. 2003. Expression of hedgehog proteins in the human thymus. *J Histochem Cytochem,* 51**,** 1557-66.

SADARANGANI, A., PINEDA, G., LENNON, K. M., CHUN, H. J., SHIH, A., SCHAIRER, A. E., COURT, A. C., GOFF, D. J., PRASHAD, S. L., GERON, I., WALL, R., MCPHERSON, J. D., MOORE, R. A., PU, M., BAO, L., JACKSON-FISHER, A., MUNCHHOF, M., VANARSDALE, T., REYA, T., MORRIS, S. R., MINDEN, M. D., MESSER, K., MIKKOLA, H. K., MARRA, M. A., HUDSON, T. J. & JAMIESON, C. H. 2015. GLI2 inhibition abrogates human leukemia stem cell dormancy. *J Transl Med,* 13**,** 98.

SAKAGUCHI, S., SAKAGUCHI, N., ASANO, M., ITOH, M. & TODA, M. 1995. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol,* 155**,** 1151-64.

SALDANA, J. I., SOLANKI, A., LAU, C. I., SAHNI, H., ROSS, S., FURMANSKI, A. L., ONO, M., HOLLANDER, G. & CROMPTON, T. 2016. Sonic Hedgehog regulates thymic epithelial cell differentiation. *J Autoimmun,* 68**,** 86-97.

SALIMI, M., BARLOW, J. L., SAUNDERS, S. P., XUE, L., GUTOWSKA-OWSIAK, D., WANG, X., HUANG, L. C., JOHNSON, D., SCANLON, S. T., MCKENZIE, A. N., FALLON, P. G. & OGG, G. S. 2013. A role for IL-25 and IL-33-driven type-2 innate lymphoid cells in atopic dermatitis. *J Exp Med,* 210**,** 2939-50.

SALIMI, M. & OGG, G. 2014. Innate lymphoid cells and the skin. *BMC Dermatol,* 14**,** 18.

SAMPATH, K., CHENG, A. M., FRISCH, A. & WRIGHT, C. V. 1997. Functional differences among Xenopus nodal-related genes in left-right axis determination. *Development,* 124**,** 3293-302.

SANCHEZ RODRIGUEZ, R., PAULI, M. L., NEUHAUS, I. M., YU, S. S., ARRON, S. T., HARRIS, H. W., YANG, S. H., ANTHONY, B. A., SVERDRUP, F. M., KROW-LUCAL, E., MACKENZIE, T. C., JOHNSON, D. S., MEYER, E. H., LOHR, A., HSU, A., KOO, J., LIAO, W., GUPTA, R., DEBBANEH, M. G., BUTLER, D., HUYNH, M., LEVIN, E. C., LEON, A., HOFFMAN, W. Y., MCGRATH, M. H., ALVARADO, M. D., LUDWIG, C. H., TRUONG, H. A., MAURANO, M. M., GRATZ, I. K., ABBAS, A. K. & ROSENBLUM, M. D. 2014. Memory regulatory T cells reside in human skin. *J Clin Invest,* 124**,** 1027-36.

SANDILANDS, A., SUTHERLAND, C., IRVINE, A. D. & MCLEAN, W. H. 2009. Filaggrin in the frontline: role in skin barrier function and disease. *J Cell Sci,* 122**,** 1285-94.

SANDILANDS, A., TERRON-KWIATKOWSKI, A., HULL, P. R., O'REGAN, G. M., CLAYTON, T. H., WATSON, R. M., CARRICK, T., EVANS, A. T., LIAO, H., ZHAO, Y., CAMPBELL, L. E., SCHMUTH, M., GRUBER, R., JANECKE, A. R., ELIAS, P. M., VAN STEENSEL, M. A., NAGTZAAM, I., VAN GEEL, M., STEIJLEN, P. M., MUNRO, C. S., BRADLEY, D. G., PALMER, C. N., SMITH, F. J., MCLEAN, W. H. & IRVINE, A. D. 2007. Comprehensive analysis of the gene encoding filaggrin uncovers prevalent and rare mutations in ichthyosis vulgaris and atopic eczema. *Nat Genet,* 39**,** 650-4.

SANTOS, M., PARAMIO, J. M., BRAVO, A., RAMIREZ, A. & JORCANO, J. L. 2002. The expression of keratin k10 in the basal layer of the epidermis inhibits cell proliferation and prevents skin tumorigenesis. *J Biol Chem,* 277**,** 19122-30.

SASAKI, H., NISHIZAKI, Y., HUI, C., NAKAFUKU, M. & KONDOH, H. 1999. Regulation of Gli2 and Gli3 activities by an amino-terminal repression domain: implication of Gli2 and Gli3 as primary mediators of Shh signaling. *Development,* 126**,** 3915-24.

SATHER, B. D., TREUTING, P., PERDUE, N., MIAZGOWICZ, M., FONTENOT, J. D., RUDENSKY, A. Y. & CAMPBELL, D. J. 2007. Altering the distribution of Foxp3(+) regulatory T cells results in tissue-specific inflammatory disease. *J Exp Med,* 204**,** 1335-47.

SCHIMMANG, T., LEMAISTRE, M., VORTKAMP, A. & RUTHER, U. 1992. Expression of the zinc finger gene Gli3 is affected in the morphogenetic mouse mutant extra-toes (Xt). *Development,* 116**,** 799-804.

SCHMID-WENDTNER, M. H. & KORTING, H. C. 2006. The pH of the skin surface and its impact on the barrier function. *Skin Pharmacol Physiol,* 19**,** 296-302.

SCHMIDT, A., OBERLE, N. & KRAMMER, P. H. 2012. Molecular mechanisms of treg-mediated T cell suppression. *Front Immunol,* 3**,** 51.

SCHMUTH, M., NEYER, S., RAINER, C., GRASSEGGER, A., FRITSCH, P., ROMANI, N. & HEUFLER, C. 2002. Expression of the C-C chemokine MIP-3 alpha/CCL20 in human epidermis with impaired permeability barrier function. *Exp Dermatol,* 11**,** 135-42.

SCHNEIDER, S., THURNHER, D., KLOIMSTEIN, P., LEITNER, V., PETZELBAUER, P., PAMMER, J., BRUNNER, M. & EROVIC, B. M. 2011. Expression of the Sonic hedgehog pathway in squamous cell carcinoma of the skin and the mucosa of the head and neck. *Head Neck,* 33**,** 244-50.

SCHNOPP, C., RAD, R., WEIDINGER, A., WEIDINGER, S., RING, J., EBERLEIN, B., OLLERT, M. & MEMPEL, M. 2007. Fox-P3-positive regulatory T cells are present in the skin of generalized atopic eczema patients and are not particularly affected by medium-dose UVA1 therapy. *Photodermatology Photoimmunology & Photomedicine,* 23**,** 81-85.

SEGRE, J. A. 2006. Epidermal differentiation complex yields a secret: mutations in the cornification protein filaggrin underlie ichthyosis vulgaris. *J Invest Dermatol,* 126**,** 1202-4.

SHAH, D. K., HAGER-THEODORIDES, A. L., OUTRAM, S. V., ROSS, S. E., VARAS, A. & CROMPTON, T. 2004. Reduced thymocyte development in sonic hedgehog knockout embryos. *Journal of Immunology,* 172**,** 2296-2306.

SHEN, E., ZHAO, K., WU, C. & YANG, B. 2011. The suppressive effect of CD25+Treg cells on Th1 differentiation requires cell-cell contact partially via TGF-beta production. *Cell Biol Int,* 35**,** 705-12.

SHENG, H., GOICH, S., WANG, A., GRACHTCHOUK, M., LOWE, L., MO, R., LIN, K., DE SAUVAGE, F. J., SASAKI, H., HUI, C. C. & DLUGOSZ, A. A. 2002. Dissecting the oncogenic potential of Gli2: deletion of an NH(2)-terminal fragment alters skin tumor phenotype. *Cancer Res,* 62**,** 5308-16.

SHEU, M. Y., FOWLER, A. J., KAO, J., SCHMUTH, M., SCHOONJANS, K., AUWERX, J., FLUHR, J. W., MAN, M. Q., ELIAS, P. M. & FEINGOLD, K. R. 2002. Topical peroxisome proliferator activated receptor-alpha activators reduce inflammation in irritant and allergic contact dermatitis models. *J Invest Dermatol,* 118**,** 94-101.

SHI, C. & PAMER, E. G. 2011. Monocyte recruitment during infection and inflammation. *Nat Rev Immunol,* 11**,** 762-74.

SHIBATA, S., TADA, Y., HAU, C. S., MITSUI, A., KAMATA, M., ASANO, Y., SUGAYA, M., KADONO, T., MASAMOTO, Y., KUROKAWA, M., YAMAUCHI, T., KUBOTA, N., KADOWAKI, T. & SATO, S. 2015. Adiponectin regulates psoriasiform skin inflammation by suppressing IL-17 production from gammadelta-T cells. *Nat Commun,* 6**,** 7687.

SHORTMAN, K. & HEATH, W. R. 2010. The CD8+ dendritic cell subset. *Immunol Rev,* 234**,** 18-31.

SIGGINS, S. L., NGUYEN, N. Y., MCCORMACK, M. P., VASUDEVAN, S., VILLANI, R., JANE, S. M., WAINWRIGHT, B. J. & CURTIS, D. J. 2009. The Hedgehog receptor Patched1 regulates myeloid and lymphoid progenitors by distinct cell-extrinsic mechanisms. *Blood,* 114**,** 995-1004.

SILVA-VARGAS, V., LO CELSO, C., GIANGRECO, A., OFSTAD, T., PROWSE, D. M., BRAUN, K. M. & WATT, F. M. 2005. Beta-catenin and Hedgehog signal strength can specify number and location of hair follicles in adult epidermis without recruitment of bulge stem cells. *Dev Cell,* 9**,** 121-31.

SINGH, V. B., SINGH, M. V., GORANTLA, S., POLUEKTOVA, L. Y. & MAGGIRWAR, S. B. 2016. Smoothened Agonist Reduces Human Immunodeficiency Virus Type-1-Induced Blood-Brain Barrier Breakdown in Humanized Mice. *Scientific Reports,* 6.

SINGH, V. B., SINGH, M. V., PIEKNA-PRZYBYLSKA, D., GORANTLA, S., POLUEKTOVA, L. Y. & MAGGIRWAR, S. B. 2017. Sonic Hedgehog mimetic prevents leukocyte infiltration into the CNS during acute HIV infection. *Scientific Reports,* 7.

SKOBE, M. & DETMAR, M. 2000. Structure, function, and molecular control of the skin lymphatic system. *J Investig Dermatol Symp Proc,* 5**,** 14-9.

SMELKINSON, M. G. 2017. The Hedgehog Signaling Pathway Emerges as a Pathogenic Target. *J Dev Biol,* 5.

SMELKINSON, M. G., GUICHARD, A., TEIJARO, J. R., MALUR, M., LOUREIRO, M. E., JAIN, P., GANESAN, S., ZUNIGA, E. I., KRUG, R. M., OLDSTONE, M. B. & BIER, E. 2017. Influenza NS1 directly modulates Hedgehog signaling during infection. *PLoS Pathog,* 13**,** e1006588.

SMITH, L. T., UNDERWOOD, R. A. & MCLEAN, W. H. 1999. Ontogeny and regional variability of keratin 2e (K2e) in developing human fetal skin: a unique

spatial and temporal pattern of keratin expression in development. *Br J Dermatol,* 140**,** 582-91.

SOLANKI, A., LAU, C. I., SALDANA, J. I., ROSS, S. & CROMPTON, T. 2017. The transcription factor Gli3 promotes B cell development in fetal liver through repression of Shh. *J Exp Med,* 214**,** 2041-2058.

SOLANKI, A., YANEZ, D. C., ROSS, S., LAU, C. I., PAPAIOANNOU, E., LI, J., SALDANA, J. I. & CROMPTON, T. 2018. Gli3 in fetal thymic epithelial cells promotes thymocyte positive selection and differentiation by repression of Shh. *Development,* 145.

SOLER, D., HUMPHREYS, T. L., SPINOLA, S. M. & CAMPBELL, J. J. 2003. CCR4 versus CCR10 in human cutaneous TH lymphocyte trafficking. *Blood,* 101**,** 1677-82.

SON, E. D., KIM, H. J., KIM, K. H., BIN, B. H., BAE, I. H., LIM, K. M., YU, S. J., CHO, E. G. & LEE, T. R. 2016. S100A7 (psoriasin) inhibits human epidermal differentiation by enhanced IL-6 secretion through IkappaB/NF-kappaB signalling. *Exp Dermatol,* 25**,** 636-41.

SON, E. D., KIM, H. J., PARK, T., SHIN, K., BAE, I. H., LIM, K. M., CHO, E. G. & LEE, T. R. 2014. Staphylococcus aureus inhibits terminal differentiation of normal human keratinocytes by stimulating interleukin-6 secretion. *J Dermatol Sci,* 74**,** 64-71.

SOUMELIS, V., RECHE, P. A., KANZLER, H., YUAN, W., EDWARD, G., HOMEY, B., GILLIET, M., HO, S., ANTONENKO, S., LAUERMA, A., SMITH, K., GORMAN, D., ZURAWSKI, S., ABRAMS, J., MENON, S., MCCLANAHAN, T., DE WAAL-MALEFYT RD, R., BAZAN, F., KASTELEIN, R. A. & LIU, Y. J. 2002. Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. *Nat Immunol,* 3**,** 673-80.

SPERGEL, J. M., MIZOGUCHI, E., OETTGEN, H., BHAN, A. K. & GEHA, R. S. 1999. Roles of TH1 and TH2 cytokines in a murine model of allergic dermatitis. *J Clin Invest,* 103**,** 1103-11.

SRENATHAN, U., STEEL, K. & TAAMS, L. S. 2016. IL-17+ CD8+ T cells: Differentiation, phenotype and role in inflammatory disease. *Immunol Lett,* 178**,** 20-6.

ST-JACQUES, B., HAMMERSCHMIDT, M. & MCMAHON, A. P. 1999. Indian hedgehog signaling regulates proliferation and differentiation of chondrocytes and is essential for bone formation. *Genes Dev,* 13**,** 2072-86.

STANDING, A. S. I., YANEZ, D. C., ROSS, R., CROMPTON, T. & FURMANSKI, A. L. 2017. Frontline Science: Shh production and Gli signaling is activated in vivo in lung, enhancing the Th2 response during a murine model of allergic asthma. *J Leukoc Biol,* 102**,** 965-976.

STECCA, B., MAS, C., CLEMENT, V., ZBINDEN, M., CORREA, R., PIGUET, V., BEERMANN, F. & RUIZ, I. A. A. 2007. Melanomas require HEDGEHOG-GLI signaling regulated by interactions between GLI1 and the RAS-MEK/AKT pathways. *Proc Natl Acad Sci U S A,* 104**,** 5895-900.

STEINMAN, L. 2007. A brief history of T(H)17, the first major revision in the T(H)1/T(H)2 hypothesis of T cell-mediated tissue damage. *Nat Med,* 13**,** 139-45.

STOCKIS, J., COLAU, D., COULIE, P. G. & LUCAS, S. 2009. Membrane protein GARP is a receptor for latent TGF-beta on the surface of activated human Treg. *Eur J Immunol,* 39**,** 3315-22.

STOITZNER, P., TRIPP, C. H., EBERHART, A., PRICE, K. M., JUNG, J. Y., BURSCH, L., RONCHESE, F. & ROMANI, N. 2006. Langerhans cells cross-present antigen derived from skin. *Proc Natl Acad Sci U S A,* 103**,** 7783-8.

STONE, D. M., HYNES, M., ARMANINI, M., SWANSON, T. A., GU, Q., JOHNSON, R. L., SCOTT, M. P., PENNICA, D., GODDARD, A., PHILLIPS, H., NOLL, M., HOOPER, J. E., DE SAUVAGE, F. & ROSENTHAL, A. 1996. The tumour-suppressor gene patched encodes a candidate receptor for Sonic hedgehog. *Nature,* 384**,** 129-34.

STRACHAN, D. P., WONG, H. J. & SPECTOR, T. D. 2001. Concordance and interrelationship of atopic diseases and markers of allergic sensitization among adult female twins. *J Allergy Clin Immunol,* 108**,** 901-7.

STRAFACE, G., APRAHAMIAN, T., FLEX, A., GAETANI, E., BISCETTI, F., SMITH, R. C., PECORINI, G., POLA, E., ANGELINI, F., STIGLIANO, E., CASTELLOT, J. J., JR., LOSORDO, D. W. & POLA, R. 2009. Sonic hedgehog regulates angiogenesis and myogenesis during post-natal skeletal muscle regeneration. *J Cell Mol Med,* 13**,** 2424-35.

STRID, J., HOURIHANE, J., KIMBER, I., CALLARD, R. & STROBEL, S. 2004. Disruption of the stratum corneum allows potent epicutaneous immunization with protein antigens resulting in a dominant systemic Th2 response. *Eur J Immunol,* 34**,** 2100-9.

SUFFIA, I., RECKLING, S. K., SALAY, G. & BELKAID, Y. 2005. A role for CD103 in the retention of CD4+CD25+ Treg and control of Leishmania major infection. *J Immunol,* 174**,** 5444-55.

SUGAYA, M., MIYAGAKI, T., OHMATSU, H., SUGA, H., KAI, H., KAMATA, M., FUJITA, H., ASANO, Y., TADA, Y., KADONO, T., OKOCHI, H. & SATO, S. 2012. Association of the numbers of CD163(+) cells in lesional skin and serum levels of soluble CD163 with disease progression of cutaneous T cell lymphoma. *J Dermatol Sci,* 68**,** 45-51.

SUTTON, C. E., LALOR, S. J., SWEENEY, C. M., BRERETON, C. F., LAVELLE, E. C. & MILLS, K. H. 2009. Interleukin-1 and IL-23 induce innate IL-17 production from gammadelta T cells, amplifying Th17 responses and autoimmunity. *Immunity,* 31**,** 331-41.

SVARD, J., HEBY-HENRICSON, K., PERSSON-LEK, M., ROZELL, B., LAUTH, M., BERGSTROM, A., ERICSON, J., TOFTGARD, R. & TEGLUND, S. 2006. Genetic elimination of Suppressor of fused reveals an essential repressor function in the mammalian Hedgehog signaling pathway. *Dev Cell,* 10**,** 187-97.

SYN, W. K., WITEK, R. P., CURBISHLEY, S. M., JUNG, Y., CHOI, S. S., ENRICH, B., OMENETTI, A., AGBOOLA, K. M., FEARING, C. M., TILG, H., ADAMS, D. H. & DIEHL, A. M. 2009. Role for hedgehog pathway in regulating growth and function of invariant NKT cells. *Eur J Immunol,* 39**,** 1879-92.

SZEGEDI, A., BARATH, S., NAGY, G., SZODORAY, P., GAL, M., SIPKA, S., BAGDI, E., BANHAM, A. H. & KRENACS, L. 2009. Regulatory T cells in atopic dermatitis: epidermal dendritic cell clusters may contribute to their local expansion. *Br J Dermatol,* 160**,** 984-93.

TAIPALE, J., CHEN, J. K., COOPER, M. K., WANG, B., MANN, R. K., MILENKOVIC, L., SCOTT, M. P. & BEACHY, P. A. 2000. Effects of oncogenic mutations in Smoothened and Patched can be reversed by cyclopamine. *Nature,* 406**,** 1005-9.

TAIPALE, J., COOPER, M. K., MAITI, T. & BEACHY, P. A. 2002. Patched acts catalytically to suppress the activity of Smoothened. *Nature,* 418**,** 892-7.

TAKABA, H. & TAKAYANAGI, H. 2017. The Mechanisms of T Cell Selection in the Thymus. *Trends Immunol,* 38**,** 805-816.

TAKEBE, N., MIELE, L., HARRIS, P. J., JEONG, W., BANDO, H., KAHN, M., YANG, S. X. & IVY, S. P. 2015. Targeting Notch, Hedgehog, and Wnt pathways in cancer stem cells: clinical update. *Nat Rev Clin Oncol,* 12**,** 445-64.

TAKIMOTO, T., WAKABAYASHI, Y., SEKIYA, T., INOUE, N., MORITA, R., ICHIYAMA, K., TAKAHASHI, R., ASAKAWA, M., MUTO, G., MORI, T., HASEGAWA, E., SAIKA, S., HARA, T., NOMURA, M. & YOSHIMURA, A. 2010. Smad2 and Smad3 are redundantly essential for the TGF-beta-mediated regulation of regulatory T plasticity and Th1 development. *J Immunol,* 185**,** 842-55.

TANG, Q. & BLUESTONE, J. A. 2008. The Foxp3+ regulatory T cell: a jack of all trades, master of regulation. *Nat Immunol,* 9**,** 239-44.

TE WELSCHER, P., FERNANDEZ-TERAN, M., ROS, M. A. & ZELLER, R. 2002. Mutual genetic antagonism involving GLI3 and dHAND prepatterns the vertebrate limb bud mesenchyme prior to SHH signaling. *Genes Dev,* 16**,** 421-6.

TEGLUND, S. & TOFTGARD, R. 2010. Hedgehog beyond medulloblastoma and basal cell carcinoma. *Biochim Biophys Acta,* 1805**,** 181-208.

TEMPE, D., CASAS, M., KARAZ, S., BLANCHET-TOURNIER, M. F. & CONCORDET, J. P. 2006. Multisite protein kinase A and glycogen synthase kinase 3beta phosphorylation leads to Gli3 ubiquitination by SCFbetaTrCP. *Mol Cell Biol,* 26**,** 4316-26.

TENZEN, T., ALLEN, B. L., COLE, F., KANG, J. S., KRAUSS, R. S. & MCMAHON, A. P. 2006. The cell surface membrane proteins Cdo and Boc are components and targets of the Hedgehog signaling pathway and feedback network in mice. *Dev Cell,* 10**,** 647-56.

TEPERINO, R., ABERGER, F., ESTERBAUER, H., RIOBO, N. & POSPISILIK, J. A. 2014. Canonical and non-canonical Hedgehog signalling and the control of metabolism. *Semin Cell Dev Biol,* 33**,** 81-92.

TEPERINO, R., AMANN, S., BAYER, M., MCGEE, S. L., LOIPETZBERGER, A., CONNOR, T., JAEGER, C., KAMMERER, B., WINTER, L., WICHE, G., DALGAARD, K., SELVARAJ, M., GASTER, M., LEE-YOUNG, R. S., FEBBRAIO, M. A., KNAUF, C., CANI, P. D., ABERGER, F., PENNINGER, J. M., POSPISILIK, J. A. & ESTERBAUER, H. 2012. Hedgehog partial agonism drives Warburg-like metabolism in muscle and brown fat. *Cell,* 151**,** 414-26.

THODY, A. J. & SHUSTER, S. 1989. Control and function of sebaceous glands. *Physiol Rev,* 69**,** 383-416.

TOKURA, Y. 2010. Extrinsic and intrinsic types of atopic dermatitis. *J Dermatol Sci,* 58**,** 1-7.

TOMURA, M., HONDA, T., TANIZAKI, H., OTSUKA, A., EGAWA, G., TOKURA, Y., WALDMANN, H., HORI, S., CYSTER, J. G., WATANABE, T., MIYACHI, Y., KANAGAWA, O. & KABASHIMA, K. 2010. Activated regulatory T cells are

the major T cell type emigrating from the skin during a cutaneous immune response in mice. *J Clin Invest,* 120**,** 883-93.

TOSTAR, U., MALM, C. J., MEIS-KINDBLOM, J. M., KINDBLOM, L. G., TOFTGARD, R. & UNDEN, A. B. 2006. Deregulation of the hedgehog signalling pathway: a possible role for the PTCH and SUFU genes in human rhabdomyoma and rhabdomyosarcoma development. *J Pathol,* 208**,** 17-25.

TRAN, D. Q. 2012. TGF-beta: the sword, the wand, and the shield of FOXP3(+) regulatory T cells. *J Mol Cell Biol,* 4**,** 29-37.

TRAN, D. Q., ANDERSSON, J., WANG, R., RAMSEY, H., UNUTMAZ, D. & SHEVACH, E. M. 2009a. GARP (LRRC32) is essential for the surface expression of latent TGF-beta on platelets and activated FOXP3+ regulatory T cells. *Proc Natl Acad Sci U S A,* 106**,** 13445-50.

TRAN, D. Q., GLASS, D. D., UZEL, G., DARNELL, D. A., SPALDING, C., HOLLAND, S. M. & SHEVACH, E. M. 2009b. Analysis of adhesion molecules, target cells, and role of IL-2 in human FOXP3+ regulatory T cell suppressor function. *J Immunol,* 182**,** 2929-38.

TRAUTMANN, A., AKDIS, M., KLEEMANN, D., ALTZNAUER, F., SIMON, H. U., GRAEVE, T., NOLL, M., BROCKER, E. B., BLASER, K. & AKDIS, C. A. 2000. T cell-mediated Fas-induced keratinocyte apoptosis plays a key pathogenetic role in eczematous dermatitis. *J Clin Invest,* 106**,** 25-35.

TROWBRIDGE, J. J., SCOTT, M. P. & BHATIA, M. 2006. Hedgehog modulates cell cycle regulators in stem cells to control hematopoietic regeneration. *Proc Natl Acad Sci U S A,* 103**,** 14134-9.

TSUTSUI, H., YOSHIMOTO, T., HAYASHI, N., MIZUTANI, H. & NAKANISHI, K. 2004. Induction of allergic inflammation by interleukin-18 in experimental animal models. *Immunol Rev,* 202**,** 115-38.

VAN DEN BRINK, G. R., HARDWICK, J. C., NIELSEN, C., XU, C., TEN KATE, F. J., GLICKMAN, J., VAN DEVENTER, S. J., ROBERTS, D. J. & PEPPELENBOSCH, M. P. 2002. Sonic hedgehog expression correlates with fundic gland differentiation in the adult gastrointestinal tract. *Gut,* 51**,** 628-33.

VAN DEN HEUVEL, M. & INGHAM, P. W. 1996. smoothened encodes a receptor-like serpentine protein required for hedgehog signalling. *Nature,* 382**,** 547-51.

VAN DOP, W. A., HEIJMANS, J., BULLER, N. V. J. A., SNOEK, S. A., ROSEKRANS, S. L., WASSENBERG, E. A., WEERMAN, M. A. V., LANSKE, B., CLARKE, A. R., WINTON, D. J., WIJGERDE, M., OFFERHAUS, G. J., HOMMES, D. W., HARDWICK, J. C., DE JONGE, W. J., BIEMOND, I. & VAN DEN BRINK, G. R. 2010. Loss of Indian Hedgehog Activates Multiple Aspects of a Wound Healing Response in the Mouse Intestine. *Gastroenterology,* 139**,** 1665-+.

VAN FURTH, R., NIBBERING, P. H., VAN DISSEL, J. T. & DIESSELHOFF-DEN DULK, M. M. 1985. The characterization, origin, and kinetics of skin macrophages during inflammation. *J Invest Dermatol,* 85**,** 398-402.

VAN LOOSDREGT, J., FLESKENS, V., TIEMESSEN, M. M., MOKRY, M., VAN BOXTEL, R., MEERDING, J., PALS, C. E., KUREK, D., BAERT, M. R., DELEMARRE, E. M., GRONE, A., KOERKAMP, M. J., SIJTS, A. J., NIEUWENHUIS, E. E., MAURICE, M. M., VAN ES, J. H., TEN BERGE, D., HOLSTEGE, F. C., STAAL, F. J., ZAISS,

D. M., PRAKKEN, B. J. & COFFER, P. J. 2013. Canonical Wnt signaling negatively modulates regulatory T cell function. *Immunity,* 39**,** 298-310.

VARJOSALO, M. & TAIPALE, J. 2008. Hedgehog: functions and mechanisms. *Genes Dev,* 22**,** 2454-72.

VERHAGEN, J., AKDIS, M., TRAIDL-HOFFMANN, C., SCHMID-GRENDELMEIER, P., HIJNEN, D., KNOL, E. F., BEHRENDT, H., BLASER, K. & AKDIS, C. A. 2006. Absence of T-regulatory cell expression and function in atopic dermatitis skin. *J Allergy Clin Immunol,* 117**,** 176-83.

VESTERGAARD, C., YONEYAMA, H., MURAI, M., NAKAMURA, K., TAMAKI, K., TERASHIMA, Y., IMAI, T., YOSHIE, O., IRIMURA, T., MIZUTANI, H. & MATSUSHIMA, K. 1999. Overproduction of Th2-specific chemokines in NC/Nga mice exhibiting atopic dermatitis-like lesions. *J Clin Invest,* 104**,** 1097-105.

VIJAYANAND, P., SEUMOIS, G., SIMPSON, L. J., ABDUL-WAJID, S., BAUMJOHANN, D., PANDURO, M., HUANG, X., INTERLANDI, J., DJURETIC, I. M., BROWN, D. R., SHARPE, A. H., RAO, A. & ANSEL, K. M. 2012. Interleukin-4 production by follicular helper T cells requires the conserved Il4 enhancer hypersensitivity site V. *Immunity,* 36**,** 175-87.

VILLANOVA, F., FLUTTER, B., TOSI, I., GRYS, K., SREENEEBUS, H., PERERA, G. K., CHAPMAN, A., SMITH, C. H., DI MEGLIO, P. & NESTLE, F. O. 2014. Characterization of innate lymphoid cells in human skin and blood demonstrates increase of NKp44+ ILC3 in psoriasis. *J Invest Dermatol,* 134**,** 984-991.

VOKES, S. A., JI, H., WONG, W. H. & MCMAHON, A. P. 2008. A genome-scale analysis of the cis-regulatory circuitry underlying sonic hedgehog-mediated patterning of the mammalian limb. *Genes Dev,* 22**,** 2651-63.

VOLZ, T., NEGA, M., BUSCHMANN, J., KAESLER, S., GUENOVA, E., PESCHEL, A., ROCKEN, M., GOTZ, F. & BIEDERMANN, T. 2010. Natural Staphylococcus aureus-derived peptidoglycan fragments activate NOD2 and act as potent costimulators of the innate immune system exclusively in the presence of TLR signals. *FASEB J,* 24**,** 4089-102.

VULCANO, M., ALBANESI, C., STOPPACCIARO, A., BAGNATI, R., D'AMICO, G., STRUYF, S., TRANSIDICO, P., BONECCHI, R., DEL PRETE, A., ALLAVENA, P., RUCO, L. P., CHIABRANDO, C., GIROLOMONI, G., MANTOVANI, A. & SOZZANI, S. 2001. Dendritic cells as a major source of macrophage-derived chemokine/CCL22 in vitro and in vivo. *Eur J Immunol,* 31**,** 812-22.

WAGNER, A. J., MESSERSMITH, W. A., SHAIK, M. N., LI, S., ZHENG, X., MCLACHLAN, K. R., CESARI, R., COURTNEY, R., LEVIN, W. J. & EL-KHOUEIRY, A. B. 2015. A phase I study of PF-04449913, an oral hedgehog inhibitor, in patients with advanced solid tumors. *Clin Cancer Res,* 21**,** 1044-51.

WAHL, S. M. 2007. Transforming growth factor-beta: innately bipolar. *Curr Opin Immunol,* 19**,** 55-62.

WANG, B. L., FALLON, J. F. & BEACHY, P. A. 2000. Hedgehog-regulated processing of Gli3 produces an anterior/posterior repressor gradient in the developing vertebrate limb. *Cell,* 100**,** 423-434.

WANG, J. L., QI, Z., LI, Y. H., ZHAO, H. M., CHEN, Y. G. & FU, W. 2017a. TGFbeta induced factor homeobox 1 promotes colorectal cancer development

through activating Wnt/beta-catenin signaling. *Oncotarget,* 8**,** 70214-70225.

WANG, W., YU, X., WU, C. & JIN, H. 2017b. IL-36gamma inhibits differentiation and induces inflammation of keratinocyte via Wnt signaling pathway in psoriasis. *Int J Med Sci,* 14**,** 1002-1007.

WATANABE, R., GEHAD, A., YANG, C., SCOTT, L. L., TEAGUE, J. E., SCHLAPBACH, C., ELCO, C. P., HUANG, V., MATOS, T. R., KUPPER, T. S. & CLARK, R. A. 2015. Human skin is protected by four functionally and phenotypically discrete populations of resident and recirculating memory T cells. *Sci Transl Med,* 7**,** 279ra39.

WATANABE, Y. & NAKAMURA, H. 2000. Control of chick tectum territory along dorsoventral axis by Sonic hedgehog. *Development,* 127**,** 1131-40.

WATKINS, D. N., BERMAN, D. M., BURKHOLDER, S. G., WANG, B., BEACHY, P. A. & BAYLIN, S. B. 2003. Hedgehog signalling within airway epithelial progenitors and in small-cell lung cancer. *Nature,* 422**,** 313-7.

WATT, F. M. & FUJIWARA, H. 2011. Cell-extracellular matrix interactions in normal and diseased skin. *Cold Spring Harb Perspect Biol,* 3.

WATT, F. M. & JENSEN, K. B. 2009. Epidermal stem cell diversity and quiescence. *EMBO Mol Med,* 1**,** 260-7.

WEIDINGER, S., ILLIG, T., BAURECHT, H., IRVINE, A. D., RODRIGUEZ, E., DIAZ-LACAVA, A., KLOPP, N., WAGENPFEIL, S., ZHAO, Y., LIAO, H., LEE, S. P., PALMER, C. N., JENNECK, C., MAINTZ, L., HAGEMANN, T., BEHRENDT, H., RING, J., NOTHEN, M. M., MCLEAN, W. H. & NOVAK, N. 2006. Loss-of-function variations within the filaggrin gene predispose for atopic dermatitis with allergic sensitizations. *J Allergy Clin Immunol,* 118**,** 214-9.

WEN, L., BARBER, D. F., PAO, W., WONG, F. S., OWEN, M. J. & HAYDAY, A. 1998. Primary gamma delta cell clones can be defined phenotypically and functionally as Th1/Th2 cells and illustrate the association of CD4 with Th2 differentiation. *J Immunol,* 160**,** 1965-74.

WEN, X., LAI, C. K., EVANGELISTA, M., HONGO, J. A., DE SAUVAGE, F. J. & SCALES, S. J. 2010. Kinetics of hedgehog-dependent full-length Gli3 accumulation in primary cilia and subsequent degradation. *Mol Cell Biol,* 30**,** 1910-22.

WENZEL, J. & TUTING, T. 2008. An IFN-associated cytotoxic cellular immune response against viral, self-, or tumor antigens is a common pathogenetic feature in "interface dermatitis". *J Invest Dermatol,* 128**,** 2392-402.

WHITE, A. C., LAVINE, K. J. & ORNITZ, D. M. 2007. FGF9 and SHH regulate mesenchymal Vegfa expression and development of the pulmonary capillary network. *Development,* 134**,** 3743-52.

WILD, A., KALFF-SUSKE, M., VORTKAMP, A., BORNHOLDT, D., KONIG, R. & GRZESCHIK, K. H. 1997. Point mutations in human GLI3 cause Greig syndrome. *Hum Mol Genet,* 6**,** 1979-84.

WONG, R., GEYER, S., WENINGER, W., GUIMBERTEAU, J. C. & WONG, J. K. 2016. The dynamic anatomy and patterning of skin. *Exp Dermatol,* 25**,** 92-8.

WOO, S. H., STUMPFOVA, M., JENSEN, U. B., LUMPKIN, E. A. & OWENS, D. M. 2010. Identification of epidermal progenitors for the Merkel cell lineage. *Development,* 137**,** 3965-71.

WOO, W. M., ZHEN, H. H. & ORO, A. E. 2012. Shh maintains dermal papilla identity and hair morphogenesis via a Noggin-Shh regulatory loop. *Genes Dev,* 26**,** 1235-46.

WOOD, L. C., JACKSON, S. M., ELIAS, P. M., GRUNFELD, C. & FEINGOLD, K. R. 1992. Cutaneous barrier perturbation stimulates cytokine production in the epidermis of mice. *J Clin Invest,* 90**,** 482-7.

WOODWARD, A. L., SPERGEL, J. M., ALENIUS, H., MIZOGUCHI, E., BHAN, A. K., CASTIGLI, E., BRODEUR, S. R., OETTGEN, H. C. & GEHA, R. S. 2001. An obligate role for T-cell receptor alpha beta T+ cells but not T-cell receptor gamma delta T+ cells, B cells, or CD40/CD40L interactions in a mouse model of atopic dermatitis. *Journal of Allergy and Clinical Immunology,* 107**,** 359-366.

XIAO, C., FENG, R., ENGEVIK, A. C., MARTIN, J. R., TRITSCHLER, J. A., SCHUMACHER, M., KONCAR, R., ROLAND, J., NAM, K. T., GOLDENRING, J. R. & ZAVROS, Y. 2013. Sonic Hedgehog contributes to gastric mucosal restitution after injury. *Lab Invest,* 93**,** 96-111.

XIAO, Y., THORESEN, D. T., WILLIAMS, J. S., WANG, C., PERNA, J., PETROVA, R. & BROWNELL, I. 2015. Neural Hedgehog signaling maintains stem cell renewal in the sensory touch dome epithelium. *Proc Natl Acad Sci U S A,* 112**,** 7195-200.

XIE, J., MURONE, M., LUOH, S. M., RYAN, A., GU, Q., ZHANG, C., BONIFAS, J. M., LAM, C. W., HYNES, M., GODDARD, A., ROSENTHAL, A., EPSTEIN, E. H., JR. & DE SAUVAGE, F. J. 1998. Activating Smoothened mutations in sporadic basal-cell carcinoma. *Nature,* 391**,** 90-2.

YAMAMOTO, J., ADACHI, Y., ONOUE, Y., ADACHI, Y. S., OKABE, Y., ITAZAWA, T., TOYODA, M., SEKI, T., MOROHASHI, M., MATSUSHIMA, K. & MIYAWAKI, T. 2000. Differential expression of the chemokine receptors by the Th1- and Th2-type effector populations within circulating CD4+ T cells. *J Leukoc Biol,* 68**,** 568-74.

YAMANAKA, K. & MIZUTANI, H. 2011. The role of cytokines/chemokines in the pathogenesis of atopic dermatitis. *Curr Probl Dermatol,* 41**,** 80-92.

YANG, Q., MONTICELLI, L. A., SAENZ, S. A., CHI, A. W., SONNENBERG, G. F., TANG, J., DE OBALDIA, M. E., BAILIS, W., BRYSON, J. L., TOSCANO, K., HUANG, J., HACZKU, A., PEAR, W. S., ARTIS, D. & BHANDOOLA, A. 2013. T cell factor 1 is required for group 2 innate lymphoid cell generation. *Immunity,* 38**,** 694-704.

YE, F., BRESLOW, D. K., KOSLOVER, E. F., SPAKOWITZ, A. J., NELSON, W. J. & NACHURY, M. V. 2013. Single molecule imaging reveals a major role for diffusion in the exploration of ciliary space by signaling receptors. *Elife,* 2**,** e00654.

YESIL, P., MICHEL, M., CHWALEK, K., PEDACK, S., JANY, C., LUDWIG, B., BORNSTEIN, S. R. & LAMMERT, E. 2009. A new collagenase blend increases the number of islets isolated from mouse pancreas. *Islets,* 1**,** 185-90.

YOO, J., OMORI, M., GYARMATI, D., ZHOU, B., AYE, T., BREWER, A., COMEAU, M. R., CAMPBELL, D. J. & ZIEGLER, S. F. 2005. Spontaneous atopic dermatitis in mice expressing an inducible thymic stromal lymphopoietin transgene specifically in the skin. *J Exp Med,* 202**,** 541-9.

YOSHIKI, R., KABASHIMA, K., HONDA, T., NAKAMIZO, S., SAWADA, Y., SUGITA, K., YOSHIOKA, H., OHMORI, S., MALISSEN, B., TOKURA, Y. & NAKAMURA, M. 2014. IL-23 from Langerhans cells is required for the development of imiquimod-induced psoriasis-like dermatitis by induction of IL-17A-producing gammadelta T cells. *J Invest Dermatol,* 134**,** 1912-1921.

YURCHENCO, P. D. & SCHITTNY, J. C. 1990. Molecular architecture of basement membranes. *FASEB J,* 4**,** 1577-90.

ZACHARIAS, W. J., LI, X., MADISON, B. B., KRETOVICH, K., KAO, J. Y., MERCHANT, J. L. & GUMUCIO, D. L. 2010. Hedgehog Is an Anti-Inflammatory Epithelial Signal for the Intestinal Lamina Propria. *Gastroenterology,* 138**,** 2368-U226.

ZARDOYA, R., ABOUHEIF, E. & MEYER, A. 1996. Evolution and orthology of hedgehog genes. *Trends Genet,* 12**,** 496-7.

ZENG, X., GOETZ, J. A., SUBER, L. M., SCOTT, W. J., JR., SCHREINER, C. M. & ROBBINS, D. J. 2001. A freely diffusible form of Sonic hedgehog mediates long-range signalling. *Nature,* 411**,** 716-20.

ZHANG, X., REDDY, J., OCHI, H., FRENKEL, D., KUCHROO, V. K. & WEINER, H. L. 2006. Recovery from experimental allergic encephalomyelitis is TGF-beta dependent and associated with increases in CD4+LAP+ and CD4+CD25+ T cells. *Int Immunol,* 18**,** 495-503.

ZHANG, X., YOU, T., JIANG, X., ZHANG, H. & ZHANG, W. 2016. Characteristic arthroscopic signs of cartilage injuries indicating concomitant occult medial meniscal peripheral tears of posterior horn. *Int Orthop,* 40**,** 191-5.

ZHANG, X. M., RAMALHO-SANTOS, M. & MCMAHON, A. P. 2001. Smoothened mutants reveal redundant roles for Shh and Ihh signaling including regulation of L/R asymmetry by the mouse node. *Cell,* 105**,** 781-92.

ZHAO, Y., TONG, C. & JIANG, J. 2007. Hedgehog regulates smoothened activity by inducing a conformational switch. *Nature,* 450**,** 252-8.

ZHOU, L., LOPES, J. E., CHONG, M. M., IVANOV, II, MIN, R., VICTORA, G. D., SHEN, Y., DU, J., RUBTSOV, Y. P., RUDENSKY, A. Y., ZIEGLER, S. F. & LITTMAN, D. R. 2008. TGF-beta-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing RORgammat function. *Nature,* 453**,** 236-40.

ZHOU, X. Y., LIU, Z. Q., JANG, F., XIANG, C. N., LI, Y. & HE, Y. Z. 2012. Autocrine Sonic Hedgehog Attenuates Inflammation in Cerulein-Induced Acute Pancreatitis in Mice via Upregulation of IL-10. *Plos One,* 7.

ZHU, A. J. & SCOTT, M. P. 2004. Incredible journey: how do developmental signals travel through tissue? *Genes Dev,* 18**,** 2985-97.

ZHU, J., PENG, T., JOHNSTON, C., PHASOUK, K., KASK, A. S., KLOCK, A., JIN, L., DIEM, K., KOELLE, D. M., WALD, A., ROBINS, H. & COREY, L. 2013. Immune surveillance by CD8alphaalpha+ skin-resident T cells in human herpes virus infection. *Nature,* 497**,** 494-7.

ZHU, J., YAMANE, H. & PAUL, W. E. 2010. Differentiation of effector CD4 T cell populations (*). *Annu Rev Immunol,* 28**,** 445-89.

ZIEGLER, S. F. & ARTIS, D. 2010. Sensing the outside world: TSLP regulates barrier immunity. *Nat Immunol,* 11**,** 289-93.