

The Role of Intraepithelial Lymphocytes and Type 2 Immunity for Tissue Homeostasis and Carcinogenesis in the Skin

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Declaration of Originality

I declare that this thesis is my own work. Where included, work of others is acknowledged in the text.

A handwritten signature in black ink, appearing to read 'T. Alexander', written in a cursive style.

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Abstract

The skin is under constant renewal whilst frequently being exposed to challenges such as physiochemical disruption and ultraviolet-radiation. How homeostatic control is maintained in the tissue alongside protective mechanisms is poorly understood. Among the basal epithelial cells (EC) is a population of resident intraepithelial lymphocytes (IEL), which provide host-protective immune surveillance (IS). The mouse epidermis is home to a particular IEL termed the dendritic epidermal T cell (DETC). The induction and functions of IS and type 2 immunity at body barrier surfaces remains enigmatic. The aim of this thesis is to investigate the mechanism(s) by which DETC provide IS and maintain epidermal homeostasis.

I show that DETC communicate with EC *via* cytokine IL13. Skin EC are activated by DETC-derived IL13, which enables a canonical EC stress response to various topical challenges. In the absence of IL13, or canonical DETC, the skin is significantly impaired with decreased ability to restore its barrier after insult and increased susceptibility to cutaneous carcinogenesis. Mechanistically, IL13 affected the rate of EC movement through the epidermis, which may explain its importance for epidermal integrity and its suppressive effect on skin carcinogenesis. These findings demonstrate that IL13 acts as a molecular bridge between DETC and EC, and that this interaction provides an integrated local tissue response to perturbation of homeostasis. Finally, I also observed that mice lacking canonical DETC had a reduced ability to repair EC DNA damage following acute carcinogen insult; revealing a hitherto unappreciated aspect of IS.

These data support a critical host-defensive role particularly of type 2 immunity in regulating EC tissue homeostasis in multiple settings. Given the considerable and increasing burden of allergic and malignant disease, these data are highly germane to human medicine, and also contribute healthily to the exciting, nascent field studying allergy-cancer interactions; allergooncology.

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List of Abbreviations

AD	Atopic Dermatitis
AHR	Aromatic Hydrocarbon Receptor
AMP	Antimicrobial Peptides
APC	Antigen-Presenting Cell
BMZ	Basement Membrane Zone
CE	Cornified Envelope
CPD	Cyclobutane Pyrimidine Dimer
Cyp	Cyclophilin
DAMP	Damage-Associated Molecular Pattern
DC	Dendritic Cell
DDR	DNA Damage Response
DETC	Dendritic Epidermal T Cells
dLN	Draining Lymph Node
DMBA	7,12-Dimethylbenz[a]anthracene
DN	Double Negative
DSB	Double-Stranded (DNA) Breaks
EC	Epithelial Cell
EDC	Epithelial Differentiation Complex
HR	Homologous Recombination
IEL	Intraepithelial Lymphocyte
ILC	Innate Lymphoid Cell
IS	Immune Surveillance
K(x)	Keratin x
KC	Keratinocyte
LC	Langerhans Cell
LN	Lymph Node
LSS	Lymphoid Stress Surveillance
MRN	Mre11-Rad50-Nbs1 (complex)

NER	Nucleotide Excision Repair
NHEJ	Non-Homologous End Joining
NK	Natural Killer
NK T	Natural Killer T
NMF	Natural Moisturising Factors
PAH	Polycyclic Aromatic Hydrocarbon
PALP	Phosphotyrosine-rich Aggregates Located on Projections
PAMP	Pathogen-Associated Molecular Pattern
PARP	Poly ADP Ribose Polymerase
PMA	Phorbol 12-Myristate 13-Acetate
PXR	Pregnane X Receptor
rIL13	Recombinant IL13
SC	Stratum Corneum
sdLN	Skin Draining Lymph Node
TCR	T Cell Receptor
TEC	Transit Amplifying Cell
TEWL	Transepidermal Water Loss
TG	Transglutaminase
TPA	12- <i>O</i> -Tetradecanoylphorbol-13-acetate
UV	Ultraviolet
WT	Wild Type
XAR	Xenobiotic Activated Receptor

Chapter 1 | General Introduction

The immune system must be able to sense the nature of a threat and elicit an appropriate response to eliminate microbes and in so doing restore tissue homeostasis. This process is invariably crucial to the life of the animal, and we categorize the main branches of immunity for dealing with different threats generally as ‘type 1’, ‘type 2’ or ‘type 17’. Mossman and Coffman first identified the CD4⁺ Th cell subsets Th1 and Th2, and characterized them based on cytokine secretion¹. Notably IL2 and IFN γ (Th1), IL3 and a then-unidentified growth factor now known to be IL4 (Th2). This picture of distinct immune compartments fits well with the two main and divergent microbial threats to the animal. Namely, microorganisms such as bacteria and viruses, which replicate and disseminate rapidly, and large slowly-replicating macroparasites, which cause considerable tissue damage as well as comprising the fecundity of the host².

Type 1 immunity is conferred by Th1 and Th17 cells. Effector Type 1 cells include CD8⁺ CTLs, M1 macrophages and neutrophils, and soluble components such as IgG antibody. By contrast, type 2 immunity is mediated by Th2 cells, IgE and IgG1 antibody, and a litany of granulocytic innate immune cells of particular preponderance at barrier tissues. These include mast cells, basophils, eosinophils and alternatively-activated macrophages³. A shared feature of the conventional T cell compartment of both immunities is the *de novo* clonal expansion of cells expressing cognate antigen-specific receptors. B cells secrete their antigen receptors as antibody, which have also undergone somatic hypermutation, affinity maturation, and clonal expansion, generating a pool of antigen-specific, efficacious antibody. These mechanisms form the basis of ‘adaptive immunity.’ The innate immune system, by contrast, relies on the recognition of predictable moieties indicative of pathogens (pathogen-associated molecular patterns; PAMP) or tissue stress (damage-associated molecular patterns; DAMP) – a hypothesis first espoused by Janeway with his seminal characterization of pattern recognition receptors (PRR). This distinction between innate and adaptive immunities, however, is being blurred with exciting discoveries of lymphoid-derived cells not constrained at all by their antigen receptors, such as innate lymphoid cells (ILC) as well as a burgeoning family of non-conventional T cells with restricted TCR such as NKT cells, MAIT cells and innate-like $\gamma\delta$ T

cells. Many of these T cells reside constitutively at the interface with the external world in epithelial tissue, and are hence termed intraepithelial lymphocytes. These lymphoid-derived cells seem to play non-redundant roles in various models of injury and tissue dysregulation. Recently, immunological memory, thought to be a defining characteristic of adaptive immunity, is being described in innate cells, hence further blurring the distinction between innate and adaptive immune compartments. In addition to this, the role of type 1 and 2 immunity in various settings is being expanded. Type 1 players such as CD8⁺ CTLs and IFN γ have clinically significant effects on viral infections and cancer - and therefore have been enormously studied over the past decades. The induction, regulation and role of type 2 immunity, by contrast, remains conspicuously understudied, and is routinely interpreted in the clinic solely as an immune modality that inappropriately causes allergic and atopic disease. While the immune system clearly evolved to combat pathogens, it is known that type 2 mechanisms, which promote anti-parasite defense, also contribute significantly to tissue health and wound healing. With respect to cancer, type 2 immunity is being expanded outside of the classically-considered pejorative role in a nascent field termed ‘AllergoOncology’⁴. Given these continuing discoveries, axioms that have been with us since the inception of immunology are slowly being dismantled, and replaced with a more holistic view of immunity being intricately integrated with tissue health and homeostasis.

The Skin Stroma and Epithelium

This thesis will be concerned with tissue immunology, and is focused on the skin. The skin is a large organ with a surface area of about 2 m² in adult humans⁵. It is divided anatomically into two main compartments, the epidermis and dermis, separated by a thin basement membrane zone (BMZ) (Figure 1.0). The BMZ is a tight meshwork of proteins derived from both the epidermal basal keratinocytes (KC), and dermal fibroblasts. These include several types of collagen, laminins, integrins and proteoglycans. The dermis contains a cocktail of cells; immunocytes which are mostly concentrated at the peri-vascular region, and a large number of dermal fibroblasts which generate connective tissue. The epidermis consists of several layers of stratified squamous epithelium, most of which is comprised of KC. The basal cells secrete most of the components of the basement membrane, including the major component laminin V. They remain anchored to it at least in part *via* hemidesmosomes – transmembrane complexes that connect extracellular matrix proteins to KC intracellular filaments, such as keratin (K). Basal KC are typified by expression of the K5/K14

heterodimer, including K15 in the embryo, and include a subset of proliferative stem cells that are a continual source of new KC in the epidermis. This is a tightly regulated program of maturation and differentiation, with cells slowly displaced up through several layers (which can be characterized based on gene expression and cell morphology) – basal, suprabasal, spinosum and granular. The source stem cells are a small proportion of the basal population, which divide asymmetrically to yield a further stem cell, and a ‘transit-amplifying cell’. These cells enter the differentiation program to form part of the epidermal barrier, downregulating K5/K14 and upregulating differentiation markers such as K1 and K10. This cell is then slowly pushed upwards as it enters terminal differentiation, ultimately becoming anuclear, dead cells whose intracellular contents are extruded to form the lipid and protein-rich, crosslinked ‘cornified envelope’ (CE) in the stratum corneum (SC)⁵. The SC is a major physiochemical barrier to insult, as well as regulator of water and loss of solutes. During terminal differentiation, many genes in the epidermal differentiation complex (EDC) locus, including loricrin, involucrin and keratin intermediate filaments are expressed and their products are linked to the nascent CE. Polymerization of various proteins within the SC is catalyzed by a family of transglutaminase (TG) enzymes – including TG1, 2, 3 and X in the human⁶. (Pro)-Filaggrin, also a component of the EDC, is expressed and the enzyme caspase 14 processes filaggrin to add to the important collection of natural moisturizing factors (NMF)⁷. This family of hygroscopic compounds, along with the hydrophobic lipid component of the SC, forms a barrier to transepidermal water loss (TEWL)⁸. This description of the SC is often termed the ‘bricks and mortar’ model⁶ – where the bricks are corneocytes, and the mortar is the space-filling protein and lipid rich extracellular material. The terminal step in epidermal differentiation is the shedding of cells – in fact a highly regulated process termed desquamation. Calcium-dependent proteases reduce SC cohesion particularly by degrading intercellular junctions such as corneodesmosomes. In mice, the initial development of the epidermis (termed stratification) occurs between embryo day 8.5 and 18.5. The embryo epidermis however does not resemble a neonatal epidermis – the newly seeded embryonic basal cells produce a unique suprabasal layer termed the periderm, which is shed before birth. Additionally, a layer of ‘intermediate cells’ forms between the basal layer and periderm in the embryo⁹. Therefore, the epidermis represents a highly complex tissue, in which defects either intrinsically (barrier defects) or extrinsically (immune defects) predispose the animal to disease.

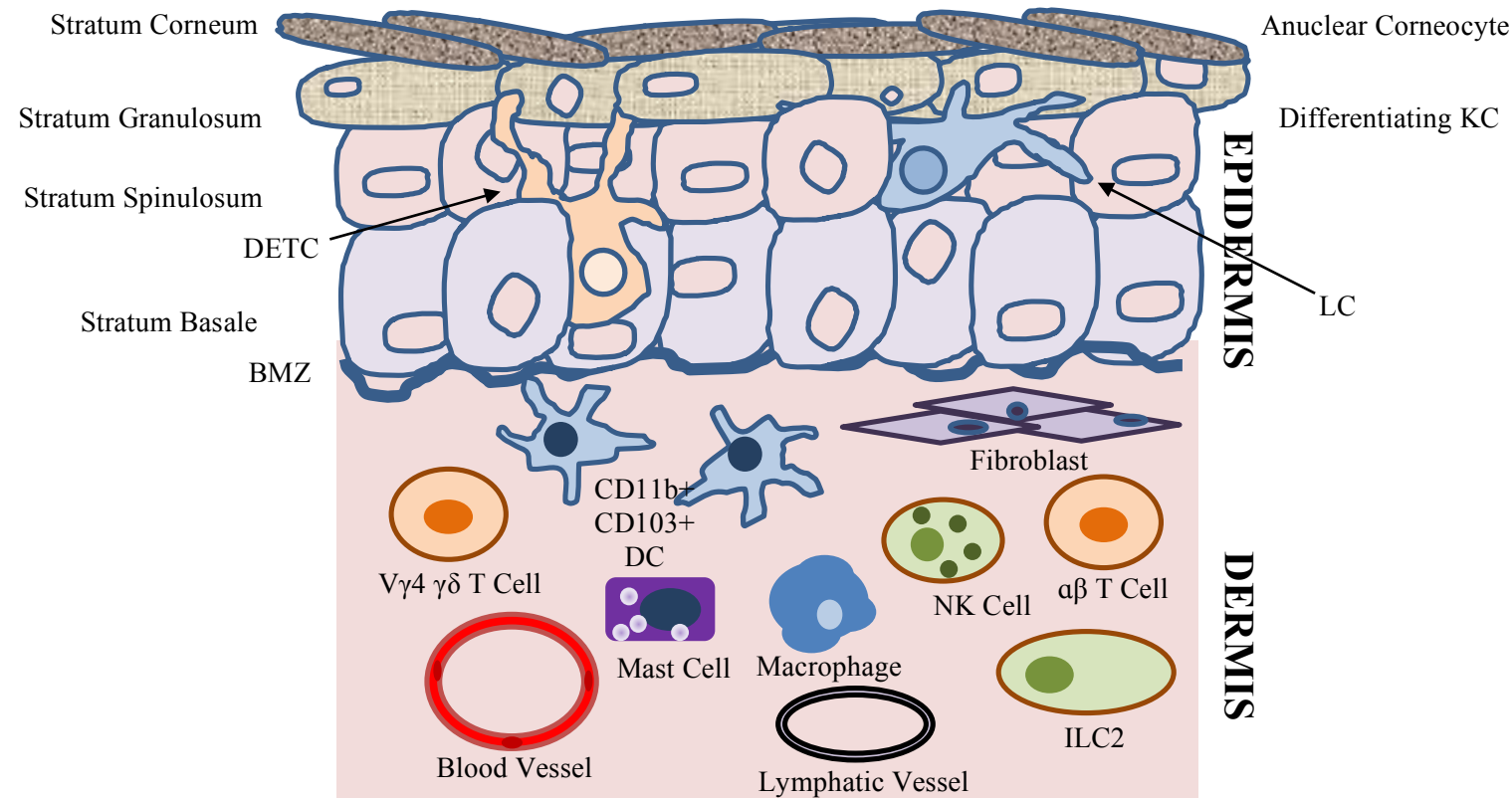


Figure 1.0 | Skin anatomy and skin-resident immunocytes in the mouse

Within the epidermis, the stratum basale gives rise to the proliferating source of KC. These maturing, differentiating cells move upwards and become anuclear, corneocytes in the outermost layer, the SC. The DETC cell body resides in the basal epidermis and interdigitates upwards through the granular layers with dendrites; the LC resides above the DETC. The basal KC are attached to the basement membrane, a delineating zone that separates the epidermis from the underlying dermis. The dermis is a vascularized tissue and is home to a numerous array of lymphoid cells and myeloid-derived granulocytes at steady state; including the IL17-producing V γ 4 $\gamma\delta$ T cell, the ILC2 and a heterogeneous DC population in the mouse. Also present are $\alpha\beta$ T cells, including CD4⁺ T_{EM}, T_{RM}, and Treg¹⁰. Adapted from “Skin Immune Sentinels in Health and Disease” F. Nestle *et al.* (2009). Nat. Rev. Immunol.

The Epimmunome

EC are the main constituent of tissues lining body surfaces such as the skin, intestine, lungs, and genitourinary tract. They regulate crucial life processes including micronutrient absorption, gaseous exchange, thermo- and hydro-control and provide a physiochemical barrier to the external environment against microbes and a plethora of non-microbial stressors. Disruption of epithelial surfaces may therefore result in dysregulated body processes and penetrance to deeper tissues by microbes or noxious moieties. EC are extremely dynamic and versatile cells and it is becoming increasingly clear that they are themselves also intimately involved in the induction and regulation of local tissue and systemic immune responses. Likewise, in close association with EC the epithelial tissues are home to several specialized subsets of tissue-resident immunocytes, including the IEL. The direct response of EC to tissue disruption strongly affects resident immunocytes and their subsequent regulation of both local and systemic immunity. Given the multifaceted biological actions of EC and the multitude of challenges imposed on epithelial tissues, it is reasonable to think that EC in conjunction with tissue-resident immunocytes possess mechanisms, both immunological and non-immunological, to maintain healthy barrier homeostasis and to minimize inflammation and cellular dysregulation.

EC are now known to be highly immunomodulatory by virtue of the cytokines, chemokines, damage-associated molecules (DAMP) and major histocompatibility (MHC) gene products they express; a repertoire that has collectively been termed the “epimmunome”¹¹. EC express a variety of PRRs, and NK receptor ligands, enabling them to respond to a wide variety of microbial and non-microbial (including self) moieties and disseminate the response to immunocytes. The NLR–inflammasome complex allows EC to respond to non-microbial sterile stress elicited by toxins, irritants, and (for skin KC) ultraviolet (UV) light¹²; the most pervasive environmental DNA-damaging agent¹³. Thus, EC express a suite of sensors for detecting differing insults and challenges at the body surfaces, and an armory of soluble and cell-surface molecules to direct an appropriate, restorative response. These epithelial-driven responses in health sculpt and modulate tissue homeostasis and local tissue immunity. In health this is done in a manner that aids morphological tissue homeostasis, restoration of the epithelial barrier following injury, and elimination or expulsion of microbial and non-microbial insults.

EC-derived cytokines include IL1, IL6, IL10, IL18, IL25, IL33, TNF α , and TSLP – of which the most robustly expressed cytokines upon any EC insult are IL25, IL33, and TSLP. In common, these three cytokines can drive type 2 immune responses, which thus impart a particular propensity of epithelial tissues to induce type 2 immunity. IL25, also known as IL17E, is a member of the IL17 cytokine family. Despite bearing some amino acid sequence homology to the best-characterized IL17 cytokines, IL17A and IL17F, IL25 has divergent biological functions and promotes Th2 rather than Th17 responses *in vivo*. IL25 directly amplifies expression of the Th2 mediators IL4, IL5, and IL13, and supports production of Th2 serum immunoglobulins¹⁴. It was found by multiple groups to be important in type 2-mediated immunity to enteric parasites, such as *Trichuris muris*¹⁵, and is upregulated in the gut upon EC-sensing of commensal bacteria¹⁶. The mouse gut parasite *Heligmosomoides polygyrus bakeri* elicits the EC-derived cytokine, IL1 β , which suppresses IL25 and IL33 and promotes pathogen chronicity by attenuating expulsive type 2 responses¹⁷, suggesting that IL25 is particularly important in maintaining immunity to gut pathogens. Similarly, mice and humans subjected to parenteral nutrition have impaired mucosal immunity, due to reduced gut luminal levels of antimicrobial effectors, but administration of exogenous IL25 to parenteral nutrition-fed mice was found to be protective against enteric bacterial invasion¹⁸. In allergic models, IL25 expression is upregulated upon exposure to allergens both in murine or human lung EC lines and in primary murine lung EC¹⁹. Elevated protein levels have also been found in tissues of patients with allergic disease in the lung and skin²⁰. IL25 has been found to drive tissue (airway) remodeling, and expression of the other major EC cytokines IL33 and TSLP in a house dust mite model of allergy²¹, and drive pulmonary fibrosis by inducing IL13 expression from lung ILCs in mice challenged with lung *Schistosoma mansoni* eggs²². In addition to production by EC, dermal DC have been reported to be a major source of IL25 in atopic dermatitis (AD) patients²³, while IL25 and IL33-activated ILCs in mouse skin promote AD-like inflammation²⁴. These reports and others highlight an interesting crosstalk and autocrine regulation of EC-derived effectors, as well as a role for IL25 in augmenting epithelial barrier immunity, or conversely promoting pathological Th2 tissue inflammation, in differing settings.

IL33 is a multi-functional protein. The full-length protein is localized in the nucleus but following cleavage the c-terminal fragment acts as a cytokine that binds the receptor ST2. IL33 was recently discovered as an IL1 family member with type 2-promoting functions similar to IL25. It is expressed by EC, macrophages, DC, and mast cells *in vivo* and its

cytokine function drives IL4, IL5, and IL13 expression and differentiation of Th2 CD4⁺ T cells²⁵. IL33-induced IL4 production appears to be mainly from innate cells and together these two cytokines will induce proliferation of B cells and amplify IgE synthesis²⁶. Similar to IL25, IL33 acts in an autocrine fashion to promote TSLP expression by EC, particularly in response to gut nematodes, where IL33 mRNA can be detected rapidly following colonization²⁷. Interestingly, the efficacy of IL33 in this infection model (and others) seems to be highly time-dependent, with administration of exogenous IL33 at late time points post-infection being ineffective in promoting type 2 responses that would otherwise resolve infection. IL33 is highly expressed by intestinal EC and inflammatory infiltrates in ulcerative colitis, with IL33 cleavage products being detected in the serum. IL33 is also rapidly released and detectable in bronchoalveolar lavage fluid following lung allergen exposure in humans, suggesting it is a rapid type 2 mediator in sites additional to the gut²⁸. Protective as well as immunopathological roles of EC-derived IL33 have been described in the skin. Transgenic over-expression of IL33 in mouse skin, driven by a KC-specific promoter, induces a spontaneous dermatitis-like disease and activates ILCs in the dermis²⁹. Similarly in human inflammatory conditions, IL33 has been reported to be upregulated in clinical psoriatic lesions and the serum of skin sclerosis patients³⁰. Conversely, mice treated with exogenous IL33, following full-thickness skin wounding, demonstrate dramatically improved wound-healing, collagen deposition, and expression of extracellular matrix proteins indicative of tissue repair³¹. These reports suggest a particularly rapid and acute role for IL33 in cutaneous homeostasis and gut integrity whereas constitutive, late, or dysregulated expression may be involved in a variety of chronic inflammatory conditions. This temporally coordinated aspect fits well with current thinking of IL33 as an “alarmin,” whereby its immediate release from intranuclear stores by damaged, apoptotic, or necrotic cells rather than a classic Golgi-mediated secretion pathway facilitates a rapid and restorative response to tissue damage.

TSLP is produced almost exclusively by EC of the lung, tonsils, intestine, and skin and is upregulated in response to tissue damage³², various TLR ligands and infection, or exposure to type 2 cytokines such as IL4, IL13, IL25, and IL33³³. A protective role of TSLP in intestinal immunity to *T. muris* has been well described; mice which are knockouts for IKKb fail to produce TSLP in response to infection, and subsequently develop chronic intestinal inflammation. Mechanistically, EC-derived TSLP suppresses p40 and upregulates OX40L expression in DCs, a costimulatory molecule with a propensity to license Th2 responses in CD4⁺ T cells. TSLP also augments Th2 cytokine production by direct effects on CD4⁺ T

cells and has indirect, agonist effects on a variety of granulocyte populations including mast cells and basophils³⁴. Similar to IL25 and IL33, inappropriate expression or dysregulation of TSLP is implicated in a number of inflammatory diseases including the triad of atopic diseases; asthma, allergic rhinitis, and AD. TSLP is required for allergic lung inflammation in mice exposed to inhaled antigen, and TSLP receptor knockout animals do not develop lung inflammation in this model. Interestingly, these animals do develop strong Th1 responses with high IFN γ , IL12, and IgG2a³⁵, highlighting how a single epithelial-derived molecule can skew adaptive immune responses in response to tissue-challenge. In humans, AD sufferers show high TSLP expression in lesional skin³⁶, and mice with induced expression of TSLP in the epidermis develop spontaneous AD-like pathology³⁷. Production of TSLP is however critically important for resistance to skin carcinogenesis in mouse models^{38,39}, highlighting a protective role of TSLP in settings outside of atopic disease.

The Local Neighbourhood: Intraepithelial Lymphocytes

IEL are found in all epithelial tissues, but have most notably been studied in the intestine and skin. IEL are adaptive T cells carrying RAG-dependent rearranged T cell receptors (TCR), nevertheless they are often MHC non-restricted cells and express many innate receptors allowing them to react to stress antigen with “innate-like” response kinetics. The IEL are a mixture of $\alpha\beta$ and $\gamma\delta$ T cells of which the $\gamma\delta$ TCR⁺ IEL is of particular relevance to this thesis. The discovery of $\gamma\delta$ T cells began more than 30 years ago with cloning of the TCR δ chain genes⁴⁰, which had until that point been misidentified as part of the TCR α chain due to their position buried within the α locus. This finding completed the picture of a tripartite arrangement of immune cells with somatically-rearranged (V(D)J-based) antigen receptors (namely $\alpha\beta$ T cells, $\gamma\delta$ T cells and B cells) – an arrangement that has been conserved to some degree or another for the last 500 million years⁴¹.

Both $\alpha\beta$ and $\gamma\delta$ T cells arise from a common, double negative (DN) progenitor in the thymus⁴². $\gamma\delta$ T cells develop very early within the life of an animal and, unlike conventional naïve $\alpha\beta$ T cells, many home directly to and seed barrier tissue sites forming part of the IEL compartment. In the mouse, the V γ 5 chain is the first to be rearranged and selected for in the thymus. This first wave of T cells exclusively migrate to the skin epidermis, which is the first site to be seeded by T cells - the V γ 5⁺V δ 1⁺ IEL as early as embryo day 16 (E16). The skin and the thymus are thus the only sites where this particular TCR chain usage can be found.

These first T cells are then rapidly followed by a selection of other V γ chains, which form the IEL repertoires at other epithelial body barrier surfaces. $\alpha\beta$ T cells only start being selected for after these early $\gamma\delta$ IEL populations are in place. The murine epidermis is very quickly colonized during ontogeny with a TCR-restricted IEL. This T cell exhibits a dendritic morphology and is hence termed a Dendritic Epidermal T Cell (DETC).

This seeding of tissue sites by TCR restricted T cells was initially found puzzling given the conventional role of the TCR in sampling diverse, DC-delivered antigen – promoting clonal expansion only following recognition of cognate antigen. It was subsequently shown that many $\gamma\delta$ TCR⁺ IEL were essentially monoclonal, which may suggest that they recognize predictable, perhaps tissue-specific, pathogen-derived or self-encoded moieties – with the assistance of secondary, co-stimulatory receptors such as NKG2D. $\gamma\delta$ T cells thus differ from conventional $\alpha\beta$ T cells not only in their recognition capabilities and activation modality – but also in kinetics of effector function – allowing for extremely rapid, local immunity to be elicited in a manner not dependent on slow, clonal-expansion of an antigen-specific effector T cell. The benefits of having a rapid, non-clonally-induced immunocyte population at barrier sites seem clear – given obstruction of penetrance of pathogens and maintenance of tissue homeostasis are invariably crucial to the life of the animal.

During development, the $\gamma\delta$ T cell population can be characterised into two discrete sub-populations. The first arises rapidly in the foetal animal from liver progenitors and are termed ‘innate’ $\gamma\delta$ T cells – mainly due to their ability to elicit effector functions without undergoing a conventional activation process (cognate antigen:self MHC signal 1, costimulation signal 2, appropriate cytokine milieu signal 3). The second $\gamma\delta$ T cell population develops from bone marrow-derived progenitors post-partum, giving rise to more conventional cells in the periphery with adaptive-like kinetics. In both cases viable rearrangement of the $\gamma\delta$ TCR locus in the thymus is driven by SOX13, a transcription factor crucial for promoting $\gamma\delta$ TCR rearrangement while opposing $\alpha\beta$ T cell development⁴³. Failure at this stage to form the $\gamma\delta$ TCR allows for β -selection, double-positivity, and eventual development of a conventional $\alpha\beta$ T cell. Hence, T cell development sequentially attempts to form a $\gamma\delta$ T cell, and should this be unsuccessful the $\alpha\beta$ programme is followed. The ratio of $\gamma\delta$ T cells : $\alpha\beta$ T cells dynamically changes with age, with the latter becoming increasingly dominant post-partum⁴⁴, suggesting a particular importance of $\gamma\delta$ T cells in the young animal (at a time when the

adaptive $\alpha\beta$ T cell and DC compartments are immature). This ratio is also highly dependent on tissue site, species and health status. Cattle and chickens, for instance, have large numbers of $\gamma\delta$ T cells in the peripheral blood at steady state (~30%), compared to 4% in human peripheral blood – rising up to 60% during some infections. Despite $\gamma\delta$ T cells often being MHC non-restricted cells, the TCR δ locus has the greatest potential for diversity in the complementarity determining region 3 (CDR3) loop, with 10^{16} combinations possible in the mouse⁴⁴, exceeding that of $\alpha\beta$ T cell and immunoglobulin.

Innate $\gamma\delta$ T cells

In the foetus, liver precursor-derived DN4 cells undergo ‘pre-programming’ – consigning them to become one of two subpopulations of ‘innate’ $\gamma\delta$ T cells, categorized based on; i) differential production of IFN γ /IL13 or IL17, ii) differential expression of SOX13 and iii) canonical T helper cell transcription factors. The first subpopulation consists of SOX13-low, CD27-positive, IFN γ (and IL13)-producing innate $\gamma\delta$ T cells – of which the DETC is a member. The second subpopulation consists of SOX13-positive, ROR γ t-positive, IL17 producing innate $\gamma\delta$ T cells. It was initially proposed that a strong, TCR-mediated agonist signal concurrent with co-stimulation induced the IFN γ /IL13-producers – while the IL17 producers represented a ‘default’ position for innate $\gamma\delta$ T cells that was maintained in the absence of such a signal⁴⁵. More recent work has described a requirement of strong, TCR-agonist selection for both subsets, with IL17 not representing a default programme⁴⁶. It is interesting that a strong, TCR signal seems a requirement of innate $\gamma\delta$ T cell development, given such a signal is usually a deletion-cue during negative selection for $\alpha\beta$ T cells. Another T cell that has similar requirements is the Treg – and innate $\gamma\delta$ T cells are often described, like Tregs, as immunomodulatory. In the case of the DETC, restraining inappropriate cutaneous inflammation⁴⁷.

Other Tissue Resident Immunocytes

In addition to the “unconventional” T cells such as the DETC in the epithelium, more conventional CD8⁺ $\alpha\beta$ T cells have been shown to rapidly accumulate in tissues upon infection, where they can become resident memory T cells and provide protective antigen-specific responses. This was elegantly shown in the skin following local infection with herpes simplex virus or vaccinia virus⁴⁸, which induced a rapid influx of antigen-specific CD8⁺ $\alpha\beta$ T cells both into the epidermal layer and the underlying dermis. Interestingly, these infiltrating

CD8⁺ αβ T cells were shown to populate the entire skin and provide long-lasting protection against re-infection as a continuing tissue-resident memory T cell population. In steady state, the subepithelial layer of most tissues contains a diverse set of immunocytes that can all contribute to skin immunity (Figure 1.0). These include tissue-specific resident populations of myeloid cells, such as DC, macrophages and mast cells, lymphoid cells such as CD4⁺ or CD8⁺ αβ T cells, γδ T cells, and ILCs, as well as stromal fibroblasts. In fact, it is becoming increasingly apparent that different tissues constitutively harbor a variety of specialized immunocytes in the subepithelial space. For example, in human skin a population of IL22 and growth factor producing T cells (Th22) can rapidly enter the epithelium upon challenge and be involved in epidermal remodeling⁴⁹. Similarly, the gut contains a resident IL22 producing population of NK-like cells⁵⁰.

ILCs are a recently-characterized family of bone marrow-derived lymphoid effector cells which lack RAG-rearranged antigen receptors. They have been described to have crucial roles in tissue homeostasis and repair, infection and lymphoid organogenesis⁵¹. In recent years, an array of different ILCs has been discovered that are resident in the subepithelial tissue layer. Different subsets of ILCs dominate in particular tissues and their specialized functions are starting to be elucidated; many of them contribute to both homeostatic and pathophysiological conditions in the tissue they inhabit⁵². These subepithelial immunocytes can respond to epithelial cues and be recruited into the epithelium upon damage – in addition systemic immune cells can be recruited both to the subepithelial and epithelial layer. Notably the type 2 ILC, termed ILC2, reside in the dermis and are not activated by conventional antigen-receptor mediated stimulation, but rather the epithelial products IL25, IL33 and TSLP along with lipid mediators such as prostaglandin D₂. In response they produce large amounts of IL5 and IL13, and hence are thought of as boosting and amplifying type 2 immunity in the tissue⁵³. ILC2s have been described to be involved in disease- and homeostatic processes at many different tissue sites, including the skin, lung, and GI tract. ILC2s in the skin of mice can be divorced from ILC2s from other body sites by the expression of integrin CD103. Skin ILC2 numbers increase after exposure to allergen (house dust mite), or by forced IL33 overexpression. With regards to the common atopic skin disease AD, ILC2s have been detected at higher levels in the skin of AD patients suggesting that may be involved in pathogenesis if not directly than indirectly⁵⁴.

In addition to the conventional MHC-restricted T cells, there are several T cells with

specificities for ligands other than MHC-peptide. The Natural Killer T cell (NKT cell) are restricted to recognizing ligands expressed by CD1d, a MHC-like non-polymorphic protein that presents microbial – or host-derived glycolipids. Therefore, the antigen receptor of these cells, despite being a T cell receptor, functions phenotypically like a PRR. Recently, butyrophilins (which are a member of the greater immunoglobulin family) have been described as having key roles in numerous aspects of immunity. Human $V\gamma 9^+V\delta 2^+$ T cells are known to react to phosphoantigens – which can be derived from microbes or host metabolic pathways as a result of cellular stress. It has recently been shown that prenyl phosphoantigens can bind to or modulate butyrophilin molecule BTN3A1 in antigen presenting cells (APC), in such a way as to sensitize the cell to scrutiny by $V\gamma 9^+V\delta 2^+$ T cells and directly activate the T cells⁵⁵. Ergo, the consequences of cellular stress directly activate non-conventional T cells, in this case $\gamma\delta$ T cells. The ability of T cells, or lymphoid-derived cells, to react to self moieties seems to suggest an important role of the immune system in responding to dysregulation of tissue homeostasis, given the consequence of this so often seems to be the preponderance of ‘stress ligands.’ In sum, the epithelium and body surface tissues are home to an intricate array of immunocytes, which can interact and integrate activities in numerous complex ways that likely differ substantially depending on the anatomical site and the challenge encountered. Interestingly, there is a common theme emerging of non-conventional lymphoid cells recognizing self encoded- rather than pathogen-encoded moieties. This suggests that sensing of cellular stress by these cells represents a key function of this compartment.

Type 2 Immunity in the Skin in Health and Disease

Type 2 immunity is elicited in response to a broad array of different antigens, allergens and environmental challenges. The responses to allergens form the mechanistic basis of allergic disorders including asthma, allergic rhinitis, food allergy and AD. Indeed, inappropriate type 2 responses likely underlie or exacerbate many disease states at body surface tissues. Likewise, disordered type 1 and 17 immunity may result in autoimmunity and metabolic disease. Despite being understudied, there are now many routes described to the induction of type 2 immunity. Allergens predominantly induce type 2 immunity; these include peanut, shellfish, pollen, nickel, bee venom, latex, house dust mite and penicillin. Why these antigens, in sensitized individuals, induce type 2 immunity is not understood – there seems to be little in common between them in terms of chemical structure or origin. However, many triggering insults do share a feature of causing considerable tissue damage, and hence

dysregulation of tissue homeostasis. In this setting, absence of type 1-inducing PAMP potently drives type 2 immunity. Indeed, all type 2 immune responses converge with effector mechanisms at body barrier surfaces.

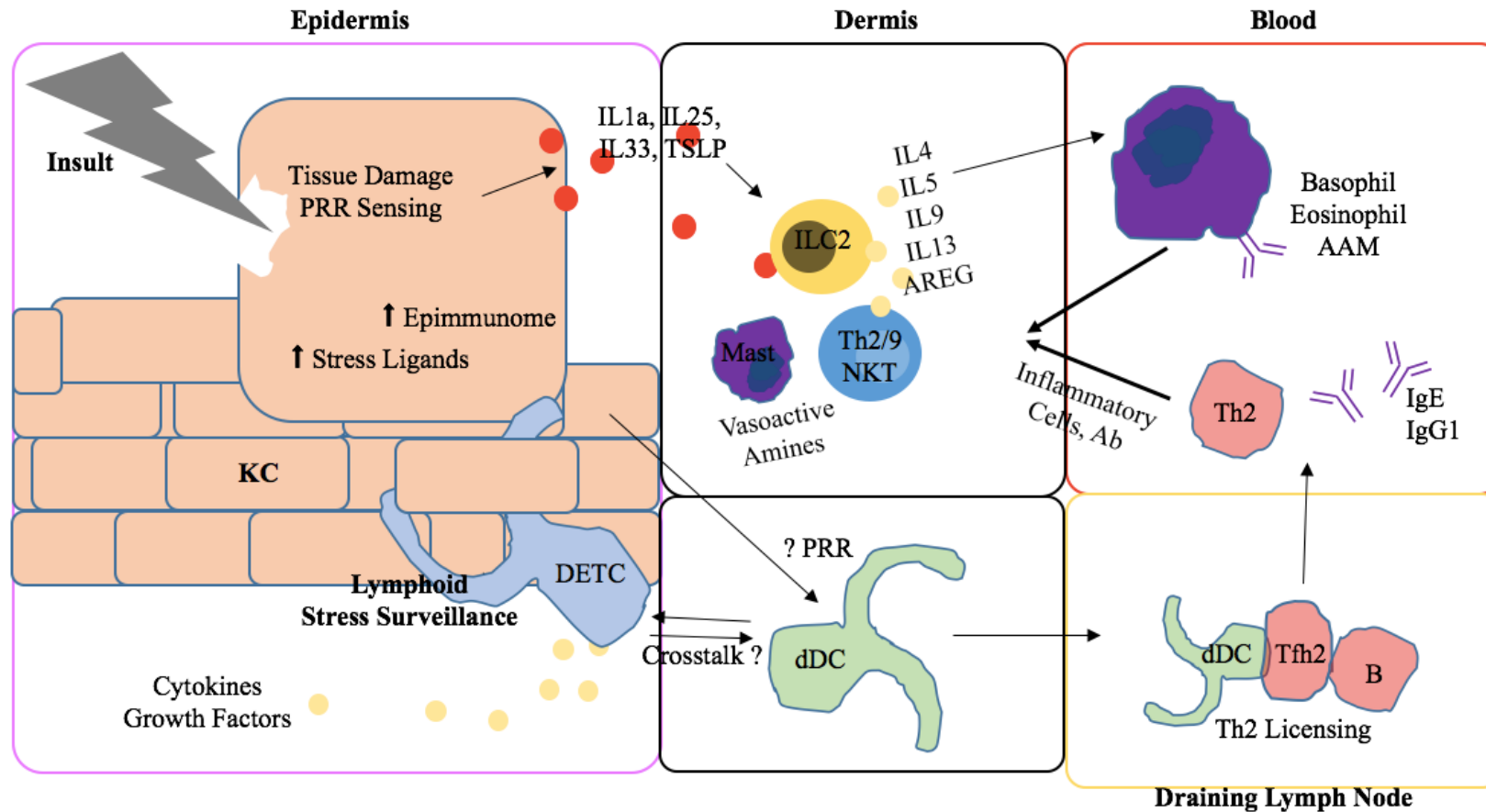


Figure 1.1 | Simplified schematic of induction of type 2 immunity in the skin

Tissue damage induces expression of epithelial cytokines and stress ligands, which can be directly sensed by the DETC. The dermis is home to heterogeneous population of immunocytes, and influx of Th2 inflammatory cells and granulocytes underlies allergic disease. Licensing of Th2 responses in skin-draining lymph nodes induces IgE and IgG1 production. Where; PRR, Pattern Recognition Receptor; KC, Keratinocyte; DETC, Dendritic Epidermal T Cell; ILC2, Innate Lymphoid Cell 2; AREG, Amphiregulin; NKT, Natural Killer T cell; dDC, dermal Dendritic Cell; AAM, Alternatively-Activated Macrophage; Tfh2, T Follicular Helper 2

The sensing of insult and induction of type 2 immunity in the skin is driven by a complex interplay between stromal, myeloid and lymphoid cells, and potent soluble mediators, in the skin, draining lymphatic and blood compartments (Figure 1.1). The challenges faced at the body barrier are diverse, with threats ranging from nanometer sized particles to meter-long worms. Insults include bacteria (and their products LPS, PGN), viruses, helminths, allergens (food, pollen, animal dander and saliva) and venoms (bee and some snake venom), irritants and chemicals (detergents), toxins and noxious xenobiotics. Additionally, compounds that typically function as adjuvants for inducing type 2 responses are extrinsic factors, such as alum, silica and titanium. Intrinsic cellular mechanisms for inducing type 2 immunity including a considerable array of PRR sensors (toll-like receptors (TLR), NOD-like receptors (NLR), RIG-I-like receptors (RLR), products of metabolism and DAMP (DNA, uric acid, ATP, ROS). Therefore, type 2 immunity can be triggered by a bewildering array of extrinsic and intrinsic factors, and in health these signals need to be integrated to induce an appropriate, restorative response to tissue damage that is not overly zealous.

The epithelial response to these threats includes direct responses to tissue damage (such as release of IL33 alarmin from pre-formed pools) and an induced upregulation of self-encoded ligands indicative of tissue dysregulation ('stress ligands'). DETC are capable of sensing stress ligands with cell surface immunoactivating receptors such as NKG2D, which in cohort with other signals induces rapid production of cytokines and growth factors – a protective mechanism that is examined closely in Chapter 3. Given there is no requirement for clonal expansion or recruitment of other cells from the blood or lymphatics, the DETC is in a prime position to elicit the most rapid responses *in situ* to tissue stress. As previously described, other immunocytes in the dermis such as ILC2s are activated by potent epithelial cytokines, and can amplify the type 2 response in the tissue by producing IL5 and IL13. Chemotaxins recruit granulocytes from the blood compartment. Mast cells are resident in the dermis at steady state and are usually coated in IgE specific to previously encountered antigen – underlying the inductive mechanism of allergic type 1 hypersensitivity. Mast cell-derived vasoactive amines promote type 2 inflammation in the skin and scavenge further IgE from blood vessels⁵⁶. These vessels are made more leaky which combined with chemotaxin release induces blood-derived granulocytes such as basophils, eosinophils and macrophages to begin entering the tissue. Monocytes also infiltrate

the tissue and differentiate into macrophages *in situ*. Macrophages activated under certain microenvironmental conditions become type 2 alternatively-activated macrophages. These cells contribute heavily to the wound healing responses and promote the lay-down of extracellular matrix material. Such a response also helps to restrict, contain and expel insults, as will be described later. Of note is the presence of numerous DC subsets in the dermis, and the LC population in the epidermis. The specific molecular signals responsible for inducing adaptive, type 2 immunity remain enigmatic. However, a requirement for CD301b⁺, IRF4-dependent dermal DCs has been shown in inducing robust Th2 cell responses to subcutaneous antigen (OVA), infection (*N. brasiliensis*) and chemicals (topical acetone and FITC)⁵⁷. DC trafficking to skin draining lymph nodes (LN) primes a classic Th2 response, with clonal expansion of CCR10⁺ skin-homing T cells and production of IgE and IgG1 antibody.

Type 2 immunity in the skin therefore represents a fascinating series of molecular interactions. There is the capability to generate extremely rapid, potent immune responses due to the presence of un-restrained non-conventional immunocytes (such as DETC, ILC2) and ‘memory’ in the form of the clonally-raised IgE coated granulocytes, and persistent memory cell populations as previously described. In a clinical setting, type 2 skin immunity is mostly appreciated in terms of allergic skin disease – notably AD. Acute AD lesions are characterized by harmful type 2 inflammation and IL4 expression. In chronic AD, macrophages and eosinophils dominate, and damage to the epithelium induces further type 2 mediators. Atopic skin inflammation, particularly in children, is often observed to lead to an increasing prevalence of allergic symptoms at other tissue sites. This is termed the ‘atopic march’⁵⁸. There are two competing hypotheses to describe the aetiology of AD: The ‘inside-out’ hypothesis suggests that an immunological defect predisposes certain individuals to atopic responses and sensitization, resulting in an immune-caused barrier defects and manifestation of allergic disease in the skin. The ‘outside-in’ hypothesis suggests that an intrinsic barrier defect of the skin, particularly the epidermal barrier, results in greater penetrance of noxious environmental moieties and hence destructive tissue immunity. Indeed, many studies have demonstrated a link between polymorphisms in genes for skin barrier components and multiple different skin disorders including AD⁵⁹. Loss-of-function polymorphisms in the skin structural

gene *FLG* (Filaggrin) are strongly associated with AD, and inherited mutations in collagen XVII (a skin structural protein) are associated with blistering disease⁵. Therefore, dysregulation of the epithelial barrier and/or its cytokines clearly exacerbates pathology, while type 2 immunity, when inappropriately triggered, results in disease at body barrier sites characterized by destructive, chronic tissue remodeling such as in uncontrolled asthma and fibrotic disease. The data to date mostly suggests that the type 2 cytokines, expressed abundantly in allergic disease settings, have barrier-disrupting functions – particularly with reference to the epithelium. This theme will be expanded in Chapter 4. Crucially, however, a pejorative effect of a mediator in a chronic inflammatory state does not necessarily imply that it is pejorative in all settings at all times. Indeed, there are likely to be spatiotemporally coordinated aspects of cytokines, for instance, which are protective in some settings, and immunopathological in others – a common feature to all immunity. The point at which a host-protective type 2 response goes from protective to immunopathological following continual perturbations remains an open question. However, once a certain level of tissue damage is reached, a perpetual type 2 response is detrimental.

Immune Surveillance

This capacity of the immune system to sense cellular dysregulation and respond by activating a stress response to restore homeostasis has been termed ‘immune surveillance.’ This continued “quality control” mechanism has most commonly been applied to and studied in relation to cancer. The cancer immune surveillance hypothesis was first proposed by Ehrlich in 1909 when he predicted that the immune system could repress or destroy the outgrowth of tumors that arise spontaneously on a continued basis⁶⁰. This proposal initiated a century of debate over the immune systems role in controlling neoplasia. The idea of a natural immune response against neoplasms or pre-malignant and dysregulated cells was revisited and expanded by Burnet and Thomas in the 1950s⁶¹. They proposed that lymphocytes form the basis of a “cancer immune surveillance” process that protects immunocompetent hosts against primary tumor development. Although the hypothesis grew in recognition with the expansion of knowledge about the immune system and tumor-antigen recognition, the architects themselves pointed to “the problem with the idea of immune surveillance is that it cannot be shown to exist in experimental animals”⁶² – and it is of course rarely appreciated in a clinical setting. By the early 1990s, little attention was paid to the

idea that natural immunity could control tumor establishment *de novo*. However, by the mid-1990s and onward several observations were made that rekindled the interest in this early aspect of tumor immunity. In short, the physiological importance of immune surveillance was well revealed by the pathological consequences of its failure: the neutralization of IFN γ with antibodies⁶³ and later the use of mice lacking IFN γ responsiveness was shown to enhance tumor growth⁶⁴. Lymphocytes were unequivocally proven to play an essential role in immune surveillance by seminal observations in *rag2*^{-/-} mice⁶⁵ and subsets of lymphocytes such as NK, NKT cells.

The original idea of cancer immune surveillance has more recently been expanded and described by the modern ‘immunoediting’ hypothesis, which postulates a three-stage series of interactions between malignant cells and the immune system; namely i) elimination, ii) equilibrium and iii) escape. During ‘elimination’, NK cells, CTLs and $\gamma\delta$ T cells target and destroy transformed cells *via* recognition of tumour antigens, assisted by the presence of ‘danger signals’ such as DAMP. ‘Equilibrium’ represents a period of tumour dormancy, with immune effector mechanisms preventing outgrowth, invasion or metastasis, while slowly sculpting the tumour to a less immunogenic variant. This is the longest phase, the tumour remaining subclinical for as long as it is restrained by the immune system. ‘Escape’ defines the point at which the malignancy evades immune control, owing to the combination of a poorly immunogenic tumour and an immunosuppressed host. Thus, the immune surveillance model incorporates not just the targeting and elimination of cancerous cells by effector immune cells and molecules, but the gradual selection for and sculpting of a poorly immunogenic tumour; which ultimately may result in immune evasion and clinical disease (cancer)⁶⁶.

Immune Surveillance, Tissue Health and Type 2 immunity

The afferent phase of immune surveillance (sensing of dysregulated self) applies to many other stresses than purely oncogenic stress. Hence, immune surveillance is not only a mechanism to control the development and outgrowth of tumors but as will be argued extensively in this thesis – a crucial regulator of tissue health and homeostasis. The implication of immune surveillance mechanisms in the maintenance and re-establishment of tissue homeostasis thus broadens its scope significantly. Immune surveillance can function by many (non-exclusive) mechanisms including (1)

recognize and remove damaged, stressed, senescent, and (pre)-malignant cells, (2) remove damaging substances, waste, and dead cells, (3) facilitate re-establishment of homeostasis by repair mechanisms, (4) neutralize potential harmful environmental substances, or (5) dampen detrimental inflammatory reactions.

Epithelial surfaces have a conspicuous propensity to drive type 2 immunity (rather than type 1) upon non-invasive/non-penetrating challenge or stress. Indeed as described previously, the most robustly expressed epithelial effectors are type 2 cytokines IL25, IL33 and TSLP. Type 2 immune mediators are thus well poised to play a role in early immune surveillance as well as homeostatic tissue regulation. What is known so far of the physiological role of type 2 responses in general is that their host-protection properties converge in different forms of barrier defenses. IL13 is the best-characterized inducer of mucus production and goblet cell hyperplasia in the respiratory and intestinal mucosa. In the skin, transgenic over-expression of IL13 induces skin remodeling, which is primarily driven by TSLP⁶⁷. In both circumstances, hyperplasia results in improved resistance to damage and damaging substances at the body barrier either *via* production of mucus at the mucosal surfaces or thickening of the skin.

Removal or expulsion is another host defense strategy induced by type 2 immunity. These mechanisms can directly protect against noxious toxins or parasites, as well as limiting their systemic dissemination. The removal/expulsive actions of allergic and type 2 immunity through sneezing/coughing/itching/vomiting/diarrhea are partly induced by EC-derived mediators including TSLP, which acts directly on sensory neurons in the skin triggering itching, and by the effect of mast cell-derived histamine on smooth muscles. Type 2 immune mediators can also confer host-protection by inactivation, neutralization, and destruction of noxious substances. This is most notably shown by the requirement for mast cells in the detoxification of snake and bee venom and the evidence that mast cell proteases can specifically attack snake venom at the structures required for toxicity and thereby neutralizing it⁶⁸⁻⁷⁰. Recent data strongly suggest that IgE mediates or at least contributes to protection against venoms as for example the protective responses against re-challenge with high doses of bee venom is abrogated in mice lacking B cells, FcεRI, or IgE. It is likely that this protection is partly *via* the very rapid IgE-mediated degranulation of mast cells.

Encapsulation and restriction is another layer of barrier defense regulated by type 2 mediators, which can help prevent the spread of noxious substances if elimination or expulsion has been insufficient. Endothelial leakage and exudate formation can be induced by mast cell-derived products and such local tissue edema may impede parasite invasion. For example it has been shown that the oedema caused by IgE-mediated mast cell degranulation is important in the defense against macroparasites such as ticks⁷¹. Another restriction mechanism, which may restrict the spread of noxious substance as well as macroparasites, involves sequestration through granuloma formation. Type 2 immune responses protect the host during infection with schistosomiasis by inducing granulomas that sequester the tissue-damaging toxins from the parasite eggs⁷².

Perhaps most importantly, much of type 2 immunity seems dedicated to tissue repair and promoting tolerance to damage. Indeed, it has been hypothesized that type 2 immunity has evolved to direct innate wound repair mechanisms². The rationale for the induction of tissue repair as a part of type 2 immune defense is obvious. It may also explain the extreme urgency of some type 2 responses (which is not easily explained if directed only toward a slow replicating macroparasite), as damage control may well be more important than pathogen control. In evolutionary terms, it makes sense to be able to quickly expel or neutralize noxious substances as well as rapidly repair the life-essential body barrier. EC-derived cytokine alarmins and cell-ligands can activate and direct the resident tissue cells to promote repair responses; IEL rapidly sense stress and can produce growth factors locally in the absence of further inflammation⁷³. Indeed, almost all of the cells associated with type 2 immunity are also associated with the wound-healing response. As stated, alternatively activated macrophages produce vascular endothelial growth factor (VEGF), arginase 1, and IGF-1; Eosinophils store preformed growth factors, matrix metalloproteinases, and lipid mediators, all of which can mediate wound healing⁷⁴. Amphiregulin is produced by mast cells following FcεRI signaling, potentially also linking IgE responses to wound repair⁷⁵.

In sum, the effects of type 2 immunity at our body surface tissues play important roles in eliminating, restricting, and neutralizing noxious environmental substances as well as repairing the damage caused and minimizing inflammation – as such this type of

immunity is critical for tissue homeostasis and responses to challenges that have breached the epithelial barrier.

Cancer Immune Surveillance, and type 2 immunity

In terms of early cancer immune surveillance, the role of type 2 immunity has been little explored. However, the effects of rapid type 2 immune responses as outlined previously could indeed play a prominent role in cancer immune surveillance. It has been demonstrated that the same tissue-resident IEL act as key components of tumor resistance and potent inducers of type 2 immunity and IgE antibodies. The humoral component of this lymphoid stress-surveillance response may limit tissue damage by targeting noxious foreign substances, such as toxins that may be the root cause of the tissue dysregulation. The IgE effector response may promote toxin expulsion and limit their systemic dissemination. Simultaneously, the cellular response can direct cytotoxicity toward dysregulated cells as well as promoting repair of the damaged tissue and dampening inflammation. To limit the likelihood of cancer, it is clearly important to repair a wound or breached barrier quickly and efficiently as is demonstrated by the close association between chronic tissue damage and cancer. Less efficient wound repair may lead to inefficient immune surveillance against (pre-) malignant cells with damaged cells being allowed to stay longer in the tissue before being replaced. Additionally, slow repair of tissue damage may lead to inflammation, which as discussed can have potent pro-carcinogenic effects. There is a considerable link between inflammation and cancer, a phenomenon first described by Virchow, who observed that tumours were more likely to arise at sites of chronic inflammation. Subsequently, supporting evidence for a link between inflammation and carcinogenesis comes from numerous experimental and clinical observations including many inflammatory conditions that predispose to cancer and that inflammatory cytokines are often correlated with a poorer prognosis⁷⁶. Likewise, although one can imagine a role for type 2 immunity and its regenerative capacity in early cancer immune surveillance this may indeed be a double-edged sword in further development of cancer. Failure of the type 2 response to adequately contain or eliminate the initiating substance may lead to a chronic wound-healing response and exacerbation of inflammation. Such continued tissue damage, repair, and regeneration may ultimately result in fibrosis. Fibrotic tissue is a highly permissive environment for tumor formation and it is also well established that continuous wound-healing

responses and tumorigenesis are two processes that rely on similar molecular mechanisms⁷⁷. As such it is perhaps not surprising that the literature on type 2 responses and cancer is somewhat bewildering.

Lymphoid Stress Surveillance

The sole immunocytes present in the murine epidermis in health are the DETC and the LC. The established model for immune surveillance at body surfaces describes a process whereby myeloid cells such as DCs detect PAMP or DAMP whereupon they traffic to draining LNs. After several days, cognate antigen-specific $\alpha\beta$ T cells are primed, clonally expand and home to affected tissues, where they elicit effector and regulatory functions informed by microenvironmental cues. A small portion of these cells enter the memory pool for responding to secondary challenge. Notwithstanding the strengths of this model, it is now clear that tissue-resident immunocytes, such as the DETC in the murine epithelium, are capable of directly sensing epithelial dysregulation and inducing local tissue and systemic immunity. This process is termed lymphoid stress surveillance (LSS), and represents a fascinating, novel aspect of immune surveillance. In health, DETC are resident in close proximity to the skin EC, keratinocytes (KC), with which they make continuous molecular interactions. As will be described in detail in Chapter 3, as a consequence of perturbation of homeostasis these molecular interactions are perturbed, and the constellation of receptor:ligand interactions between KC and DETC provides discrete information pertaining to tissue health. Under conditions of physiochemical tissue disruption or barrier perturbation¹¹, infection⁷⁸, genotoxic stress⁷⁹, sterile inflammation, or heavy proliferation⁸⁰, EC respond by upregulating self-encoded cell-surface markers, which are often termed “stress antigens” as they are indicative of a dysregulated state of the epithelium. One of the most important and best-characterized families of stress-induced EC ligands includes Rae-1, H60, and MULT1 (mouse), MICA, MICB and ULBPs (human). These are members of the larger family of MHC class Ib molecules and are reported upregulated on EC by stresses such as heat-shock, UV-irradiation, DNA-damage, viral and bacterial infection, and autoimmunity. These unconventional MHC molecules engage the activating lectin-type receptor NKG2D, which is constitutively expressed by tissue-resident DETC, NK and NKT cells but is also expressed on activated CD8⁺ T cells and in some circumstances subsets of CD4⁺ T cells.

The NKG2D pathway has proven importance in numerous settings of cell dysregulation, such as cancer⁸¹, infection⁸², autoimmunity⁸³, and transplantation⁸⁴, and its key role in immune surveillance is supported by the plethora of strategies tumors and viruses have adopted to evade it^{85,86}. In relation to cancer, NKG2D-ligands are expressed by most epithelial tumors and the NKG2D-pathway is strongly associated with anti-tumor responses in both humans and mice⁸⁷. NKG2D ligands are often upregulated early upon cellular dysregulation or transformation, it has however been controversial whether immune cells could be activated by such self-moieties alone. By generating transgenic mice where an autologous NKG2D-ligand, Rae-1, could be upregulated on KC by administration of doxycycline it was shown that even in the absence of any overt microbial stress (or overt tissue/cellular dysregulation as in a tumor setting) engagement of NKG2D on the DETC activated these cells and caused profound changes in the local immune compartment⁸⁸. This DETC-modulated tissue immunity was found to be highly type 2 in character. Antigen administered epicutaneously concurrently with Rae1-expression induced antigen-specific type 2 immunoglobulins, particularly IgE and IgG1, along with type 2 cytokine expression in the skin and draining LNs⁸⁸.

Therefore, DETC-immune surveillance, at least *via* the NKG2D-Rae1 axis, induces type 2 immunity in the tissues. $\gamma\delta$ T cells were earlier shown to play a crucial role in protecting against cutaneous malignancy in seminal work by Girardi and others in 2001; and more generally $\gamma\delta$ T cells have been shown to play prominent roles in the control of malignancy in murine models of B cell lymphoma, prostate cancer, transplantable models of melanoma and adenocarcinoma⁸⁹. In the skin, the same cell that provides critical tumour protection thus also initiates potent type 2 immunity⁹⁰. These findings seem incompatible, given that type 2 immunity is classically considered deleterious for tumour eradication. Therefore, my thesis is focused on examining the mechanisms by which DETC-elicited type 2 immunity may modulate homeostasis in the skin. LSS-mediated sensing of stress antigens and the atopic mechanisms that ensue provide a direct molecular link between allergic-type immunity at body surfaces and regulation of tissue homeostasis. Indeed, as will be expanded on in Chapter 5 introduction, numerous studies have long suggested an inverse correlational link between allergy and cancer⁹¹, of particular significance at

tissues that interface with the external environment. These data suggest that resident immunocytes can recognize and act solely on alterations in autologous stress antigens and thus survey the “health-status” of a given KC, in a manner highly conducive to tissue health in multiple settings – including malignancy.

Thesis Hypothesis

Tissue-resident immunocytes are in a unique position to carry out a continued maintenance function such as tissue stress surveillance. DETC have the capacity to recognize antigens that are displayed in tissues following a variety of stressors, and they can respond rapidly in large numbers without requiring clonal expansion. This process has been termed LSS and falls under the rubric of immune surveillance. The very early stages of an immune response – the afferent phase – are conventionally ascribed to myeloid cells or NK cells. DETC however can also be afferent sensors of cellular dysregulation, the consequences of which will be examined in this thesis. In light of the host-protective LSS response, I hypothesise that DETC-elicited type 2 immune surveillance is a crucial regulator of tissue homeostasis and carcinogenesis.

Thesis Aims

Aim 1

To examine the DETC response to acute tissue dysregulation. I will characterize DETC morphology and effector functions – notably cytokine production. I will examine the KC epimmunome response to epithelial damage, and construct a model for how the KC and DETC responses may be integrated in health to maintain tissue homeostasis (Chapter 3).

Aim 2

To scrutinise the contribution of DETC and their effector molecules to physiological models of tissue injury and dysregulation; mild skin abrasion *via* tape stripping, full thickness skin wounding, and epithelial carcinogenesis (Chapter 4 and 5).

Aim 3

To analyse the contribution of DETC to acute genotoxic insult in the skin. To determine whether KC DNA-damage repair pathways are directly influenced by the DETC or its products (Chapter 6).

Chapter 2 | Materials and Methods

See Table 2.2 for list of reagents used, Table 2.1 for list of primers, and Table 2.0 for list of antibodies for flow cytometry.

Mice and Animal Husbandry

Transgenic mice were typed *via* PCR to confirm their genotype. Wildtype (WT) and transgenic mice were sex- and age-matched and all mice were >6 weeks old at the start of experiments. For most experiments, WT animals were purchased from an external company (Charles River or Harlan). Neonatal mice were bred in-house for experiments requiring neonatal skin, and were culled between 2 and 7 days post-partum. Mice were maintained in individually ventilated cages under specific pathogen-free conditions; with food and water provided *ad libitum*. All studies complied with institutional guidelines and Home Office regulations, and were carried out under PPL 70/7850.

To produce bone marrow chimeras, BALB/c and IL13eGFP mice were irradiated with 1 dose of 750 Rads. They were subsequently reconstituted by intravenous injection with 8×10^6 donor bone marrow cells and left for 8 weeks. Mouse drinking water was supplemented with antibiotic Baytril throughout. Four groups of chimeras were made. WT recipients animals received WT bone marrow and were hence IL13 sufficient in all compartments. IL13eGFP recipients received IL13eGFP bone marrow and were IL13eGFP in all compartments. IL13eGFP recipients received WT bone marrow, and were IL13 sufficient in the hematopoietic compartment and IL13eGFP in the skin and DETC. WT recipients received IL13eGFP bone marrow, and were IL13eGFP in the hematopoietic compartment and IL13 sufficient in the skin and DETC.

Skin Treatments and Cutaneous Carcinogenesis

Experiments requiring ear treatments with chemicals, mice were first anaesthetised with inhalation isoflurane. For treatments on dorsal back skin, mice were anaesthetised and the area shaved with a surgical blade to remove hair. Mice were left for a

minimum of 1 week, and hair regrowth during experiments was gently removed by clipping with trimmers.

Two cutaneous chemical carcinogenesis models were used, i) 'DMBA Complete' or ii) 'DMBA + TPA'. For DMBA complete, animals were initiated with topical application of 600 nmol DMBA (BALB/c background animals) or 200 nmol DMBA (FVB background animals), dissolved in 100 μ L 100% acetone. Animals were then left 1 week, and given once weekly topical applications of 200 nmol DMBA for a minimum of 10 weeks. For the DMBA+TPA protocol, initiation was the same as DMBA Complete. However, animals were given twice weekly applications of 20 nmol TPA (BALB/c background animals) or 5 nmol TPA (FVB background animals) dissolved in 100% ethanol for a minimum of 10 weeks in place of further DMBA. Cutaneous tumours were counted and measured with calipers once a week. All chemical carcinogenesis protocols were performed in a Class 2 Microbiological Safety Cabinet, within a Biohazard Class 2 facility.

Subcutaneous Tumour Inoculation

The right flank of mice was carefully shaved the day before inoculations. CT.26 cells were grown in RPMI culture medium + 10% FCS, supplemented with penicillin and streptomycin antibiotics. 1×10^5 CT.26 cells were injected subcutaneously into the flank in 100 μ L sterile PBS. Subsequently the flank was palpated daily and the tumour mass measured with calipers.

Epidermal Sheet or Cell Suspension Isolation

Mice were euthanized *via* an appropriate Schedule 1 method. Body wall skin was removed with scissors and collected directly into sterile PBS, along with ears if required. Subcutaneous fat was removed from body wall skin by scraping with rounded forceps. The skin was then cut into strips 1 cm in width. Ears were split into dorsal and ventral sides. For isolation of intact epidermal sheets for immunohistochemistry or isolation of RNA or DNA, skin was floated dermis side-down in 0.5 M NH_4SCN (ammonium thiocyanate) for 40 minutes, 37°C, 5% v/v CO_2 . Subsequently, intact epidermal sheets were carefully peeled away from the dermis

with rounded forces, and the sheets were washed by immersion in PBS for 5 minutes. Sheets were then submersed into an appropriate buffer for experimentation.

For isolation of epidermal cell suspensions, normally for flow cytometry, skin was floated dermis side-down in filter-sterilized, TrypLE Express solution for 2 hours at 37°C, 5% v/v CO₂. Following digestion, intact epidermis was carefully peeled away from the dermis with rounded forceps and placed in a fresh tube with filter-sterilized pH 7.5 epidermal digestion buffer (TrypLE Express solution supplemented with 200 µg/mL DNase I and 1x DNase buffer (1.21 g/L Tris Base, 0.5 g/L MgCl₂, 0.073 g/L CaCl₂) on a rotator for 30 minutes, 37°C with 175 RPM agitation. Cell suspensions were vortexed at moderate speed for several seconds and then pipetted through a 40 µm cell strainer directly into 1 volume of PBS. Suspensions were centrifuged at 2000 RPM, 1 minute, 4°C. Epidermal cell pellets were then resuspended in an appropriate buffer for experimentation.

Tape Stripping and Transepidermal Water Loss Measurement

Mice were anaesthetized with inhalation isoflurane. Clear adhesive tape was firmly applied to the ear skin and then ripped from the skin in one smooth motion. This procedure was performed six times per ear. For measurement of TEWL, a tewameter probe (Tewameter TM300; Ck Electronic, Germany) was used to record skin water evaporation rate. Mice were kept anaesthetized and the probe placed directly onto the skin. For measurement of ear TEWL, the ear was first spread onto the surface of a gloved finger to allow the probe to make a seal around the entire ear. Probe readings were analyzed and water evaporation rate reported with MPA software (MPA software; Ck Electronic, Germany) as g/hm² (where g = water loss in grams, h = time in hours, m² = meters squared). For reconstitution experiments, 50 ng rIL13 in PBS was aliquoted and spread onto the surface of the ear with a sterile pipette tip. The opposing ear was treated with vehicle PBS in the same manner.

Full Skin Thickness Wounding and Healing Measurement

For full thickness wounding, mice were anaesthetized with inhalation isoflurane and transferred onto a heat block with an anesthetic nose-cone to maintain anesthesia throughout the procedure. Buprenorphine local anesthetic (Vetergesic) was

administered subcutaneously to the area to be wounded. A single, full-thickness 5mm wound in the skin was made using a disposable sterile punch biopsy tool at the midline of the lower back. The mouse was observed until recovery and returned to an individually ventilated cage. Paper bedding and wet food was provided for 7 days following wounding. Wound closure was measured with calipers daily for the first 2 days, and then once every 2 days until complete wound closure. The wound sagittal (x) and transverse (y) plane was measured and these were applied to the ellipse area formula to calculate wound area:

$$(\text{area} = \pi (\text{radius } x)(\text{radius } y))$$

BrdU Immunohistochemistry on Paraffin Sections

Ears were collected into PBS, and a 1 cm by 1 cm square of tissue was then cut from the center of each ear and placed into a histological cassette. The cassettes were then stored for at least 24 hours in neutral buffered formalin at RT. These fixed tissue samples were then sent to the Histology service at the South Kensington Campus of Imperial College London to be embedded in paraffin wax, and 5 µm sections cut onto slides. Following this, the slides were returned to the investigator and stored at RT in the dark. Prior to staining, slides were de-waxed and cleared by being immersed in xylene two times for 5 minutes each, then 100% EtOH two times for 5 minutes each, and finally washed under flowing water in the sink for 5 minutes. Antigen retrieval for BrdU staining was performed by incubating the de-waxed and cleared slides in a 95°C waterbath for 15 minutes, immersed in pH 6.0 sodium citrate solution (2.94 g/L tri-sodium citrate dehydrate, 0.5 mL/L Tween-20. Following this slides were water washed in the sink for 5 minutes.

Tissue endogenous peroxidase activity was blocked by incubation in 0.37% H₂O₂ for 15 minutes at RT, with gentle agitation on a rotator, followed by a wash in wash buffer (PBS + 0.25% Triton X-100) for 5 minutes at RT. A hydrophobic barrier was drawn around the tissue sections on the slides with a hydrophobic pen and the sections were blocked with serum of the same species as the secondary antibody and fish skin gelatin; both diluted in wash buffer (secondary blocking serum from VECTASTAIN Elite ABC Kit kit), 1% fish skin gelatin. Slides were left to block for 30 minutes at RT and then excess block was drained onto tissue paper. Endogenous

avidin and biotin sites were blocked with avidin reagent for 10 minutes and then biotin reagent for 10 minutes at RT with a Avidin/biotin blocking kit. Slides were then washed for 10 minutes in wash buffer with gentle agitation.

All staining was performed in a foil-wrapped, humidified chamber, in the dark. For BrdU staining, a rat IgG2a anti-BrdU (clone BU1/75, ab6326; abcam) purified antibody was used with a VECTASTAIN *Elite* ABC Kit for Rat IgG as per manufacturer's instructions. The slides were first incubated with the primary anti-BrdU antibody (1/50 in wash buffer with 1% fish skin gelatin) for 30 minutes at RT in the dark. The slides were then washed three times for 5 minutes each in wash buffer with gentle agitation. Then they were incubated for 30 minutes at RT with the secondary biotinylated antibody diluted in wash buffer with secondary blocking serum, as per manufacturers instructions. The slides were then washed as before, and incubated for 30 minutes at RT with streptavidin-biotin-horseradish peroxidase conjugate diluted in wash buffer. The conjugate had been prepared at least 30 minutes prior to use as per manufacturers instruction. Slides were then washed as before, and developed for 2 minutes in the dark with DAB substrate and chromogen solution (1 drop of DAB chromogen) from supplied dispenser bottle was added to 1.5 mL of DAB substrate, vortexed and then dispensed onto the slides). Following this slides were water washed in the sink for 10 minutes. Washed slides were then stored in tap water at RT, for at least 24 hours. Slides were dipped into Harris' haematoxylin solution for 30 seconds, briefly rinsed under tap water for several seconds, and then briefly dipped into 1% acid-alcohol solution (0.3% HCl, 70% EtOH) for less than 1 second. Following this slides were water washed in the sink for 5 minutes. Slides were then de-hydrated and cleared (3x ethanol and 2x xylene incubations this time for 3 minutes each). Slides were then air dried, and mounted with pertex mounting medium. A coverslip was applied and finished slides were visualized with a light microscope or stored in the dark.

Standardized Flow Cytometry and Antibodies

Epidermal cell suspensions from naïve or treated skin were re-suspended in 1/300 viability dye in PBS (LIVE/Dead fixable dead cell stain) in PBS in the dark for 30 minutes, 4°C. 1 volume of FACS buffer (PBS with 2% FCS, 2mM EDTA) was then

added to the suspension and it was centrifuged at 2000 RPM, 1 minute, 4°C. For staining of cell surface markers, cells were first blocked in blocking solution (2% Fc γ receptor block (2.4G2 hybridoma supernatant, sourced in-house), 2% normal rat serum, 2% FCS), for at least 15 minutes at 4°C. Cells were then washed, and resuspended in a staining solution mastermix, with conjugated monoclonal antibodies diluted typically to 1/100 or 1/200 in FACS buffer. Most antibodies for flow cytometry were fluorochrome conjugated, rat monoclonals. Cells were incubated for at least 30 minutes at 4°C and then washed twice in FACS buffer. Usually, stained cells were analyzed the same day on the Flow Cytometer (BD FACSVerse™, BD Biosciences; NJ, USA). When this was not possible cells were fixed with 2% PFA for 15 minutes at RT, washed, resuspended in FACS buffer and stored wrapped in aluminum foil in the fridge at 4°C for up to 3 days.

Staining for Intracellular Cytokines

Staining for intracellular cytokines for flow cytometry was performed using the Intracellular Fixation & Permeabilization Buffer Set as per manufacturer's instructions. Briefly, after staining cells with a fixable dead cell stain, cells were fixed with the kit's fixation buffer and washed. Subsequently, cells were blocked with blocking serum diluted in kit-supplied permeabilization buffer. Staining was performed as before, with all antibodies also diluted in permeabilization buffer. Following staining, cells were washed in fresh permeabilization buffer, and resuspended in FACS buffer for flow cytometric analysis.

γ H2AX Staining by Flow Cytometry

For intranuclear γ H2AX staining, following staining with dead cell stain the cell pellet was drip re-suspended in ice-cold 70% EtOH while slowly vortexing. The suspension was then placed in a -20°C freezer for 2 hours. Subsequently, 1 volume of blocking solution (FACS buffer supplemented with 2% Fc receptor block, 2% normal mouse serum, 2% normal rat serum, and 2% normal rabbit serum) was added to the suspension which was then centrifuged at 1200 RPM, 5 minutes, 4°C. Cell pellet was re-suspended in blocking solution and 50 μ L aliquoted per well into a 96 well plate. The plate was placed in the fridge for 15 minutes at 4°C. 50 μ L of 2x staining solution or 50 μ L of isotype control staining solution (see Table 2.0 for flow cytometry

antibodies), was aliquoted directly into wells. The plate was incubated in the dark for 45 minutes at room temperature. The plate was then centrifuged at 1200 RPM, 5 minutes, 4°C and the wells washed with PBS before being centrifuged again. The wells were then resuspended in 200 µL FACS buffer and this volume transferred to FACS tubes for analysis.

γH2AX and p53 Staining on Epidermal Sheets

Epidermal sheets were prepared as per (Epidermal Sheet or Cell Suspension Isolation). Subsequently, epidermal sheets were placed on slides. Slides were fixed in ice-cold acetone at -20C for 15 minutes. Slides were rehydrated in PBS for 10 minutes at RT. The slides were then immersed in block buffer (PBS, 2% normal serum of the same species as the secondary antibody, 0.25% BSA, 0.3% Triton X-100) for 30 minutes at RT. Slides were washed in PBS for 5 minutes. Slides were then incubated in block solution with the primary antibodies (either; purified mouse IgG1 anti-γH2AX (Clone JBW301; Millipore) or purified rabbit polyclonal anti-p53 (Millipore)) at 1/100, overnight at 4°C in the dark. All staining was performed in a sealed humidified chamber. The next day slides were washed three times in wash buffer (PBS with 0.3% Triton X-100) for 5 minutes each time with gentle agitation. Slides were then incubated in block solution with the secondary antibodies (goat anti-rabbit-AF488 or goat anti-mouse AF555 (both ThermoFisher Scientific) for 1 hour at RT. Slides were washed as before. Slides were then mounted with DAPI solution and analyzed immediately on the fluorescence microscope with the FITC and PE filters.

Table 2.0 | Flow Cytometry Antibodies

Specificity	Species & Isotype	Clone	Fluorochrome	Catalogue Number	Manufacturer
CD45	Rat IgG2b	30-F11	APC	17-0451-82	eBioscience
IFN γ	Rat IgG1	XMG1.2	AF488	53-7311-82	eBioscience
IFN γ	Rat IgG1	XMG1.2	PE	12-7311-82	eBioscience
IL13	Rat IgG1	eBio13A	PE	12-7133-82	eBioscience
IL13R α 1	Rat IgG2a	13MOKA	PE	12-2130-80	eBioscience
IL17a	Rat IgG2a	eBio17B7	FITC	11-7177-81	eBioscience
IL4	Rat IgG1	11B11	PE	12-7041-82	eBioscience
Keratin 5	Mouse IgG1	D516B4	FITC	FCMAB291F	Millipore
p53	Mouse IgG2b	DO-7	PE	645805	Biolegend
TCRV γ 5*	SH* ² IgG	536	APC	137506	Biolegend
TCR $\gamma\delta$	AH* ³ IgG	eBioGL3	e450	48-5711-82	eBioscience
γ H2AX	Mouse IgG1	JBW301	FITC	16-202A	Millipore

N.B. *V γ 3 by other nomenclature. *² syrian hamster. *³ armenian hamster.

Comet Assay

Neutral Comet Assays were carried out as per manufacturers instructions with the kit Comet Assay HT. Epidermal cells purified from skin *ex vivo* were resuspended to 2×10^5 cells/mL in ice-cold PBS. All buffers and medium were ice-cold and calcium/magnesium-free. Cells were mixed with proprietary agarose (1 part cell suspension to 10 parts agarose) and immediately spread over the surface of wells on a 20 well slide; which corresponded to approximately 500 cells per well. The slide was then immersed in proprietary lysis solution overnight at 4°C. The following day the slide was immersed in 1x filter-sterilized, pH 9.0, electrophoresis buffer (12 g/L Tris base, 40 g/L sodium acetate) for 30 minutes at 4°C. The slide was then placed into a horizontal electrophoresis tank, equidistant between the two electrodes. 1x electrophoresis buffer pre-cooled to 4°C was added to the tank up to a level 0.5 cm above the slide surface, and then adjusted to yield a 300 mA current. Constant voltage was applied at 20 V for 1 hour.

The slide was then immersed in DNA precipitation solution (1M NH₄Ac in 95% EtOH) overnight at 4°C. The slide was then transferred into reagent-grade 70% EtOH for 30 minutes at RT, before being dried for 15 minutes at 37°C in a box containing a desiccant sachet. SYBR Gold-DMSO was diluted 1/30,000 in Tris-EDTA buffer and 50 µL added to each well of the slide. The slide was left for 30 minutes at RT in the dark and then immersed fully in ice-cold distilled water for 30 minutes to wash. The slide was then air-dried for 20 minutes at 37°C and analyzed with a Comet Assay IV microscope with the fluorescein filter. Comets were scored with Comet Assay IV software (Perceptive Instruments; UK) and data analysed in Graph Pad Prism 6.

Primary Keratinocyte Culture

Body wall skin from neonatal mice less than 1 week old was removed by cutting with scissors and collected into sterile PBS. Whole skin was incubated overnight at 4°C in 5 U/mL Dispase in PBS supplemented with 2x antibacterial and antimycotic solution. The following day, the epidermis was peeled away from the dermis with sterile forceps. The epidermis was transferred into a fresh tube of TrypLE Express solution supplemented with 200 µg/mL DNase I and 1x DNase buffer (1.21 g/L Tris Base, 0.5 g/L MgCl₂, 0.073 g/L CaCl₂) and placed back into the incubator with gentle rotational agitation for a further 45 minutes at 37°C, 5% v/v CO₂. The tube was gently

vortexed for a few seconds and the suspension pipetted through a cell-strainer directly into 1 volume of culture medium (defined keratinocyte serum-free medium with supplements). These isolated epidermal cells were then pelleted *via* centrifugation at 2000 RPM, 1 minute, 4°C, and resuspended in 2 mL cell culture medium. A cell count was performed with a CASY Model TT Cell Counter and Analyzer (Innovatis, Roche).

Culture vessels were pre-coated with 60 µg/mL rat tail-derived Collagen I. Collagen was dissolved in 20 mM acetic acid and coating was performed for 1 hour at RT. The wells/flasks were then washed 3 times in PBS and culture vessels used the same day. Cells were seeded at 3×10^5 cells/cm² vessel surface area in culture medium supplemented with 1x antibacterial/antimycotic solution. The following day (1 day post-seed) culture medium was gently aspirated and replaced with fresh culture medium supplemented with 1x antibacterial/antimycotic solution for experimentation. For treatments involving recombinant IL13, 20 ng rIL13 was added to each well in a 12 well plate.

PMA & Ionomycin *In Vitro* Stimulations

Epidermal cell suspensions were seeded at a density of at least 1×10^6 cells/mL into 96 well, round-bottomed plates in Complete medium (RPMI-1640 + 10% FCS). Cells were incubated for 5 hours with either; i) medium alone + Brefeldin A at 10 µg/mL, or ii) medium + Brefeldin A + PMA and ionomycin. For some experiments Brefeldin A was substituted with Monensin which was used at the manufacturers recommended concentration. One of two different concentrations was used for PMA and ionomycin. A low-PMA concentration for DETC stimulation in epidermal cell suspensions was PMA at 6.25 ng/mL and ionomycin at 3.5 µg/mL. The standard concentration was PMA at 25 ng/mL and ionomycin at 1 µg/mL. Following stimulation, cells were washed in PBS and resuspended for staining for intracellular cytokines for flow cytometry.

RNA Extraction

A RNeasy Mini Kit was used for all extractions, as per manufacturer's instructions. Epidermis, or tumours excised from culled animals were immediately fully submersed in RNAlater solution. Tissue was left to stabilize overnight in RNAlater in the -20°C

freezer, and then stored at -80°C for archival or immediately processed as follows. Tissue was removed from RNAlater with forceps and gently blotted on a clean piece of tissue paper. It was then transferred into a fresh eppendorf tube, which contained 600 µL of B-Mercaptoethanol-supplemented Buffer RLT, and a 5 mm stainless steel RNase-free bead. Tissue was then mechanically disrupted and homogenized with a TissueLyzer II instrument at 30 Hz for 3 minutes. Tubes were then visually examined to ensure tissue was sufficiently disrupted. If not, tubes were put through the TissueLyzer II again. These lysates were then centrifuged at 13,000 RPM for 3 minutes, and the supernatant transferred into a fresh eppendorf.

1 volume of reagent grade 70% EtOH was added to the lysate, and the tube mixed by pipetting. Up to 700 µL of this sample was aliquoted directly on top of a RNeasy spin column, which was centrifuged for 15 seconds at 13,000 RPM. The eluent was discarded, and 700 µL Buffer RW1 was added to the spin column, which was centrifuged for 15 seconds at 13,000 RPM. The eluent was discarded and 500 µL Buffer RPE was added to the spin column, which was then centrifuged for 15 seconds at 13,000 RPM. The eluent was discarded, and 500 µL Buffer RPE was again added to the spin column, which was centrifuged for 2 minutes at 13,000 RPM. The eluent was discarded, and the RNeasy spin column was centrifuged dry for 1 minute at 13,000 RPM. The eluent was discarded, and 20 µL RNAase-free water was added to the spin column, which was then centrifuged for 1 minute at 13,000 RPM to elute the RNA. Following this, these 20 µL volumes of RNA were stored in the -80°C freezer for archival, or used immediately for cDNA synthesis. RNA was analyzed *via* UV-vis spectroscopy with a Nanodrop instrument to ensure sufficient yield (A260/A280) and purity (A260/A230).

cDNA Synthesis

iScript cDNA Synthesis kit was used for all cDNA synthesis reactions. 1 µg of RNA was mixed in a 20 µL final volume with proprietary components; iScript reaction mix, iScript reverse transcriptase, nuclease-free water; as per manufacturer's instructions. Tubes were then incubated on a standard thermal cycler with the following protocol; i) 5 minutes at 25°C, ii) 30 minutes at 42°C, iii) 5 minutes at 85°C, iv) hold at 4°C.

cDNA was stored undiluted in the -80°C freezer for archival, or used immediately for qRT-PCR.

qRT-PCR

cDNA was first diluted to 1/20 in fresh ddH₂O, and 2 uL aliquoted into wells in a 384 well plate. Primers were single-stranded DNA oligonucleotides sourced from Sigma, diluted in ddH₂O to a concentration of 4 µM, all of which were intron-spanning as verified by the NCBI Primer-Blast tool (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). For SYBR Green-based detection of product, a mastermix was made for each gene to be analyzed with the following volumes per well; i) 5 µL 2x SYBR Green, ii) 1 µL forward primer, iii) 1 µL reverse primer and iv) 1 µL ddH₂O. Final volume per well was 10 µL; 2 µL of which was cDNA template and 8 µL mastermix. Gene-specific qRT-PCR products were continuously measured with a ViiA 7 Real-Time PCR system (Applied Biosystems; CA, USA) up to 40 cycles at 60°C. The following controls were performed to ensure specificity of signal; i) a non-template control omitting cDNA was included for each mastermix on all runs, ii) melt curves were examined for each mastermix on all runs for a single peak. Ct values of genes of interest were normalised against Ct values of a suitable housekeeping gene, and relative expression calculated using the $2^{-\Delta C_T}$ method. For the skin, cyclophilin was used as the housekeeping gene (for skin Taqman assays, it was substituted with beta actin, an inventoried assay).

DNA Extraction and *hras* qRT-PCR.

Starting material was either epidermal sheets isolated *via* ammonium thiocyanate digestion of skin or whole tumours excised from an animal. Epidermis or tumours were digested with a Gentra Puregene Tissue Kit as per manufacturers instructions for fixed tissue samples. Briefly, tissue was digested overnight in proprietary cell lysis solution with proteinase K digestion at 55°C. Following this, tissue was treated with RNase A to destroy RNA. Proteins were removed from the solution with protein precipitation solution, and DNA was precipitated out of solution with isopropanol. DNA was then incubated in DNA hydration solution for 1h at 37°C, and left overnight at RT with gentle agitation. DNA samples were stored at -80°C for archival, or used immediately for qRT-PCR. DNA samples were nanodropped to ensure adequate purity and yield. DMBA-induced transversion mutation in *hras*

(codon 61, CAA → CTA) were quantified with a forward primer, mutant-specific reverse primer, and a custom generated TaqMan 6'-FAM conjugated probe for visualising product. DNA was diluted to 50 ng/μL in fresh ddH₂O, and 6.75 μL aliquoted into wells in a 96 well plate (total 337.5 ng DNA/well). Primers were single-stranded DNA oligonucleotides (Sigma) diluted in ddH₂O to a concentration of 10 μM. The 6'-FAM conjugated probe was shipped pre-diluted to a concentration of 5 μM. For Taqman probe-based detection of product, a mastermix was made for with the following volumes per well; i) 12.25 μL 2x Taqman Gene Expression Mastermix, ii) 2.25 μL forward primer, iii) 2.25 μL reverse primer, iv) 1.50 μL probe. For quantification of ACTB housekeeping gene, an inventoried 20x ACTB Taqman gene expression assay was used (Mm00607939_s1; Life, CA, USA), as per manufacturers instructions. The ACTB primers and probe lie within a single exon and hence detect a genomic DNA target. Final volume per well for hras or ACTB qPCR was 25 μL; 6.75 μL of which was 337.5ng DNA template and 18.25 μL mastermix. PCR programme, controls, and gene expression analysis performed as previously described for cDNA qRT-PCR.

Table 2.1 | Primers for qRT-PCR

Target	Forward Sequence (5' to 3')	Reverse Sequence (5' to 3')
<i>Atm</i>	CTGCCGGCCAAGAAGTAGAA	TCACCCTGAAAAGACTGGCAT
<i>Bcl2</i>	AGCCTGAGAGCAACCCAATG	ATAGTTCCACAAAGGCATCCCAG
<i>Cldn1</i>	GCCATCTACGAGGGACTGTG	CACTAATGTCGCCAGACCTGAA
<i>Cyc</i>	CAAAATGCTGGACCAACACAA	CCATCCAGCCATTTCAGTCTTG
<i>IgE</i>	CAAGTCAGCCAAGAAGTTCAC	GACAGGTCGAAGTAGGATAGTC
<i>IgG1</i>	TTGCATATGTACAGTCCCAG	GTCAGAGTAATGGTGAGCAC
<i>IgG2a/c</i>	CTCTGGACAACCATCACCA	GAAGATCCACTTTACCTTGAAGAG
<i>IgM</i>	AAGACTGGAATAACAGGAAGGA	GTTTGTGCACCTCATTGGG
<i>Il13</i>	GCTTATTGAGGAGCTGAGCAACA	GCCAGGTCCACACTCCATA
<i>Il13ra1</i>	AGAGGTTGAAGAGGACAAATGCC	GCGACAAAGACTGGAATGGTGAG
<i>Il13ra2</i>	CCGAAATGTTGATAGCGACAGC	CCAAGCCCTCATACCAGAAAAAC
<i>Il1a</i>	TTGGTTAAATGACCTGCAACA	GAGCGCTCACGAACAGTTG
<i>Il33</i>	CACATTGAGCATCCAAGGAA	AACAGATTGGTCATTGTATGTACTCAG
<i>Il4ra1</i>	CCAATCAGACAGATACCAGATG	CCAGGTCAGCAGCCATTC
<i>Il6</i>	TGATGGATGCTACCAAAGTGG	TTCATGTACTCCAGGTAGCTATGG
<i>K15</i>	GGAAGAGATCCGGGACAAA	TGTCAATCTCCAGGACAACG
<i>K5</i>	CATGTCTCGCCAGTCCAGTG	GGAACCGCACCTTGTCGATG
<i>Ocln</i>	TTGAACTGTGGATTGGCAGC	CAAGATAAGCGAACCTTGCGC
<i>Rae1</i>	TGGACACTCACAAGACCAATG	CCCAGGTGGCACTAGGAGT

<i>Tnfa</i>	ACTGGAGTTGTACGGCAGTG	GGCTGATCCCGTTGATTTC
<i>Tgml</i>	CCTTGAGCTCCTCATTGGAA	CCCTTACCCACTGGGATGAT
<i>Tslp</i>	TCGAGGACTGTGAGAGCAAG	TGTTTTGTCTGGGGAGTGAA
Mutant <i>hras</i>	CTAAGCCTGTTGTTTTGCAGGAC	CATGGCACTATACTCTTCTA
Hras Probe	5'6FAM-CGGAAACAGGTGGTCAT-MGB3'	

Serum IgE ELISA

Venous blood was collected from the tail vein of living mice, or cardiac puncture was performed under terminal anaesthesia at the conclusion of an experiment. Blood was collected into a cuvette containing a proprietary clotting activator, and the tube was left to stand until a visible clot had formed. Tubes were then centrifuged at 6,500 RPM for 15 minutes. Following this the serum supernatant was carefully withdrawn with a pipette, and aliquoted into a fresh tube. Serum samples were stored undiluted at -80°C and thawed when appropriate for ELISA.

For determining total serum IgE *via* capture ELISA, 96 well enzyme immunoassay plates were first coated with capture antibody. Purified Rat Anti-Mouse IgE (Clone R35.72N/A ; 553413, BD) was diluted 1/500 in CBB (carbonate bicarbonate buffer; 80 mL / L 0.2 M Na₂CO₃, 170 mL/L 0.2 M NaHCO₃, pH 9.6 with NaOH) and 100 µL aliquoted per well; the plate was then covered to prevent evaporation and incubated for 3 hours at 37°C. The plate was washed twice with PBS-Tween (PBS with 0.05% Tween-20) and banded dry against clean laboratory tissue. The plate was then blocked with blocking solution (PBS with 1% BSA, 1% normal rat serum) for 1 hour at 37°C, covered. The plate was washed three times with PBS-Tween (PBS with 0.05% Tween-20) and banded dry against clean laboratory tissue. For all plates, 12 doubling-dilutions of a suitable IgE standard were aliquoted in duplicate wells across the top of the plate. For IgE ELISA, a 2000 ng/mL IgE standard was double-diluted 12 times to 0.454 ng/mL in PBS. Serum samples were diluted 1/10 in PBS and aliquoted in duplicate wells. The plate was then incubated overnight at 4°C, covered. Following this, the plate was washed four times with PBS-Tween (PBS with 0.05% Tween-20) and banded dry against clean laboratory tissue. Biotinylated Rat Anti-Mouse IgE (Clone R35-118 ; 553419, BD) was diluted 1/500 in blocking solution and 100 µL aliquoted per well; the plate was then covered to prevent evaporation and incubated for 2 hours at 37°C. Following this, the plate was washed five times with PBS-Tween

(PBS with 0.05% Tween-20) and banded dry against clean laboratory tissue. Streptavidin-Alkaline Phosphatase conjugate was diluted 1/1000 in PBS and added to all wells. The plate was incubated for 1 hour at 37°C, covered, and washed five times with PBS-Tween (PBS with 0.05% Tween-20) and banded dry against clean laboratory tissue. Substrate solution was prepared with 1 pNPP tablet dissolved in 20 mL CBB. Once the tablet had fully dissolved, 20 μ L MgCL was added to the solution, which was then vortexed. Substrate was immediately added to all wells, and the plate was incubated for 5 minutes in the dark to develop. Subsequently, the colorimetric reaction was measured with a UV-Vis ELISA plate reader at 405 nm every 5 minutes for 15 minutes.

The standard curve for each plate was plotted semi-logarithmically (log sample concentration *v.s.* A405) to generate a sigmoidal curve. Samples were examined for where they fell on the curve, and those within the curves linear range were quantified. Samples that fell below the limit of detection were re-ran at a lesser dilution when sufficient sample volume remained. Samples that reached saturation were re-ran at a greater dilution. The background signal A405 (wells with no sample or standard added) was subtracted from all wells to be quantified. The straight-line equation was then used to derive sample Ig concentration from the standard curve linear region.

Statistical Evaluation

Statistical significance between experimental groups was determined *via* a Student's *t* test. For some data sets linear regression analysis was performed where appropriate. All analysis was performed with Prism 6 software (Graphpad; CA, USA). A *p* value of < 0.05 was considered statistically significant. Significant results are denoted on graphs with asterixs. Where; * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, **** = $P \leq 0.0001$. Where included, error bars represent standard error of the mean. Data is representative is of at least two repeat experiments unless otherwise indicated.

Table 2.2 | List of Reagents

Product Name	Catalogue Number	Company
Ammonium thiocyanate	A7149	Sigma-Aldrich
Antimicrobial Solution	A5955	Sigma-Aldrich
Avidin/biotin blocking kit	SP-2001	Vector Laboratories
Brefeldin A	B7651	Sigma-Aldrich
DAB substrate kit	ab64238	abcam
Defined keratinocyte serum-free medium with supplements	10744-019	ThermoFisher Scientific
Dispase I	354235	Corning
DMBA	D3254	Sigma-Aldrich
DNase I	11284932001	Roche
Fish skin gelatin	G7765	Sigma-Aldrich
Gentra Puregene Tissue Kit	158667	QIAGEN
Harris haematoxylin solution	HHS128	Sigma-Aldrich
IgE Standard	557079	BD
Intracellular Fixation & Permeabilization Buffer Set	88-8824-00	eBioscience
Ionomycin	I0634	Sigma-Aldrich
iScript cDNA Synthesis Kit	1708890	Bio-Rad
LIVE/Dead Fixable Aqua Dead Cell Stain	L34957	ThermoFisher Scientific
LIVE/Dead Fixable Near-IR Dead Cell Stain	L10119	ThermoFisher Scientific
Monensin	554724	BD
Neutral Comet Assay Kit	4250-050-K	Trevigen
Normal Mouse Serum	M5905	Sigma-Aldrich
Normal Rabbit Serum	R9133	Sigma-Aldrich
Normal Rat Serum	R9759	Sigma-Aldrich
OCT compound	14-373-65	ThermoFisher Scientific
PMA	P1585	Sigma
Rat tail-derived Collagen I	A1048301	ThermoFisher Scientific
Recombinant IL13	413-ML/CF	R&D Systems
RNA Later Solution	AM7020	ThermoFisher Scientific
RNEasy Mini Kit	74104	QIAGEN
Streptavidin-Alkaline Phosphatase Conjugate	554065	BD
SYBR-Gold Stain	S-11494	ThermoFisher Scientific

SYBR-Green Stain	4367659	ThermoFisher Scientific
Taqman Gene Expression Mastermix	4369016	ThermoFisher Scientific
TPA	P1585	Sigma-Aldrich
Tri-sodium citrate dihydrate	S1804	Sigma-Aldrich
Tris-EDTA Buffer	T9285	Sigma-Aldrich
Triton X-100	X100	Sigma-Aldrich
TrypLE express	12604021	ThermoFisher Scientific
Tween-20	P9416	Sigma-Aldrich
VECTASTAIN elite ABC kit (Rat IgG)	PK-6104	Vector Laboratories

Chapter 3 | DETC and Lymphoid Stress Surveillance

Introduction

DETC Thymic Development and TCR Tuning

Innate $\gamma\delta$ T cells break the conventional T cell activation paradigm, allowing them to sense infection or tissue dysregulation and act rapidly in synchrony with innate responses. Strid *et al* described a molecular axis in the epidermis for DETC-mediated sensing of epithelial stress, and subsequent induction of potent local tissue immunity, and modulation of downstream adaptive immunity, in a manner independent of the TCR^{88,90}. Additionally, innate $\gamma\delta$ T cells can be activated solely upon stimulation with IL1 β and IL23⁹², providing protection against bacterial and fungal pathogens particularly in neonates. These activation modalities are in agreement with the DETC expression of NK-like immunoactivating receptors such as NKG2D. This suggests a novel role of the TCR in innate $\gamma\delta$ T cells – an arrangement endowed during ontogeny that is likely crucial given their tissue sequestration and TCR monoclonality, as previously described. That is not to say that the TCR is unimportant – indeed, unlike ILC which seem totally unconstrained by antigen receptors, DETC may use the TCR *in situ* for novel, constitutive engagements with KC as detailed below.

During normal thymic development, DETC progenitors engage Skint1 (Figure 3.0), a butyrophilin-like protein expressed on medullary thymic epithelial cells – an interaction found to be crucial for DETC development⁹³. This strong, agonist signal induces a gene regulatory network conducive to suppression of *Roryt* and *Il17*, upregulation of *Tbet* and *Ifn γ* , downregulation of CCR6 and upregulation of skin homing genes including CCR10⁹⁴. The mechanics of this process were demonstrated beautifully by M. Wencker *et al*. A majority of DETC precursors at a crucial developmental timepoint (E15) were shown to have experienced strong, TCR stimulation (calcium flux and phosphorylation of Erk and Src) which was not the case in the absence of Skint1 – adding potent evidence for Skint-1 being a DETC TCR ligand (or at least a crucial modulation of the TCR ligand and/or its binding, by

Skint1). Strikingly, when examined one day later at E16, indices of TCR-stimulation were substantially attenuated only in Skint1-sufficient animals, suggesting this engagement, while crucial, was additionally inducing tonic hyporesponsiveness of the DETC TCR⁹⁵. In G8 transgenic mice which express a T10/T22-restricted $\gamma\delta$ TCR, thymic expression of T10/T22 was crucial for the development of IFN γ -producing $\gamma\delta$ T cells suggesting that TCR engagement is crucial during $\gamma\delta$ T cell ontogeny⁴⁵. It is important to note however, that mature DETC are not fully TCR-anergic (they can be activated by anti-V γ 5 antibody injected into the ear, for instance), but represent altered antigen-receptor responsiveness hardwired during ontogeny. Somewhat conspicuously, Skint1 is expressed on KC, in addition to mTEC – indeed, peripheral Skint1 is likely required for retention of the DETC population following seeding in a manner not yet clear (unpublished, private communication).

Interestingly, innate $\gamma\delta$ T cells in different tissue sites expresses a particular set of variable gamma and delta chains. The IEL repertoires in the tongue and reproductive tract epithelia are mainly V γ 6, in the gut they are V γ 7 and V γ 4 in the lung. The DETC expresses V γ 5⁹⁶ (termed V γ 3 in some nomenclature). In the absence of the V γ 5 chain, as in Skint-deficient mice where the DETC TCR is not selected or in mice where the V γ 5 and V δ 1 chain is deleted, the epidermis is seeded with other non-canonical TCR $\gamma\delta$ T cells – and in total $\gamma\delta$ T cell deficient animals, $\alpha\beta$ T cells take their place (JS group observations and others). This hierarchical seeding would suggest a preference for the V γ 5⁺V δ 1⁺ chains in maintenance of the epidermal population and a unique role for this population of T cells in the epidermis. The replacement IEL populations, such as the $\alpha\beta$ T cell in the $\gamma\delta$ -deficient mouse, are not well maintained in the skin and lost over the lifetime over the animal – and importantly, are not able to respond to KC damage like the DETC⁹⁷, while also maintaining qualitatively different molecular interactions with the KC⁹⁸.

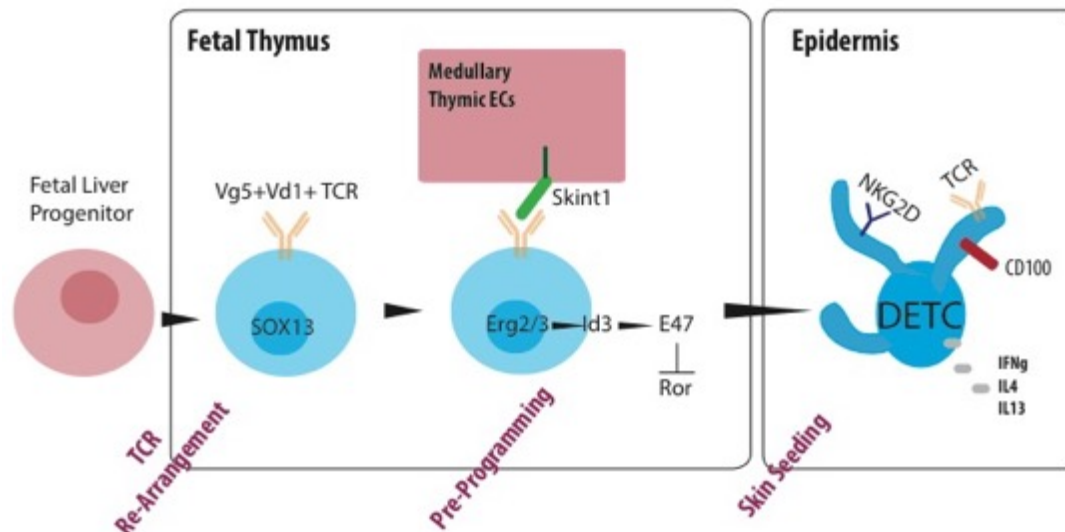


Figure 3.0 | Molecular determinants of thymic DETC development

Skint1-mediated selection induces upregulation of Egr3 (a marker of strong TCR-mediated signaling) and Egr2 – both are known to be transcriptional regulators of Id3. E47 is inhibited by Id3, which in turn attenuates Ror expression and the IL17 program in DETC precursors. Ligation of peripheral Skint1 by the DETC is crucial for persistence in the epidermis. Soluble factors secreted by the DETC under various stimulation conditions include IL13, IL4, IFN γ , keratinocyte growth factors (KGF) and insulin-like growth factors (IGF). (Adapted from “Epigenetic and transcriptional regulation of $\gamma\delta$ T cell differentiation: Programming cells for responses in time and space.” N Schmolka, B Silva-Santos *et al.* Sem. Immunol. (2015).)

Whether epidermal Skint1 constitutes a peripheral DETC TCR ligand in the epidermis remains un-verified – however, recent elegant intravital microscopy experiments by Chodaczek *et al* (described in more detail below) have shed some light on the putative ligand, among other DETC interactions with KC and neighbouring LCs. Both LC and DETC infiltrate the epithelium very early during stratification of the skin epithelium, and are long-lived and likely self-renewing immune compartments, which clearly integrate and physically interact with the EC.

DETC Interactions with their Neighbours

At steady state, the DETC maintains numerous contacts with KC through beautiful dendritic processes which interdigitate KC cell-cell junctions (Figure 3.1). Constitutive binding of a TCR ligand, likely Skint1, occurs with the specialized, immunosynapse-like ‘PALP’ (phosphotyrosine-rich aggregates located on projections) regions located at the tip of dendrites. Unlike basolateral dendrites, apical dendrites point upwards towards the external world, and interdigitate KC junctions

within the stratum granulosum. They are poorly motile and maintain contact with targets on the squamous KC⁹⁸. The ‘steady state-binding’ hypothesis suggests that the DETC TCR constitutively engages a KC ligand, which is labile under stress conditions. Although compelling with recent data, this hypothesis does however remain contentious. Skint1 expression is known to decrease under stress conditions, supporting the candidacy of Skint1 as a DETC TCR steady-state ligand, while endogenous ‘stress ligands’ such as Rae1 increase. At least one group has demonstrated V γ 5V δ 1 tetramers bind only to a KC ligand under conditions of stress and not at steady state⁹⁹ – however, work carried out by others in the group have questioned the utility of these tetramers. Adhesion of the PALP to their respective KC contacts is in part mediated by integrin CD103 to its putative ligand E-Cadherin, and a similar role has been shown for LFA-1. JAML is expressed at steady state and rapidly upregulated with stimulation. Ligation of JAML-ligand CAR induces PI3K recruitment and is a costimulatory signal for IL2 and IFN γ production. Experimental inhibition of this interaction causes reduced DETC activation and poorer healing in a skin wounding model¹⁰⁰. In at least one study NKG2D was found to be intracellular and not clustered to PALPs regions at steady state, suggesting that other signals, such as loss of TCR binding, permit this potent immunoactivating receptor to become mobilized. It is known that expression of NKG2D-ligand Rae1 alone is enough to activate DETC⁸⁸, ergo sequestration of some potent receptors may potentially represent an important checkpoint for DETC activation. CD100 binding of Plexin-B2 has shown to be particularly important for morphological changes in the DETC, most notably rounding, *via* induction of ERK kinase and cofilin¹⁰¹. Similarly to JAML interaction blockade, CD100 deficient animals demonstrate poorer wound healing, suggesting rounding may impart some functional change to the DETC.

Therefore, it seems likely that information regarding KC and epidermal tissue health is communicated to the DETC *via* an intriguing constellation of signals, in both agonist (NKG2D-ligands) and loss-of-signal modalities (TCR-ligand), as a form of epithelial immune surveillance. Although a consistent model of how these signals may coordinate to promote DETC-driven barrier surveillance remains elusive, deficiency in any one of these interactions is usually enough to promote a phenotype. Given the preponderance of DETC dendrites at inter-cellular junctions one could

suppose that the DETC is primarily looking for physical disruption of the epidermis. After all, the epidermis is a highly dynamic structure, in which a delicate balance of proliferation of basal cells, and differentiation of granular cells with complex inter-cellular junctions, maintains exclusion of most microbes and other insults in health.

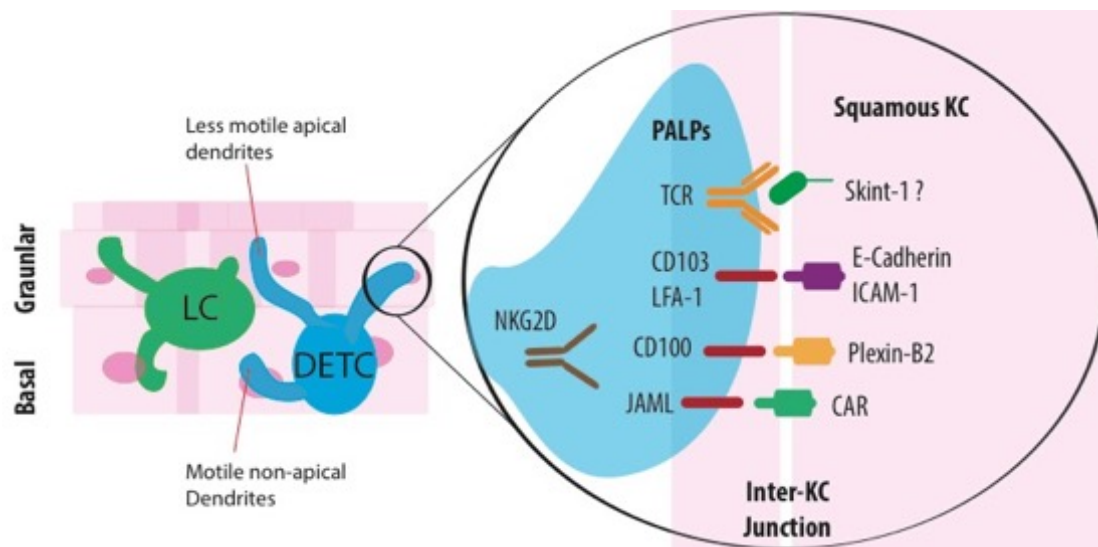


Figure 3.1 | The DETC surveys stratum granulosum inter-KC junctions via numerous molecular interactions at steady state

The DETC maintains numerous connections at steady state with proteins expressed particularly at KC cell-cell junctions in the stratum granulosum. DETC sensors are clustered in a specialized signaling apparatus termed the ‘PALP’ at the tip of apical dendrites. Changes in this constellation of signaling moieties due to a perturbation in tissue homeostasis activate the DETC. The DETC cell body remains localized to the basal epidermis at steady state, while LCs are positioned slightly higher in the granular epithelium. (Adapted from “Cross-talk between intraepithelial $\gamma\delta$ T cells and epithelial cells” Deborah A. Witherden and Wendy L. Havran. JLB. (2013).)

Hypothesis

In this opening chapter I will examine the morphology and cytokine production of the DETC following tissue injury *in vivo*, or stimulation *in vitro*. I will also scrutinise the epimunome, and how the DETC and KC compartments may interact in response to epithelial stress.

I hypothesise that:

1. DETC are acutely responsive to tissue injury, and one effector function is rapid cytokine secretion.
2. DETC cytokine secretion imparts consequential, direct molecular crosstalk with the KC.

Aims

1. To examine under which stress conditions DETC become activated and analyse which cytokines they produce.
2. To study the cytokines produced by KC following topical insult.
3. To study crosstalk between the DETC and KC as a potential molecular axis for driving protective responses to tissue injury.

The consequence of these interactions will subsequently be scrutinised in the context of epithelial homeostasis (Chapter 4), epithelial carcinogenesis (Chapter 5), and direct immune surveillance of epithelial DNA-damage (Chapters 5 and 6).

Results

The DETC were observed *in situ* in whole epidermal sheets isolated from the ears of naïve animals. In agreement with extensive literature, the DETC at steady state exhibited dendritic morphology when stained with an anti-TCR $\gamma\delta$ antibody (Figure 3.2 | A). DETC were evenly spread throughout the epidermis. Each DETC had multiple dendrites at steady state, agreeing with the literature that a wild-type DETC has on average 3 to 7 dendrites of which most are apically anchored and immotile⁹⁸. Indeed, in a single intravital confocal microscopy experiment DETC dendrites were observed in a living animal at steady state, which were almost entirely immotile over a time period of 1 hour (data not shown).

Subsequently two different models of skin perturbation were carried out – mild abrasion by tape stripping or full skin thickness wounding with a 5 mm biopsy instrument (Figure 3.2 | B). The aim was to observe DETC morphological changes in response to injury. DETC rounding in response to physical injury has been described in the literature. It was observed that tape stripping caused near total loss of DETC dendrites and rounding of the cell body throughout the tape stripped area at the timepoint examined (24 hours). Previous work in the group has shown DETC return to a dendritic morphology within 48 hours of tape stripping, consistent with tissue repair and replenishment of the SC; which is histologically normal at this time point. Thus suggesting DETC morphology is acutely responsive to tissue perturbation. DETC proximal to a full thickness skin wound edge (Figure 3.2 | B, red line, right panel) were also fully rounded.

Previous work by Dr J Strid and others observed IL13 expression in the epidermis, rapidly following various skin stressors. Following tape stripping, a clear upregulation of *Il13* is seen in the epidermis; which is absent in TCR $\delta^{-/-}$ animals (Figure 3.2 | C), suggesting that the canonical TCR $\gamma\delta^{+}$ DETC in the epidermis rapidly and transiently upregulates *Il13* in response to tissue stress. The peak of *Il13* expression occur very rapidly – within 2-6 hours following insult, and is reduced back to baseline within 18 hours. This rapid IL13 expression appear to be a conserved feature of KC stress as it is seen following a variety of skin stresses including physical perturbation such as shaving or tape stripping, topical exposure to chemicals such as TPA or DMBA and following UV-irradiation (Figure 3.2 | D).

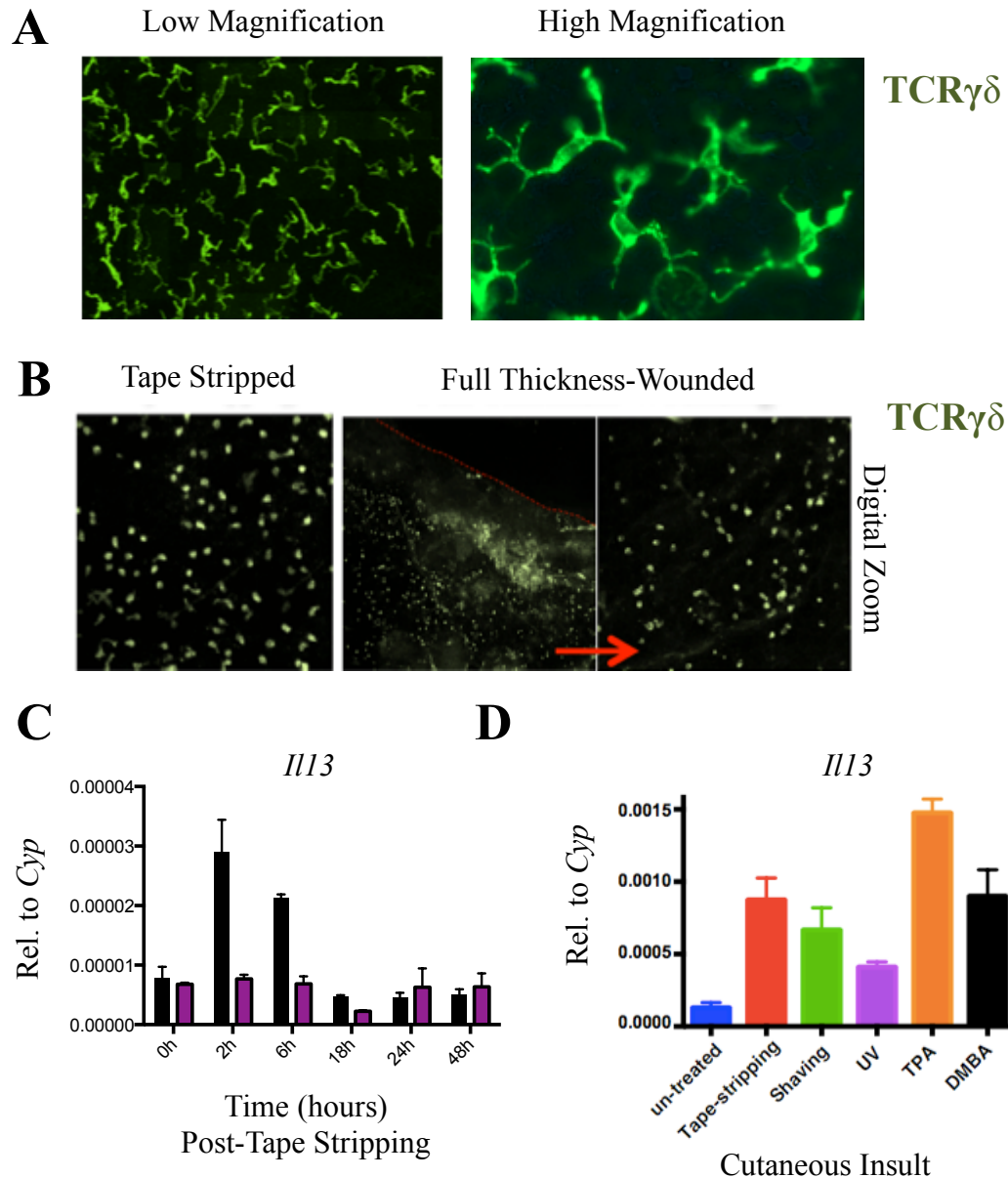


Figure 3.2 | DETC morphologically change *in situ* and produce IL13 in response to various skin stressors

(A) Immunofluorescence microscopy of a whole epidermal sheet isolated from WT FVB ear skin. DETC were visualized with purified hamster anti- $\text{TCR}\gamma\delta$ and a secondary goat anti-hamster AF488. (B) Intravital confocal microscopy of the DETC population in the dorsal ear of a living WT FVB mouse. 24 hours prior to imaging the dorsal ear was tape stripped (left panel) or a 5 mm full-thickness punch biopsy was performed (right panel). 3 hours prior to imaging DETC were labeled *in situ* with 8 μg of anti-TCR $\text{V}\gamma 5$ APC administered by tail *i.v.* injection. Red dotted line demarcates the wound/biopsy edge. Representative images shown. (C) Ears of WT FVB (black bars) or $\text{TCR}\delta^{-/-}$ (purple bars) were tape stripped and at the indicated time points epidermis was recovered for qRT-PCR. (D) qRT-PCR analysis of isolated epidermis 4h after indicated topical stressors ($n=3-6$ per condition). UVB was administered at 100 mJ/cm^2 . Data in C) and D) are expressed as mean $\pm$ 1 SEM relative to the control gene cyclophilin (*Cyp*). Panels C & D data kindly provided by Dr Strid.

Given the strong upregulation of *Il13* following diverse skin stressors, IL13 protein production by DETC was subsequently examined with the commonly used PMA and ionomycin stimulation model. Intracellular cytokines were detected by flow cytometry – following *ex vivo* stimulation of epidermal cells. Freshly isolated epidermal tissue was processed and single cell suspensions stimulated in the presence of one of two different protein transport inhibitors; brefeldin A or monensin. There are three main cell types in these epidermal cell suspensions as identified by flow; CD45⁺ cells comprising the DETC and LC, and CD45⁻ KC (Figure 3.3).

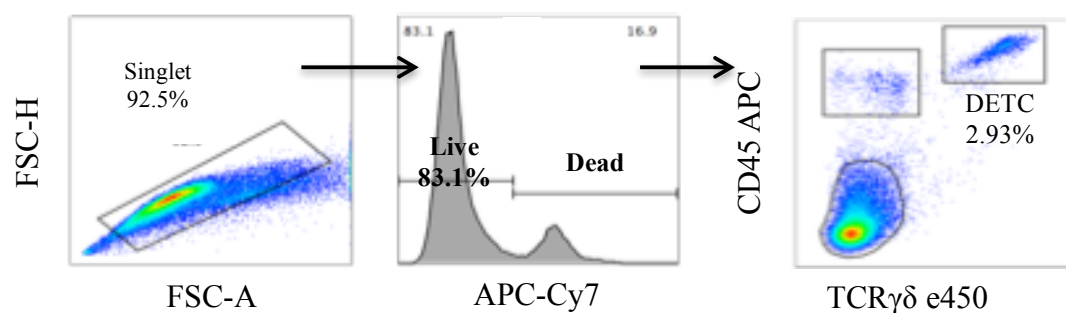


Figure 3.3 | Flow cytometry gating strategy for examining DETC in the epidermis

DETC are gated as follows. Doublet cells are excluded by forward scatter area *v.s.* height. Dead cells are excluded on the basis of uptake of a fixable, live/dead discrimination dye. Two lymphocyte (CD45⁺) populations are present in the epidermis at steady state - CD45^{hi}, TCRγδ⁺ DETC and CD45^{mid} Langerin and MHCII⁺ LCs. DETC can also be elucidated based on the expression of the Vγ5Vδ1 TCR with an anti-Vγ5 antibody. Percentages shown for gates are representative for epidermal cell suspensions in a WT FVB mouse.

When cultured with a blocking agent alone (Figure 3.4 | A) staining with anti-IL13 antibody did not detect appreciable IL13 in DETC. It could be supposed that the action of physically disrupting the epidermis into a cell suspension, and putting it into suspension culture for 4 to 5 hours may be enough of a stimulus to provoke DETC IL13 production. This did not seem to be the case – however any IL13 produced by the DETC during the process of disrupting the epidermis may have been released before cytokine secretion was blocked. Colleagues had previously determined a concentration of PMA and ionomycin that yields good stimulation of DETC with minimal cell death – defined in the figure as ‘Lo’ stimulation. The ‘Hi’ stimulation represents concentrations commonly used to stimulate splenic cell suspensions and conventional T lymphocytes. A majority proportion of DETC were observed producing IL13 protein following stimulation with low dose PMA and ionomycin using either golgi-

block (61.8 % and 66.8% for monensin and brefeldin A respectively.) A smaller IL13⁺ population was seen with the ‘Hi’ concentration compared to the Lo. The stimulation shows the potential DETC has for production of IL13 and recapitulates the *Il13* gene expression signal seen following different stressors *in situ* (Figure 3.2 | C,D).

In line with the literature IL17 was not detected (Figure 3.4 | B). As expected, when gating on specific populations in the epidermal cell suspension, only the DETC was found to produce IL13 (Figure 3.4 | D), while the LC and CD45⁺ cells were not. At steady state, and acutely following injury the only cell population in the epidermis that can produce IL13 is therefore the DETC.

The bias of IL13 production by $\gamma\delta$ T cells in the epidermis is not a general phenomenon of $\gamma\delta$ T cells. Skin-draining lymph node $\gamma\delta$ T cells when subjected to comparable stimulation were found to be more IFN γ dominant than IL13 (Figure 3.4 | E) – and dermis-resident $\gamma\delta$ T cells are IL17 producers¹⁰². Likewise, splenic $\gamma\delta$ T cells did not show a high proportion of IL13-producers.

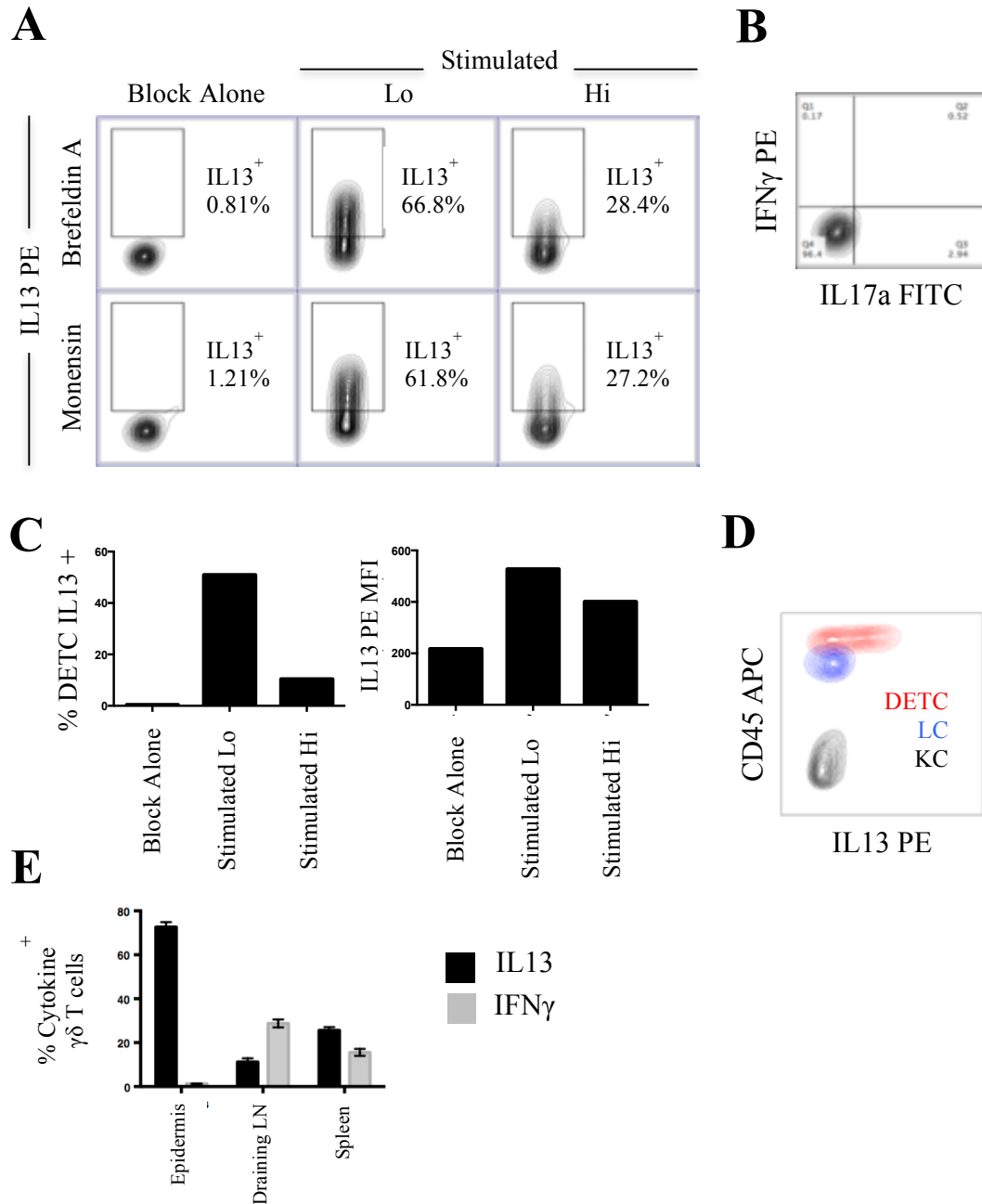


Figure 3.4 | DETC preferentially produce IL13 when stimulated *in vitro*.

(A,B) Epidermal cell suspensions from WT FVB skin were stimulated *in vitro* with PMA and ionomycin, together with either brefeldin A or monensin, for 5 hours, before being stained intracellularly for cytokines IL13, IL17a and IFN γ . All gating based on antibody isotype controls on the DETC population. (C) The proportion of DETC that were IL13⁺, and MFI of the IL13⁺ population is shown. (D) The LC population (CD45^{mid}, TCR $\gamma\delta$ ⁻) and KC (CD45⁻, TCR $\gamma\delta$ ⁻) were contemporaneously stained for intracellular IL13 in the epidermal cell suspension. (E) Comparable stimulations by PMA/Ionomycin was performed on cell suspensions from the epidermis, skin-draining lymph nodes and spleen and cells stained intracellularly for IL13 and IFN γ . Splenic cell suspensions had erythrocytes lysed (n=3). Error bars represent standard error of the mean.

DETC-production of IL13 was also examined in a different model. The IL13eGFP reporter animal was exploited to examine IL13 (eGFP) production without having to stain for it with an antibody. IL13eGFP reporter mice express eGFP under the control of the *Il13* promoter, and are a knock in-knock out model¹⁰³. Whole skin was taken from BALB/c mice and IL13eGFP mice and cell suspensions were stained for the DETC TCR-V γ 5. These suspensions were then immediately analysed on the flow cytometer. Skin from naïve, IL13eGFP animals yielded a DETC population that was uniformly eGFP⁺ (Figure 3.5 | A, left panel). In contrast, V γ 5⁻ T cells were not producing any detectable (IL13)eGFP at steady state. (Figure 3.5 | A, right panel). Following *in situ* activation, DETC upregulated IL13egfp further, as shown by an increase in eGFP MFI, whilst V γ 5⁻ T cells remained negative (Figure 3.5 | B). DETC are the only cells in the epidermis capable of producing IL13, but the dermis is home to other IL13 producing populations of cells. Using the IL13 reporter mice, the dermal ILC population and some of the dermal mast cells are also positive for IL13egfp even in resting skin (private communication, strid group colleagues). Hence, the spatial and temporal expression of IL13 may be of importance for skin homeostasis.

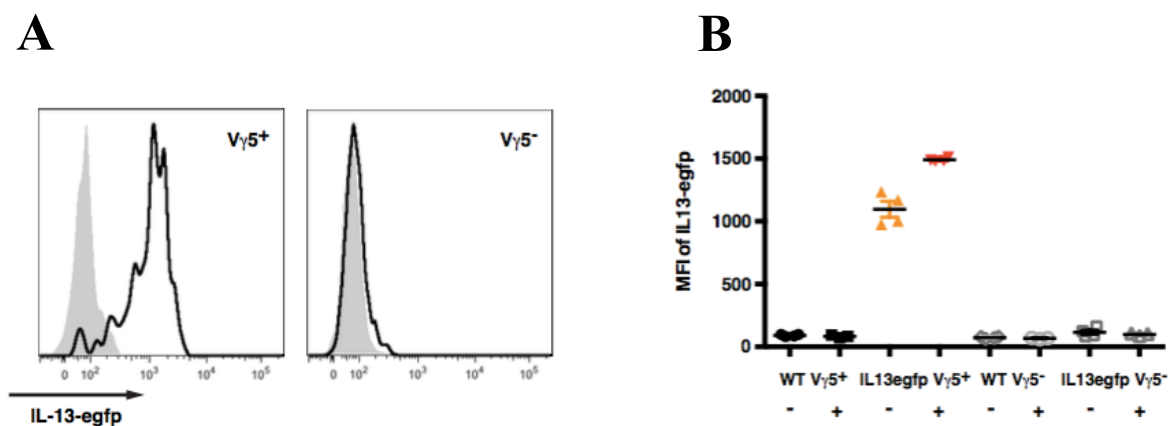


Figure 3.5 | DETC produce IL13 at steady state *in situ* – and increase production in response to skin stress

(A) Whole skin cell suspensions freshly isolated from BALB/c (grey filled histogram) or IL13eGFP reporter mice (black line histogram) were stained for TCRV γ 5 and examined for eGFP by FACS. (B) BALB/c or IL13eGFP mice were untreated (-) or treated topically with 10nmol TPA 24h hours (+) before cull. Subsequently, whole skin cell suspensions were isolated and processed as for (A). MFI of eGFP in one of four different gated populations; i) BALB/c V γ 5⁺ events, ii) IL13eGFP V γ 5⁺ events, iii) BALB/c V γ 5⁻ events or iv) IL13eGFP V γ 5⁻ events. n=4 mice per group.

IL13 mediates its effects by interacting with a complex receptor system comprised of IL4R α and two IL13 binding proteins, IL13R α 1 and IL13R α 2 (Hershey, 2003). To investigate whether KC can sense local fluctuations in IL13, KC expression of the IL13 receptors was examined. Two sets of primer pairs were used to detect expression of IL4 and IL13 receptors *via* qRT-PCR; (i) *Il4ra1*, which detects the IL4 receptor alpha 1 subunit (and thus detects both the type 1 and type 2 IL4Rs, the type two being the IL4Ra1/IL13Ra1 heterodimer which IL13 can signal through) and (ii) *Il13ra1*, which detects only the type 1 IL13 receptor.

Expression of the IL4 and type 1 IL13 receptors was detectable at steady state on both WT FVB (Figure 3.6 | A) and BALB/c (Figure 3.6 | B) backgrounds, indicating that KC can sense IL13 constitutively. In addition to fluctuations in cytokine, an important mechanism of modulating IL13 responses may be regulation at the level of its receptors. Indeed, following tape stripping, *Il4ra1* and *Il13ra1* expression increased significantly and peaked at 6 to 18 hours, and returned to baseline at 48 hours, in line with morphological tissue repair. When exposed to a different stressor, topical DMBA, both receptor subunits were also upregulated (Figure 3.6 | B). The stress-upregulation of both receptors was independent of IL13 itself as the receptors were equally upregulated in IL13-deficient mice (Figure 3.6 | B; orange bars).

To examine further which KC were expressing the IL4/IL13 receptor(s) and in which layer of the epidermis, the basal cell marker K5 was used to identify basal KC. Basal KC being those cells resident in the stratum basale, which maintain contact with the basement membrane. The stratum basale contains keratin filaments of K5 and K14 – while the spinous differentiating layers contains K1 and K10 filaments¹⁰⁴. A majority of CD45⁻ cells in the epidermis are K5⁺ - approximately 80% (Figure 3.6 | C). This is expected given that the murine stratified epidermis is mostly one-cell thick at steady state, when examined histologically. All basal K5⁺ KC expressed IL13R α 1 protein as demonstrated by FACS. (Figure 3.6 | C). Hence, in the basal epidermis, that is also home to the DETC, KC are able to detect fluctuations in IL13.

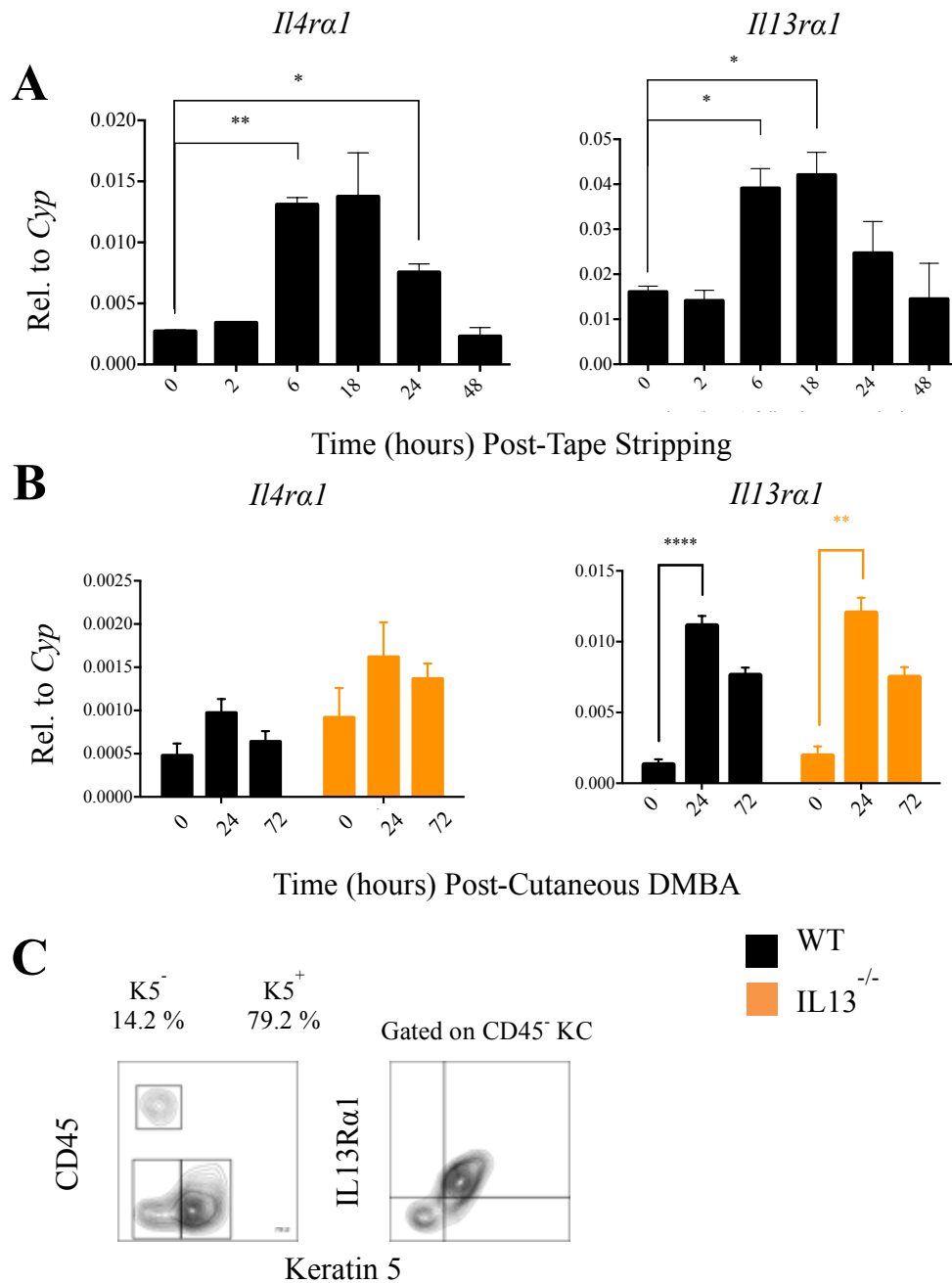


Figure 3.6 | Basal KC express stress-modulated IL4 and IL13 receptors - independently of IL13

(A) Ear skin of FVB mice was tape stripped and at the indicated time points later, epidermis was recovered for qRT-PCR (n=3 mice per time point) (B) WT and IL13^{-/-} mice were treated once cutaneously on ears with 400 nmol DMBA and at the indicated time points epidermis was recovered for qRT-PCR (n=4 mice per time point) Data for A and B are expressed as mean ± 1 SEM relative to the control gene cyclophilin (Cyp). (C) Representative example of WT Epidermal cell suspensions stained for indicated markers. CD45 gating set by eye. Keratin 5 and IL13Rα1 gating set against FMO controls. IL13Rα1 staining also verified separately with appropriate isotype control. Data representative of >2 biological repeat experiments. Statistics *via* Students T test where here; * = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001, **** = P ≤ 0.0001

In addition to sensing DETC-derived cytokines, KC are themselves capable of producing a diverse set of cytokines and chemokines as well as upregulating cell-surface antigens following injury. Thus it was subsequently examined how and to what extent KC would upregulate epimmunological genes following topical stress protocols. The tape stripping model was used for its simplicity and relevance to everyday, common physical abrasion injury at the skin. Topical DMBA treatment model was also used due to following work with carcinogen in tumour models (Chapter 5) and immune surveillance of DNA damage (Chapter 6).

Il1a shows upregulation following topical perturbation, peaking at 6 hours following DMBA and 18 hours following tape stripping (Figure 3.7). Interestingly, there was a significant decrease in *Il1a* transcripts at 2 hours post-tape stripping. This was not seen in the DMBA model and may suggest that KC in the outmost layer has been removed by tape stripping as these KC have previously been shown to harbor pre-formed IL1a. In both DMBA and tape stripping models, levels of transcript reduced to lower than $t=0$ h levels at 48 hours post-insult. *Il6* showed upregulation following insult at 2 hours post-tape stripping and 6 hours post-DMBA – it very rapidly returned to steady state levels at 18 hours post-tape stripping and 24 hours post-DMBA. IL33 and TSLP can be described as the canonical, type 2, epithelial products. Peak *Il33* upregulation was observed at 6 hours (DMBA) and 18 hours (tape stripping). *Tslp* expression in both models seems to be bimodal. There is an immediate upregulation at 6 hours (DMBA) and 2 hours (tape stripping), with a secondary upregulation at 2 days following both insults.

Therefore, a conventional epimmunome response to tissue stress is conserved with two different tissue stressors. The canonical, type 2 cytokines IL33 and TSLP show particularly robust upregulation in response to epidermal stress.

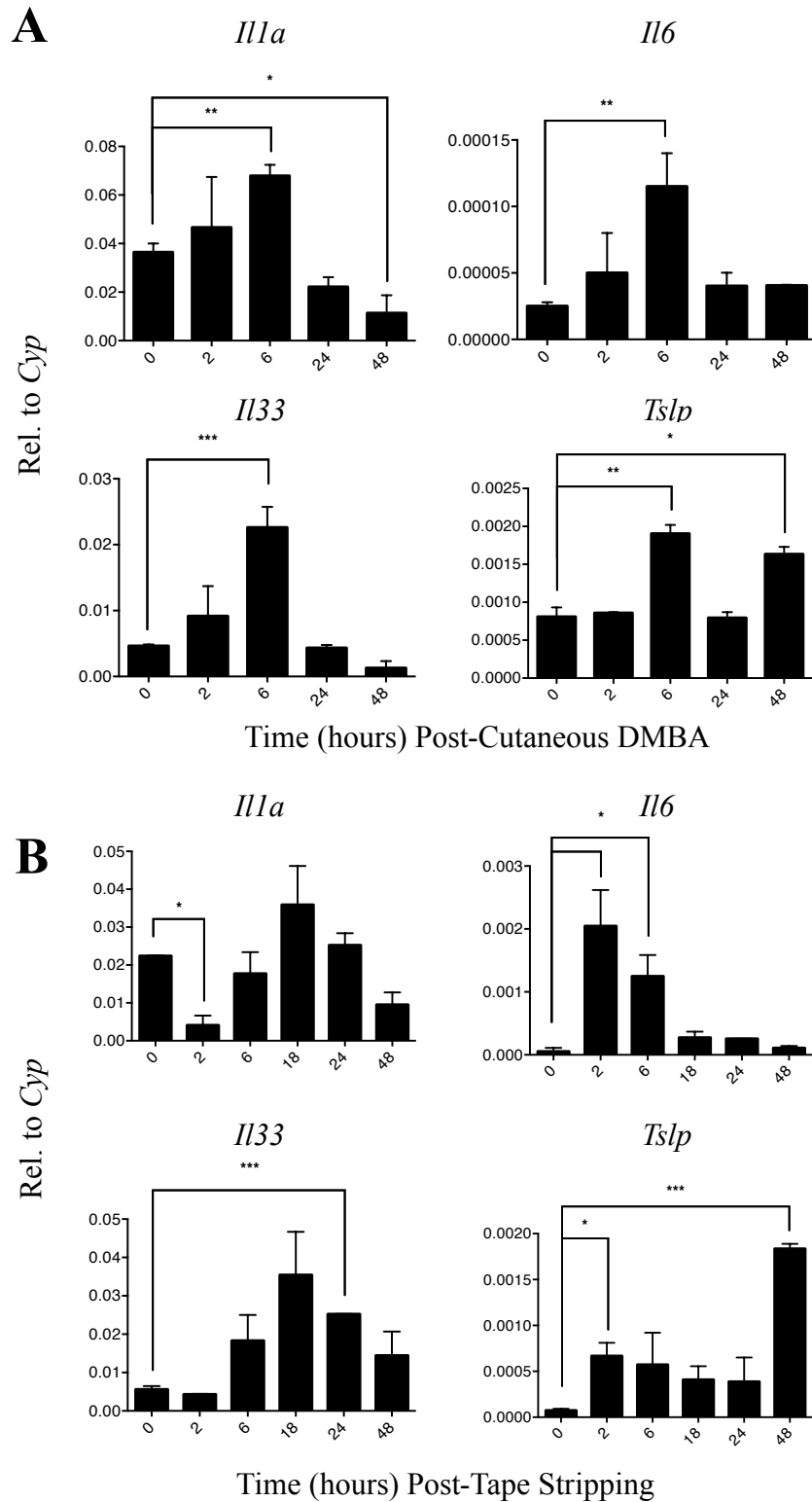


Figure 3.7 | Epidermal cytokines are upregulated by physiochemical skin stress

(A) Ear skin of FVB mice was treated topically with 400 nmol DMBA once or (B) subjected to mild tape stripping. At the indicated time points, epidermis was recovered and processed for qRT-PCR. Data are expressed as mean $\pm$ 1 SEM relative to the control gene cyclophilin (Cyp). (A,B) $n = 3$ mice per time point. Statistics *via* Students T test where here; * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, **** = $P \leq 0.0001$

Activated DETC mainly produce IL13, therefore the role of IL13 for KC responses *in vivo* and *in vitro* was examined. It was hypothesized that one effect of IL13 was to promote the production of cytokines by KC – representing a molecular crosstalk axis in the epidermis.

Tslp and *Il33* gene expression changes were examined in the epidermis at two time points – steady state and 72 hours following DMBA treatment. *Il33* and *Tslp* were upregulated 72 hours following topical DMBA was severely attenuated in IL13 deficient animals (Figure 3.8). As DETC are the only cells in the epidermis to produce IL13, these striking effects on the canonical KC stress response in the absence of IL13 was most likely due to a defect in DETC-KC interaction *via* IL13.

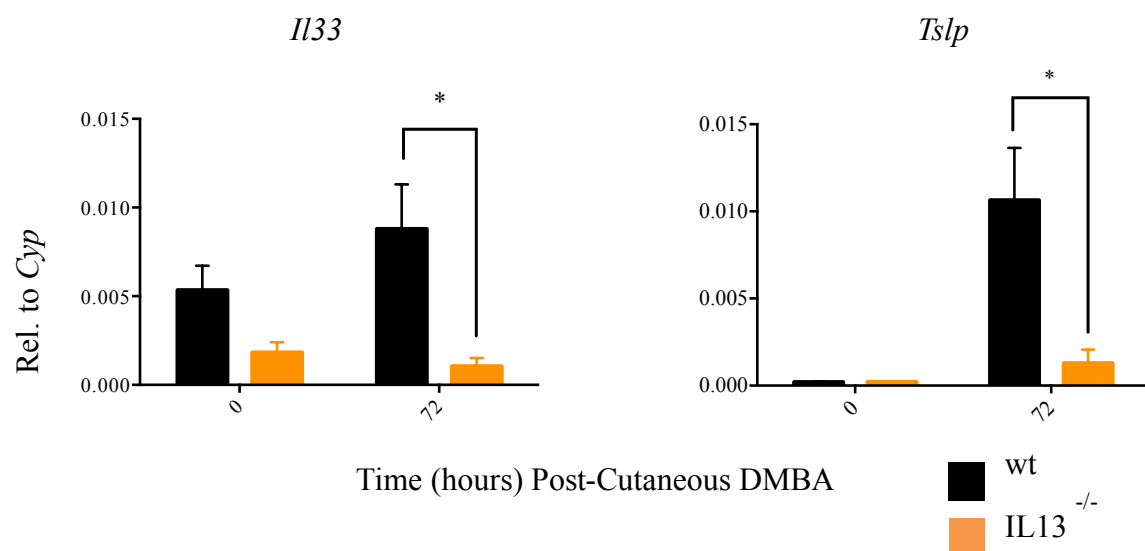


Figure 3.8 | IL13-deficient mice demonstrate a defective *Il33* and *Tslp* upregulation in response to epidermal stress

Ear skin of BALB/c or IL13^{-/-} mice was treated once topically with 400 nmol DMBA and at the indicated time-points epidermis was recovered for qRT-PCR. n = ≥ 6 mice per time point per group, across multiple repeat experiments. At the indicated time-points epidermis was recovered for qRT-PCR. Data are expressed as mean ± 1 SEM relative to the control gene cyclophilin (Cyp). Statistics *via* Students T test where here; * = P ≤ 0.05.

As stated, it is known that other cells in the skin outside of the epidermis can produce IL13, such as ILC2s in the dermis. Therefore, the importance of IL13 derived specifically from the DETC for KC stress responses was examined.

To that end, bone marrow chimeras were generated in which IL13-deficiency could be conferred upon specific compartments, exploiting the principle that the DETC and LC in the epidermis are radio-resistant. ILC are known to be radiosensitive, they also seed the skin from a skin-draining LN population that is bone-marrow derived¹⁰⁵. Four groups of chimeras were created (see Table 3.0 for terms used); WT BALB/c were irradiated and reconstituted with WT bone marrow and were hence WT. IL13eGFP recipients received IL13eGFP bone marrow and were therefore IL13-deficient in all compartments, like a IL13^{-/-} mouse. IL13eGFP recipients received WT bone marrow, and were IL13-sufficient in the hematopoietic compartment and IL13-deficient in the DETC. WT received IL13eGFP bone marrow, and were IL13-deficient in the hematopoietic compartment and IL13-sufficient in the DETC. The host animals were all CD45.1⁺, and the donor animals CD45.1⁻. Therefore, reconstitution of various compartments within the animal could be checked based on this divergent expression.

Cell suspensions from control animals' thymus, lymph nodes and skin were stained for various markers. Thymus and lymph node compartments reconstituted almost entirely with donor-derived cells (Figure 3.9 | A,B). All DETC and LC in the skin remained host-derived as expected (Figure 3.9 | C). There was no significant difference in the proportion of epidermal CD45⁺ cells that were DETC between any of the chimera groups (Figure 3.9 | D).

Table 3.0 | Key to bone marrow chimeras

Label	Host	Donor
WT	WT	WT
HPC IL13 ^{-/-}	WT	IL13 ^{-/-}
IL13 ^{-/-}	IL13 ^{-/-}	IL13 ^{-/-}
DETC IL13 ^{-/-}	IL13 ^{-/-}	WT

WT=wild type, HPC=hematopoietic compartment. IL13^{-/-} is IL13^{-/-eGFP/eGFP}.

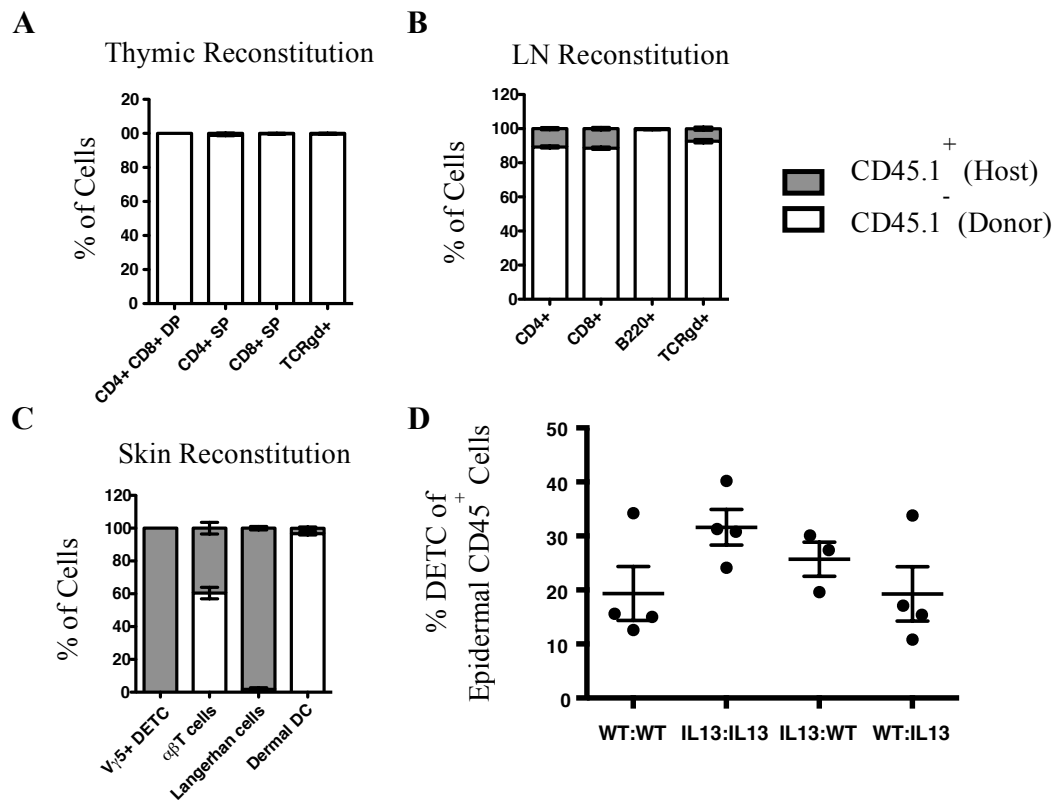


Figure 3.9 | Generating bone marrow chimera animals and checking reconstitution

Exploiting the principle that DETC are radioresistant, bone marrow chimeras were made to generate animals that were WT, IL13-deficient, IL13 deficient in the DETC compartment, and IL13 deficient in the hematopoietic compartment. Adequate reconstitution of respective compartments was checked in control animals on the basis of divergent CD45.1 expression between host (CD45.1⁺) and donors (CD45.1⁻) in the thymus (**A**), skin-draining lymph nodes (**B**) and whole skin (**C**). The proportion of DETC (gating as per Figure 3.2) in the epidermis was examined for all groups (**D**). n = 4 animals per group, per analysis. Each dot represents a different animal. Error bars are ± 1 SEM. Reconstitution data (A,B,C) kindly provided by Dr Crawford.

Epidermal cell suspensions from WT and IL13^{-/-} chimeras were treated comparably to those in Figure 3.4, to examine eGFP expression in DETC following PMA & ionomycin stimulation. As expected, the WT animals did not show GFP signal under any condition (Figure 3.10). In the IL13^{-/-} chimera animal, ~100% of DETC subjected to golgi-block alone were IL13eGFP⁺ when compared to the WT (Figure 3.10 | A, bottom left panel). When stimulated, the proportion of IL13eGFP^{hi} DETC increases from 12.5% to 70% - suggesting a clear effect of stimulation (Figure 3.10 | A, B) – while the eGFP MFI increases by more than 3-fold (Figure 3.10 | C). Only DETC from IL13^{-/-} background animals showed eGFP⁺ DETC (Figure 3.10 | D).

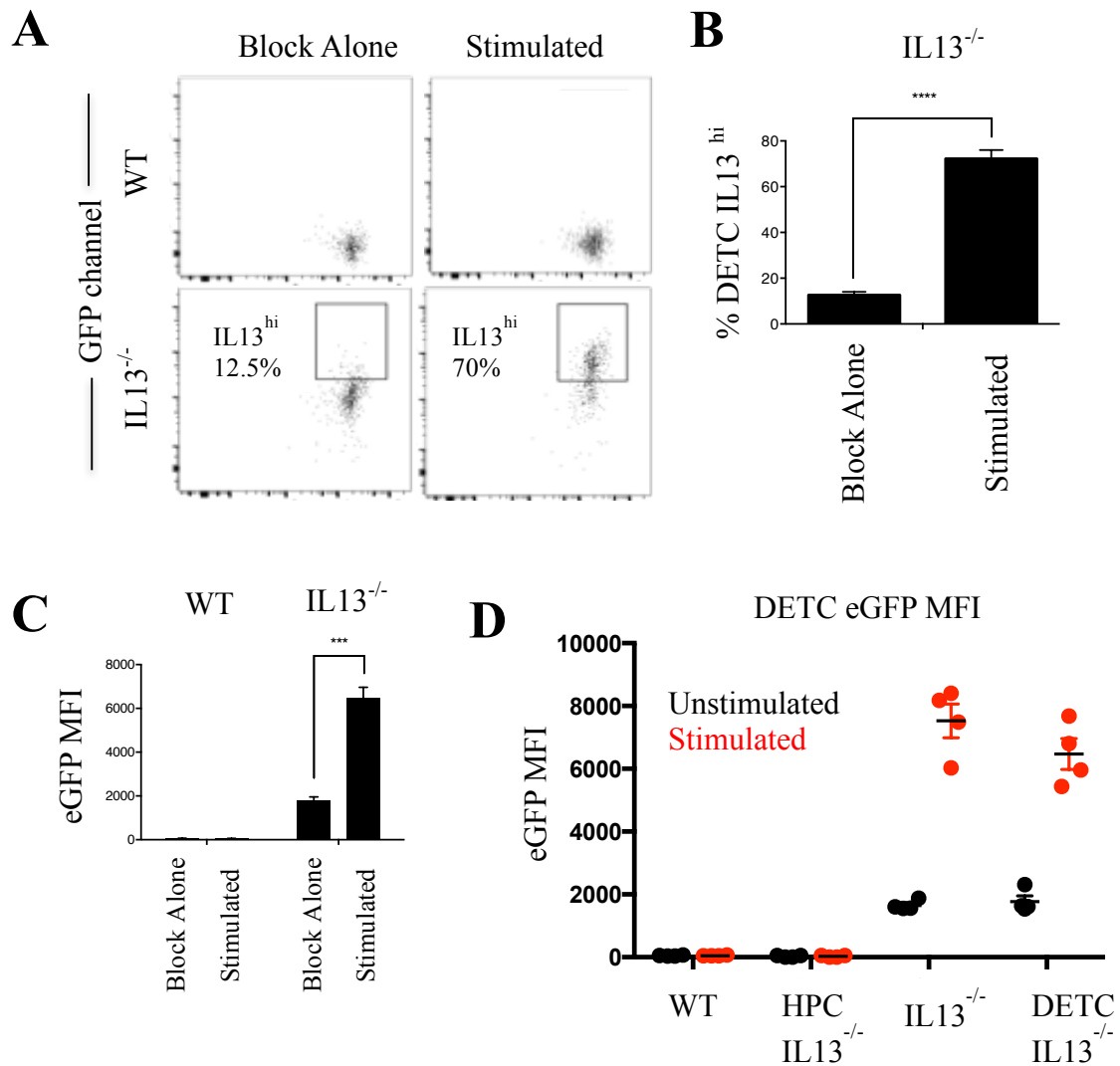


Figure 3.10 | DETC from IL13^{-/-} bone marrow chimera animals produce IL13 at steady state and in response to PMA & ionomycin stimulation

(A, B, C, D) Epidermal cell suspensions from WT chimera or IL13^{-/-} chimera were stimulated *in vitro* with PMA and ionomycin, and brefeldin A for 5 hours. Subsequently they were stained for TCR-V γ 5 and examined flow cytometrically for eGFP. The population shown is DETC. IL13eGFP^{hi} gate was set by eye against the unstimulated (block alone) condition in the reporter animal. (B) The proportion of DETC that were IL13eGFP^{hi}, and (C) MFI of the total IL13eGFP⁺ population. (D) DETC eGFP MFI in all four chimera groups, with and without stimulation. WT animals were WT animals that received WT bone marrow (n=4). IL13^{-/-} animals were IL13eGFP animals that received IL13eGFP bone marrow (n=4). HPC IL13^{-/-} were WT animals that received IL13eGFP bone marrow. DETC IL13^{-/-} were IL13eGFP that received WT bone marrow. Statistics *via* Students T test where here; * = P $\leq$ 0.05, ** = P $\leq$ 0.01, *** = P $\leq$ 0.001, **** = P $\leq$ 0.0001

Therefore, the bone marrow chimera animals reconstituted correctly, and could be used to examine IL13eGFP expression in different compartments. Using these animals, the question was asked whether IL13 from skin DETC populations specifically, was the crucial determinant of a maximal epimune response to stress. To that end WT and chimera animals were treated topically with DMBA and gene expression changes examined in the epidermis at 1 and 3 days post-treatment. The WT and HPC IL13^{-/-} animals showed a peak *Il33* upregulation at 24 hours following DMBA treatment (Figure 3.11 | A), which decreased slightly at 72 hours in both groups. There was no statistically significant difference between these two groups. The greatest *Tslp* increase was seen at 72 hours as in prior experiments, and there was no difference between the WT and HPC IL13^{-/-} groups. The total IL13^{-/-} and the DETC IL13^{-/-} both showed very poor upregulation of both *Il33* and *Tslp* compared to the WT and HPC IL13^{-/-}. This failure to maximally upregulate *Il33* and *Tslp*, compared to WT animals, recapitulates the data seen in non-chimera BALB/c v.s. IL13^{-/-} animals (Figure 3.8). Crucially, these BM chimeras show that DETC-derived IL13 is essential for a canonical KC response to stress.

A

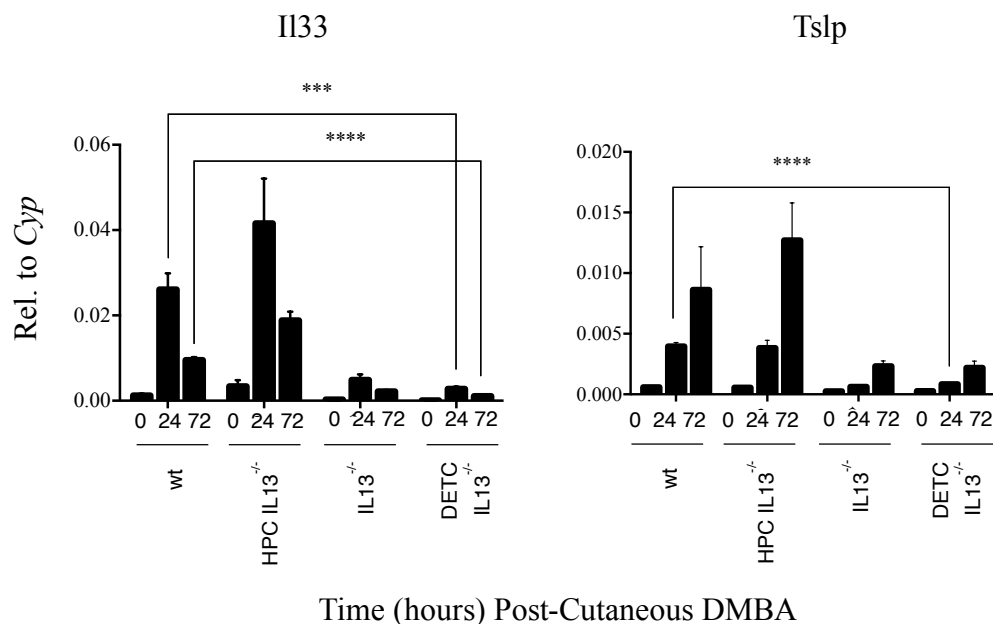
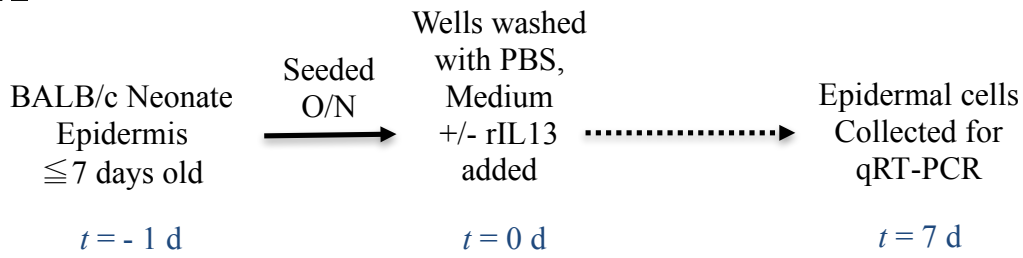
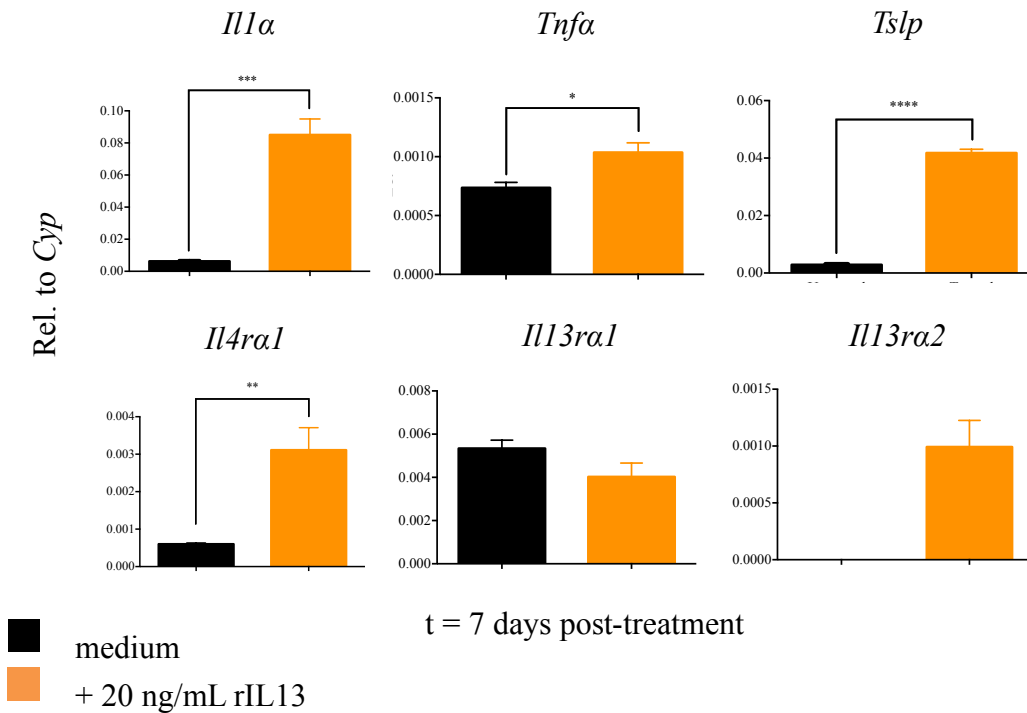


Figure 3.11 | DETC-derived IL13 is required for a maximal, canonical response to epidermal insult

(A) Bone marrow chimera mice were treated once topically on ear skin with DMBA and at the indicated time points epidermis was recovered for qRT-PCR. n = 4 mice per time point. Data are expressed as mean ± 1 SEM relative to the control gene cyclophilin (Cyp). See

Table 3.0 for a guide to the bone marrow *v.s.* peripheral IL13 status in these animals. Statistics *via* Students T test where here; * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, **** = $P \leq 0.0001$

Finally, to further examine whether IL13 can activate KC directly, primary KC were grown *in vitro* and their response to recombinant (r) IL13 cytokine examined. KC were grown from neonatal mice and 20 ng/ml rIL13 added following 1 day in culture. The addition of IL13 was shown to significantly activate KC and transcripts of *IL1a*, *Tnfa*, *Tslp* and *Il4Ra1* were all upregulated with rIL13 added to culture (Figure 3.12 | B) *Il13ra1* expression was not affected by rIL13, supporting the *in vivo* result showing that the upregulation of *Il13ra1* following stress was independent of IL13 (Figure 3.6 | B), following DMBA. In contrast, the IL13 decoy receptor subunit *Il13ra2* was strongly induced on *in vitro* grown KC by addition of rIL13.

A**B****Figure 3.12 | IL13 activates KC grown *in vitro***

(A) Schematic illustrating the timeline for neonatal KC cultures. Epidermal cell suspensions from BALB/c pups less than 7 days old were seeded into collagen-coated 12 well plates overnight. The following day, the medium was removed, the wells washed with PBS, and fresh medium with or without rIL13 (20 ng/mL) added. 7 days later the cells were processed for qRT-PCR. (B). Untreated cells shown in black bars, treated cells shown in orange bars. At least $n=6$ pups per group. The experiment was repeated twice with comparable results. For *Il13ra2* no bar is shown for untreated cells as no signal was detected without rIL13. Data are expressed as mean $\pm$ 1 SEM relative to the control gene cyclophilin (Cyp). Statistics via Students T test where here; * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, **** = $P \leq 0.0001$

Discussion

DETC produce IL13 at steady state and under conditions of epithelial stress

I began by examining how DETC are responsive to perturbation of homeostasis in the epidermis. My initial stains aimed to detect the DETC population *in situ*. My staining demonstrated that they are dendritic at steady state, and that this morphology is responsive to at least two different models of tissue injury (Figure 3.2 | A, B). Staining of the cell body seems to suggest there are TCR distributed over the membrane and not just at the apical dendrite tip. Despite this, a particular clustering of TCR and signaling apparatus indicative of TCR binding by agonist ligand (such as CD3 zeta chains, phosphorylated SFK and ZAP70) at the dendrite tip PALS regions has been described in the literature⁹⁸. The DETC rounded up in response to tape stripping or full thickness wounding. One question is whether we are truly looking at rounding of the cell under stress conditions (Figure 3.2 | B), or whether the TCR being bound by the antibody re-localizes, under stress, to the cell body. Work by others using different markers such as CD3 or IL2-p8 GFP, confirms actual rounding – while the antibody binding to the TCR itself is not a signal that induces rounding in long time-course follow up experiments. The use of the IL2-p8 GFP model is interesting, given that IL2 is generally thought to be present in activated T cells. The steady state ligand-binding hypothesis suggests that the DETC maintains dendritic outreach and morphology at steady state in part by binding a physiological ligand on the KC. This ligand is labile under conditions of tissue stress – and loss of this binding facilitates rounding. Rounding in response to tissue stress is certainly supported by this data. The literature reports that $V\gamma 5^{-/-}$ mice, which have a replacement $\gamma\delta$ T cell population with non-canonical TCR, demonstrate greater spread of rounded IEL, following skin trauma, than in WT animals – and that inhibition of LCK *in vitro* (mimicking a loss of TCR agonist stimulation) induces rounding⁹⁸, suggesting the TCR:ligand interaction acts to stabilize dendrite attachment. Curiously, preferential retraction of basolateral dendrites prior to apical dendrites in wounding has been observed by others, suggesting a spatially distinct localization of the $V\gamma 5$ ligand and/or its stability following wounding. Clearly, the dendritic morphology cannot be maintained entirely by binding to a $V\gamma 5$ ligand, as the replacement $\gamma\delta$ T cell population in $V\gamma 5^{-/-}$ animals are not entirely rounded. Additionally, rounding could be

induced by a ligand only expressed during stress – such as the NKG2D ligand Rae1. As previously stated, in a Rae1-inducible transgenic mouse, expression of Rae1 is enough to elicit DETC rounding⁸⁸, whereas DETC rounding *in vitro* has been shown to be partially dependent on NKG2D¹⁰⁶. Rae1 protein is known to be expressed following tape stripping⁹⁰ – and hence could be a rounding stimulus to the DETC in my injury models (Figure 3.2). Additionally, the interaction of DETC CD100 with Plexin B2 on KC has also been shown to be crucial for DETC rounding and wound repair¹⁰¹, while significantly fewer dendrites have been observed in mice lacking the integrin CD103, which maintains putative contacts with epithelial E cadherin. Given the role of cadherins in maintaining cell-cell adhesion, an intriguing possibility arises that the physical state of the epidermis is communicated to the DETC by surveillance of intercellular junctions. This idea is particularly well supported by the location of DETC apical dendrites at KC intercellular junctions – along with the radical changes in expression of intercellular junction proteins that occur in response to wounding (Chapter 4). E-Cadherin is also ligated by the KLRG1 receptor expressed on LCs and ILC2s in the skin – an interaction which is able to suppress proliferation and cytokine secretion²⁴. Therefore it seems likely that my observed DETC rounding in response to physical injury (tape stripping and full thickness wounding) represents a conserved response to a disruption of the normal physical arrangement of the epidermis.

The elicitors of the rounding process itself are quite varied, and the magnitude of tissue stress is known to differentially modulate rounding/dendrite retraction. High dose cutaneous TPA rapidly promotes total DETC rounding, whereas low dose TPA induced rounding followed by transient formation of new, more motile dendrites – a behavior the authors termed ‘probing’⁹⁸. An important question is how the rounding process actually affects the DETC – does it potentiate DETC production of soluble factors, for instance? Or does dendrite detachment from the apical KC sensitize the DETC to further signals located elsewhere in the epidermis? The ‘probing’ behavior demonstrated after basolateral dendrite retraction suggests that dendrite function is more complex than simply binding to a TCR ligand. Indeed, DETC possess a number of cytolytic granules¹⁰⁷ concentrated at the PALPs regions, suggesting that in addition to an afferent sensing role they may be used to deliver cytolytic payloads and kill dysregulated cells. Finally, one could ask: is there a reciprocal effect on the KC from the binding/detachment of DETC dendrites? DETC CD103, which is considerably

enriched at DETC PALP regions, and its KC ligand E-Cadherin, represent an interaction, which is relevant to DETC morphology. Cadherin-cadherin interactions are crucial for connecting the cytoskeletons of cells together – and for distributing the stresses encountered at the epidermis¹⁰⁸. There is a described role of E-Cadherin modulating gene expression *via* PI3K/AKT signaling pathways in cancer and in urothelial cells¹⁰⁹ – therefore the ligation of epithelial cadherins by DETC CD103 at intercellular junctions may have a direct effect on the KC *via* outside-in signaling. This however is yet to be explored and remains speculative. Given that the DETC are sensitive to ligands at intercellular junctions, disruption or downregulation of these proteins may represent an interesting mechanism to sensitize DETC to dysregulated KC, hence amplifying the lymphoid stress surveillance response.

Dr J Strid had previously observed a rapid upregulation of *Il13* expression within 2 hours following tape stripping, a response that was conserved across various heterogeneous tissue insults (Figure 3.2 | C, D). The rapidity of this response is interesting, as it suggests that DETC receive a signal conducive to a dysregulated state of epidermis within 2 hours of it being disrupted. This signal could either come from the KC itself, or be related to the tissues physical integrity as suggested, or in some cases it may be a direct effect on the DETC. The ‘steady state binding hypothesis’ suggests that the DETC TCR ligates a KC ligand at steady state, and this ligand is labile under conditions of stress. Loss of DETC TCR-ligand binding may therefore be an activation signal (akin to a NK cell activation modality) – along with concurrent ligation of endogenous stress ligands (such as Rael) by the immunoactivating receptor NKG2D. It is known that expression of the most plausible DETC TCR ligand, Skint-1, is highly stress labile and is quickly downregulated upon tissue stress – opposite to the regulation of NKG2D-ligands (Hayday lab, personal communication). Another plausible acute ‘signal’ is IL1 α , the protein is released within preformed pools in KC within 10 minutes of stress¹¹⁰ - at least one paper demonstrates that DETC can be activated by IL1 – albeit IL1 β – release from KC in a contact hypersensitivity model¹¹¹. Following tape stripping, KC-derived *Il6* and *Tslp* were shown to be quickly upregulated at 2 hours (Figure 3.9 | B) and may also represent DETC-stimulating signals. The NKG2D-ligand Rael is known to induce DETC rounding and IL13 expression⁸⁸ – but appreciable protein expression of Rael

does not occur until hours after tape stripping (group observations and others) – and hence in the first instance is probably not responsible for inducing the first wave of DETC-IL13. For some insults to the epidermis, a contribution of local commensal organisms on the skin in activating DETC cannot be excluded. For example, at least two of the insults tested presumably aid epidermal penetrance by physically removing the SC (tape stripping) or dissolving it (DMBA vehicle acetone). Given the large and diverse family of commensal organisms on the skin, it isn't unreasonable to suppose that these insults aid penetrance of commensals – which are known to potently modulate immune response – including in the skin¹¹². The direct mechanism of how DETC get activated *in situ* was not further pursued in my work.

To detect IL13 protein I used the PMA/ionomycin *ex vivo* stimulation model. The usual drawbacks of using the PMA/ionomycin method to examine what cytokines (T) cells produce are valid. The DETC are in an artificial environment, stimulated with a very potent mitogen, presumably no longer maintaining 'normal' contacts with KC, which would modulate their function as discussed. The golgi-transport/protein export inhibitors themselves are toxic, and they along with PMA/ionomycin may sculpt DETC behaviour in a manner dissimilar to *in vivo*. It may be that low vs. high concentration of PMA/Ionomycin does stimulate DETC differently – particularly given the previously mentioned literature demonstrating *in situ* DETC morphology differences to topical low vs. high dose TPA. With these criticisms in mind, I demonstrated that DETC are capable of producing high amounts of IL13 protein. In addition, using the IL13eGFP reporter mouse, eGFP protein (under the control of the IL13 promoter) was demonstrated at steady state in freshly isolated DETC (Figure 3.5) and this was increased further following skin perturbation. This data shows that DETC produce IL13 at steady state, but can increase this production rapidly in response to perturbation of tissue homeostasis.

I did not detect IFN γ in this model, nor a convincing IFN γ upregulation following stress in many experiments throughout my PhD – suggesting the IL13 response *in situ* in the epidermis predominates. Interestingly, however, IFN γ mRNA can be detected when DETC are stimulated *in vitro* with IL12 and alarmin IL33 (Dr O Sobolev, private communication). IL12 potentiates Type 1 responses to combat viral infection at barrier surfaces, therefore the milieu of signals the DETC receives may be

integrated to induce an appropriate cytokine (IFN γ to combat viruses, IL13 to tissue injury). This, however, is speculative.

How are epidermis-resident $\gamma\delta$ T cells biased to IL13 production? As described previously, during ontogeny the ‘pre-programing’ imparts them with a particular transcriptional program and TCR sensitivity suitable for their barrier localization. The epidermis is seeded preferentially with TCR-V γ 5⁺ T cells, which already in the thymus are capable of IL13-production (Hayday lab, personal communications). Additionally, DETC may be conditioned *in situ* by molecules that preferentially promote IL13 secretion, perhaps soluble molecules secreted by the KC and/or as a direct consequence of stress surveillance.

KC are immunologically active and express receptors for DETC cytokines

In agreement with the literature I detected clear upregulation of genes including *Il1a*, *Il6*, *Il33* and *Tslp* in the epidermis in response to two different perturbation models; DMBA and tape stripping (Figure 3.7). A common finding to all cytokines present in the epidermis following tissue stress, is that they all seem to have varied and pleiotropic effects on epithelial biology. There are often contradictory findings reported in the literature, which may also be due to differing experimental models. The epidermis is a highly complex, and heterogeneous tissue, and often something as simple as the presence or absence of calcium in culture models (for instance), can fundamentally alter the differentiation state of the cells and their response to challenge. Additionally, mono-layer cultures do not allow for exacting differentiation of KC and most granular/barrier proteins are not recapitulated faithfully *in vitro*. However, examining gene regulation differences *in situ* directly in response to skin challenge evades some of these issues.

Il1 α was clearly upregulated in response to both stressors (Figure 3.9). IL1 α and IL1 β are described as having profound effects on regulating epidermal repair to injury. IL1 α promotes lipid synthesis, and has pleiotropic effects regulating KC differentiation and the mechanical attachment of cells. IL1 β has been described to regulate intercellular junctions proteins, such as ZO-1¹¹³. Therefore it seems likely IL1 may modulate reformation of the impermeable SC removed with sticky tape –

and lipid components which may have been dissolved in vehicular acetone. *Il1α* was curiously downregulated at 2 hours following tape stripping. This may represent a true downregulation, or perhaps the superior layers of epidermis, which were removed with sticky tape, were particularly enriched for its transcripts. *Il6* peaked very rapidly following tape stripping, at 2 hours, and gradually decreased back to baseline at 48 hours, in line with morphological tissue repair. It peaked later at 6 hours following DMBA treatment, suggesting its upregulation is due directly to physiochemical damage of the epidermis. IL6 and its receptor are known to be increased in all nucleated epidermal cells following barrier disruption – and the *IL6^{-/-}* mice exhibit poorer repair following tape stripping¹¹⁴.

KC, in addition to modulating their cytokine expression during tissue stress, also upregulate expression of IL4 and IL13 receptors – thus further sensitizing them to fluctuations of IL13 (Figure 3.8). Interestingly, all basal KC are positive for IL13ra1 already in the resting tissue but this is increased upon perturbation of barrier homeostasis following tape stripping or DMBA exposure (Figure 3.6). A simple interpretation of these data would be that the KC upregulate the IL4/13 receptors, in order to sensitize themselves to receiving (DETC-derived) cytokine following injury. Expression of these receptors by KC is also described in the literature, and by cells at body barrier surfaces more generally¹¹⁵. IL13Rα1 was only expressed by KC in the epidermis, whilst LC and DETC were negative. As will be described in Chapter 4, IL13 has been described to have a role in modulating aspects of epithelial biology in practically every layer of the epidermis – from keratin expression in the basal layers, processing of Filaggrin filaments, to the final shedding of cells in desquamation¹¹⁶. Indeed, there seemed to be a positive relationship between basal KC marker K5 expression, and IL13Ra1 expression, when examined flow cytometrically (Figure 3.6). To conclude these data, KC clearly express the receptors to respond to DETC-derived IL13, and stress-modulation of these receptors suggests a key role of this axis in responding to perturbation of barrier homeostasis

IL13 is key to DETC-KC Crosstalk in the Epidermis

Next, I tested the idea that DETC-derived IL13 modulates KC responses. Upregulation of *Il33* and *Tslp* is a conserved response following tissue perturbation

and I consider this to be a canonical KC response to stress. When examining this response in IL13-deficient mice, I found that IL13 was crucial for KC to maximally upregulate *Il33* and *Tslp* following tissue injury. Following this, I used BM chimera mice to show that IL13 derived specifically from the DETC is key to inducing this canonical KC stress-response. Therefore suggesting that the spatio-temporal expression of IL13 in the skin is of importance. Indeed, the IL13-deficient mice also demonstrate structural changes in the skin (Chapter 4) such as thinner ears that also appear more shiny after tape stripping insult. Likewise, the ratio of basal:granular cells in IL13-deficient epidermis is altered. Such a change in the composition of the epidermis may also affect the *Il33/Tslp* deficiency seen here, if these cytokines are expressed by a particular layer of epidermal cells which is deficient in the IL13-deficient mouse. These structural changes and others are examined in more detail in Chapter 4.

The chimera data (Figure 3.11) suggests IL13 production by a bone marrow derived population does not play a role in modulating acute epidermal cytokine production to stress. This seems logical as we also do not see any infiltration of the epidermis at these early (1 to 3 day) time points following stress (either tape stripping or DMBA injury). Given that we know the DETC is the only epidermal population capable of producing IL13, it seems logical to suppose that specifically DETC-derived IL13 is crucial for maximal KC cytokine response. The BM chimera supports this, as when DETC are lacking IL13, but ILCs in the dermis are capable of producing IL13, the KC *Il33* and *Tslp* production is still deficient. The ILC2 population exists in steady state in the dermis, and has been described to infiltrate the skin in a IL4-dependent manner following tissue damage. Recent work has also demonstrated that at least human ILC2s acquire skin-homing properties in skin-draining LNs (such as CCR10 expression) and enter into the skin under both homeostatic and stress conditions¹⁰⁵. Interestingly, the maintenance of this population in human skin was dependent on crosstalk between CCR10⁺ ILCs and skin T cells. Crucially - these ILCs were bone-marrow derived. With regards to my model, even if a bone-marrow derived population of ILC2s infiltrates into the skin, the IL13 it putatively produces is clearly not enough to rescue the phenotype in the skin IL13^{-/-} mouse – in which bone-marrow derived ILCs are IL13-sufficient but the DETC are not. This underlines the

requirement of epidermal (DETC-derived) IL13. With regards to the dermis-resident $\gamma\delta$ T cells, in the mouse they are particularly biased to IL17 production, rather than IL13, suggesting they are not a confounding cell population.

The reduced *Tslp* upregulation following DMBA treatment in the skin IL13^{-/-} mice is particularly interesting given IL13 and TSLP interactions have been described at several barrier sites previously including the skin, where IL13 induces TSLP-driven skin fibrosis¹¹⁷. Additionally, a bronchiolar epithelial cell TSLP-IL13 feedback loop has been described as important for lung injury repair¹¹⁸. In Figure 3.7, *Tslp* expression following either tape stripping or DMBA was found to be bimodal, with a small upregulation quickly following insult (2h tape stripping, 6h DMBA), followed by a larger upregulation at 1-2 days. I speculate KC initially produce a quantity of TSLP in response to stress, which is then amplified by a wave of IL13 produced by the DETC. As an alarmin, IL33 seems to be regulated primarily by release of its protein from dead/dying cells, it may be premature to impart a functional significance of its regulation at the mRNA level. As described in the general Introduction, protective as well as immunopathological roles of EC-derived IL33 have been described in the skin. Administration of exogenous IL33 has been shown to improve outcomes in models of allograft rejection, skin injury and wound healing¹¹⁹. Therefore, one could speculate that IL13-amplified IL33 and TSLP production plays a protective role to skin injury. Reports often suggest a particularly rapid and acute role for IL33 in cutaneous homeostasis whereas constitutive, late, or dysregulated expression may be involved in a variety of chronic inflammatory conditions. It is plausible that this is common to type 2 cytokines in the skin in general. This temporally coordinated aspect fits well with current thinking of IL33 as an “alarmin,” whereby its immediate release from intranuclear stores by damaged, apoptotic, or necrotic cells rather than a classic Golgi-mediated secretion pathway facilitates a rapid and restorative response to tissue damage¹²⁰.

To demonstrate directly that IL13 has a role in promoting KC cytokine expression, I treated primary KC *in vitro* with recombinant IL13, and examined gene expression changes by qRT-PCR (Figure 3.11). KC from adult mice are notoriously difficult to grow in culture, as are curiously those from certain background strains such as FVB

and C56BL/6. I therefore chose to use BALB/c neonatal primary KC for the majority of my work. However, adult KC was used to confirm results and their responses did not differ from the neonatal KC. The timing of KC seeding and rIL13 exposure and dose are all important variables. In preliminary experiments, multiple time points were examined prior to settling on the 7 day data (Figure 3.12). I am therefore confident there is a clear and consistent effect of rIL13 in culture on KC. rIL13 induces cytokine upregulation in KC *in vitro*, of which *Tslp* is consistently and strongly upregulated. Despite the significance of IL33 regulation *in vivo*, it was not examined *in vitro* due to IL33 expression and its protein are known to be highly disordered in cultures (private communication).

I approached these experiments wary of concluding too much from KC grown and treated in an artificial setting. The complex, three-dimensional environment in which KC normally reside is poorly replicated *in vitro* – where KC adhere to collagen in a monolayer. *In vitro* grown monolayers of KC therefore do not represent the phenotypic and morphological mixture of cells found *in vivo* and importantly the cells do not mature and differentiate as *in situ*. Interestingly, I did observe with the light microscope that with increasing age, individual cells seemed to become more granular and less cuboid. This occurred particularly with those cells that were contact-inhibited and surrounded by a large lawn of neighboring KC. I grew the KC in serum and calcium-free medium, allowing the cells to proliferate and form a cobblestone lawn following seeding. Despite the reductionist nature of these KC cultures, I believe there is power in testing the direct effect of rIL13 on regulating gene expression in KC divorced from all other competing stimuli that occur *in vivo*. Importantly, the *in vitro* data recapitulated the IL13-dependent gene expression changes I observed *in vivo*.

Conclusions

I conclude this chapter by suggesting a model of a hitherto unappreciated molecular axis of communication between DETC and KC *via* IL13 in the epidermis (Figure 3.13). Based upon my main findings, I conclude that:

1. Upon topical skin perturbation, KC produce cytokines, which are known to promote barrier repair – including the canonical epimune cytokines IL33 and TSLP.
2. DETC produce IL13 at steady state, and increase production following a variety of topical skin perturbations.
3. KC express all the IL13Rs and are responsive to IL13
4. Lack of IL13 *in vivo* hinders a canonical KC stress response and specifically DETC-derived IL13 is crucial for KC responses to stress

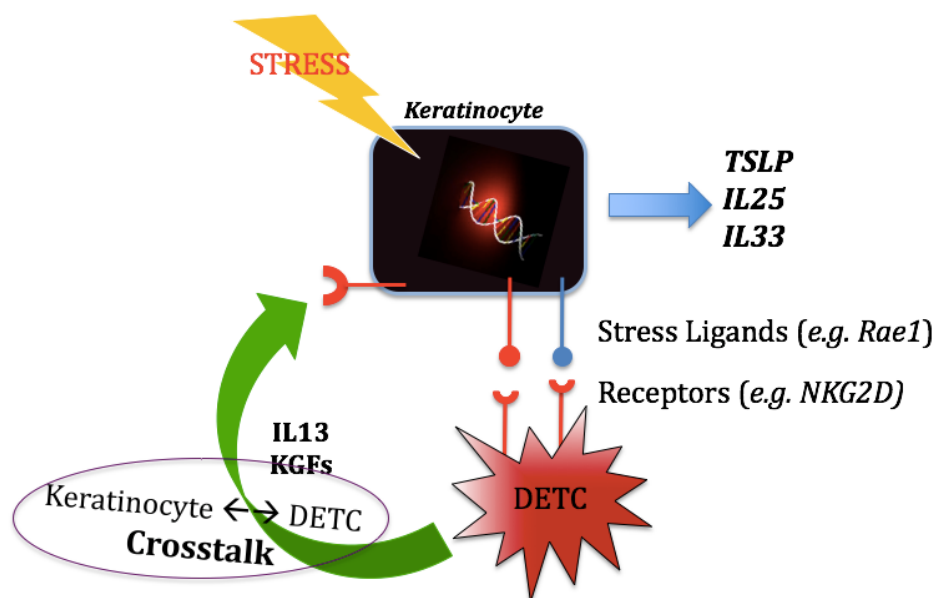


Figure 3.13 | Model of DETC and KC crosstalk

Under conditions of stress, KC respond by upregulating canonical type 2 cytokines including IL25, IL33 and TSLP, along with self-encoded stress antigens such as Rae1. The DETC detects perturbation of homeostasis at least in part through the immunoactivating receptor NKG2D ligating Rae1, and responds by rapidly producing IL13 and other soluble factors such as keratinocyte growth factors (KGF). IL13 feeds back on the KC, which expresses type 1 and 2 IL13 receptors, to amplify epimune expression – particularly cytokines TSLP and IL33.

The relevance of this molecular crosstalk between the KC and DETC is examined in Chapter 4 in physiological models of tissue injury and modulation of epidermal homeostasis.

Chapter 4 | DETC and Epidermal Homeostasis

Introduction

Cytokine Modulation of the Epithelial Barrier

EC express an armory of soluble and cell-surface molecules to direct an appropriate, restorative response – including stress ligands like Rae1 and various cytokines such as IL33 and TSLP (Chapter 3) – regulation of which is in part driven by the neighbouring DETC population. These responses in health sculpt and modulate tissue homeostasis and local tissue immunity, in a manner that aids morphological tissue homeostasis, restoration of the epithelial barrier following injury, and elimination or expulsion of microbial and non-microbial insults. KC cytokine production in response to barrier perturbation has been described since the 1980's, along with speculation on the roles they may play in restoring the barrier¹²¹. More controversial is the question of how individual cytokines affect epithelial biology, particularly given experiments of this nature are usually overtly reductionist. *In situ*, cytokines form part of a complex, spatiotemporally coordinated network often not faithfully reproduced in culture experiments, there are also likely to be summative effects of the large numbers of cytokines induced during injury¹¹⁶. Additionally, the heterogeneous structure of the epidermis likely permits cytokines to induce different effects in different layers, probably in part through differential expression of cytokine receptors on basal and granular cells as shown in Chapter 3. Indeed, key regulators of epidermal homeostasis such as p63, are shown to have opposing effects in regulating basal proliferation vs. suprabasal differentiation in late transiting cells¹²². The prominence of cytokine expression during tissue perturbation, along with the abundance of data indicating a clear role in regulating barrier homeostasis, make cytokine regulation an important area of epidermal biology albeit one that must be interpreted with great care.

Perhaps the best place to start looking at the effect of cytokines on the epidermis is the proliferating basal cells. Intradermal injection of IL21 induces epidermal proliferation and hyperplasia, and blockade of this cytokine in a psoriasis model reduces proliferation in an IFN- γ dependent fashion¹²³. IL23, a member of the IL12

family, is upregulated in psoriatic lesions, and induces stratum spinulosum hyperplasia and altered differentiation¹²⁴. *In vitro*, however, IL23 demonstrates little effect on proliferation, suggesting either a paucity of target cells in culture (the question of whether KC actually stick down and proliferate in culture is a perennial technical concern) or the mechanism *in vivo* relies on a third party cytokine or cell (the authors suggest TNF from macrophages are responsible). Likewise, transgenic expression of human IL6 in rat KC *in vivo* promoted proliferation and lymphocyte infiltration¹²⁵, and IL10-family members IL20 and IL22 promote proliferation with differential effects on differentiation (spreading of differentiation markers to the suprabasal layers, and decreased expression of them respectively)¹²⁶. IL24 demonstrates an interesting divergence in functions in normal skin compared to inflamed skin – highlighting one of the concerns in interpretation raised earlier. In normal skin, proliferation is promoted at the expense of differentiation (keratin 10 marker is decreased, while keratin 16, a hyperproliferation (and psoriasis) marker is upregulated). However, in inflamed skin IL24 synergizes with IL20 to reduce proliferation, cell migration and potentially SC biogenesis (filaggrin expression is down)¹²⁷. IL1 has a particularly large literature concerning this topic – and interestingly was one of the most strongly induced by rIL13 *in vitro* in my hands (Chapter 3). Intracutaneous injection of IL1 α has been shown to improve barrier function at steady state and following perturbation, in part by upregulating genes responsible for cell adhesion, proliferation and differentiation¹²⁸.

Effects of IL13 on KC

There is a considerable body of literature outlining effects of IL13 on individual aspects of epithelial biology (such as proliferation, differentiation, structural components). These effects are usually interpreted to be pejorative to epithelial health. Transgenic expression of IL13 (or IL4) in the epidermis is usually sufficient to drive spontaneous dermatitis pathology in the mouse along with pleiotropic changes to skin immunocytes and skin remodeling^{129,130}. These studies clearly indicate the potential these type 2 cytokines have to modulate epithelial homeostasis and biology. However, forced overexpression studies do not represent a physiological scenario and the results must be interpreted with some caution. AD lesions are typically associated with infiltration of IL4/IL13-expressing cells. However it may be that dysregulated expression or overproduction of these cytokines, in the wrong place at the wrong time

is immunopathological - rather than the cytokines themselves. Outside of the skin, a marked homeostatic defect in a lung injury model was shown in IL13R α 1^{-/-} mice, suggesting a positive role of IL13 signaling on lung epithelium. This was driven in part by poorer expression of epithelial cell-associated genes, particularly those regulating structural repair activities such as enzymes and collagens¹³¹. Additionally, the protective effect of IL13 on physical barrier immunity and restriction of parasites, for example, is well described.

A role for IL13 in modulating proliferation in lymphoid cells has been known for a long time¹³², a direct role in myeloid, epithelial and stromal cells remains less clear. Many epithelial cancers for instance, continue to express receptors for type 2 mediators such as IL4 and IL13, allowing for a direct effect on tumor growth, death, and proliferation that is independent of their effect on immunocytes. This direct effect on KC is discussed in line with my tumour work in Chapter 5. The literature describes IL13 (and IL4) as capable of suppressing gene expression in the epidermis, particularly genes important in the differentiation process and construction of the SC and CE. Filaggrin, loricrin and involucrin are all described to be downregulated by IL13, along with the enzyme responsible for processing filaggrin monomers, caspase 14¹³³. The intercellular junctions which are responsible for maintaining an impermeable barrier (tight junctions and desmosomes) are also negatively affected by type 2 cytokines¹³⁴ – although perhaps in a manner not dependent on mRNA regulation, but re-mobilization of the junction proteins. The prevailing paradigm in atopic skin disease is therefore driven by observations that type 2 cytokines cause pejorative effects on KC, particularly downregulation of EDC genes, along with the association of type 2 cytokines and skin lesions. However, many of these studies are performed on KC monolayers grown *in vitro* cultures for 2 days, in which EDC genes are dysregulated, while little work is performed *in vivo* in a physiological model system. It seems counter-intuitive to suppose that an (IL13-driven) reduction in intercellular junctions, and hence increase in barrier permeability (and potential penetration of organisms, along with loss of solutes) could be a good thing. It has been proposed however, that in some cases it allows for better penetration of important mediators such as AMP, and increases regeneration of damaged tissue by promoting turnover, or slewing off of damaged cells¹¹⁶. A mechanism of increasing

turnover of cells by shedding seems like it would only be beneficial at barrier surfaces where such cells will be quickly lost to the environment – indeed IL13 is conspicuously prevalent at body surfaces. In a gut *T. tichuria* parasite model, IL13 was found to mediate its protective effects by increasing turnover rate of EC (an ‘epithelial elevator’), hence slewing off the parasite to be excreted¹³⁵. The increased-AMP-penetration mechanism is not easily supported, however, by data suggesting IL13 also downregulates defensins and antimicrobial factors generally, such as the S100 family.

Despite these findings with AMP, cytokines thought to be barrier disrupting have been shown paradoxically to promote wound healing. IL4 antisense oligonucleotides (which downregulate gene expression by hybridizing to mRNA) decrease healing in a full-thickness mouse skin wound model¹³⁶. Although this was thought to be due to effects in the dermis rather than the epidermis, notably recruitment of inflammatory cells and modulation of extracellular matrix components – such as fibroblasts and collagen deposition. Conversely, group 1 or 2 interferons when given by various routes (topical, intradermal or systemic administration) have been shown in multiple papers to result in poorer skin wound healing – despite positive effects described in the literature such as promoting ceramide synthesis and hence the lipid envelope integrity and decreasing TEWL.

Hypothesis

Many cytokines are able to powerfully modulate various aspects of epithelial biology and hence barrier homeostasis. The DETC, resident among the basal KC, surveys the epidermis for signs of epithelial stress or dysregulation. As shown in Chapter 3, IL13 is a key cytokine produced by DETC. Given the published protective role of DETC in several scenarios of epithelial dysregulation;

I hypothesise that:

1. IL13 plays a host-protective role in epidermal homeostasis at steady state
2. IL13 produced by the DETC under conditions of stress exerts a protective effect on maintaining epidermal health - directly linking DETC-stress surveillance to tissue health

Aims

1. To examine the role of IL13 for physical skin barrier integrity at steady state and following injury
2. To investigate the effect of IL13 on KC maturation and movement in the epidermis

Results

To examine integrity of the epidermal barrier, TEWL was measured using a tewameter probe directly on the skin surface. TEWL is commonly used as a measure of barrier integrity in human and works well on mouse skin too. This tool was used to study barrier function in mutant mice lacking canonical DETC (TCR $\delta^{-/-}$) or IL13 (IL13 $^{-/-}$) and compared these to WT mice. In resting steady state skin, TCR $\delta^{-/-}$ mice had significantly elevated TEWL compared to WT controls (Figure 4.1 | A). Similarly, IL13 $^{-/-}$ also had significantly higher steady state TEWL compared to their WT counterparts (Figure 4.1 | B). Hence, without any perturbation, canonical DETC and IL13 play a role in supporting normal barrier function.

To further examine the ‘health status’ of the KC in the epidermis in the absence of canonical DETC or IL13 from the DETC, *Rae1* expression was examined, which is not normally expressed in resting epidermis. Rae-1 levels were compared in steady state epidermis from WT, TCR $\delta^{-/-}$ and bone marrow chimera animals lacking IL13 either in the hematopoietic compartment, systemically or in the DETC. *Rae1* expression was significantly increased in TCR $\delta^{-/-}$ animals (Figure 4.1 | C), and strikingly in animals that lack IL13 only in the DETC and in complete IL13 deficient mice (Figure 4.1 | D). Therefore, the increased TEWL - a physical metric of barrier function - coincides with a higher expression of an endogenous ligand associated with a dysregulated state of tissue. This demonstrates, that DETC and DETC-derived IL13 are crucial for physical skin barrier integrity.

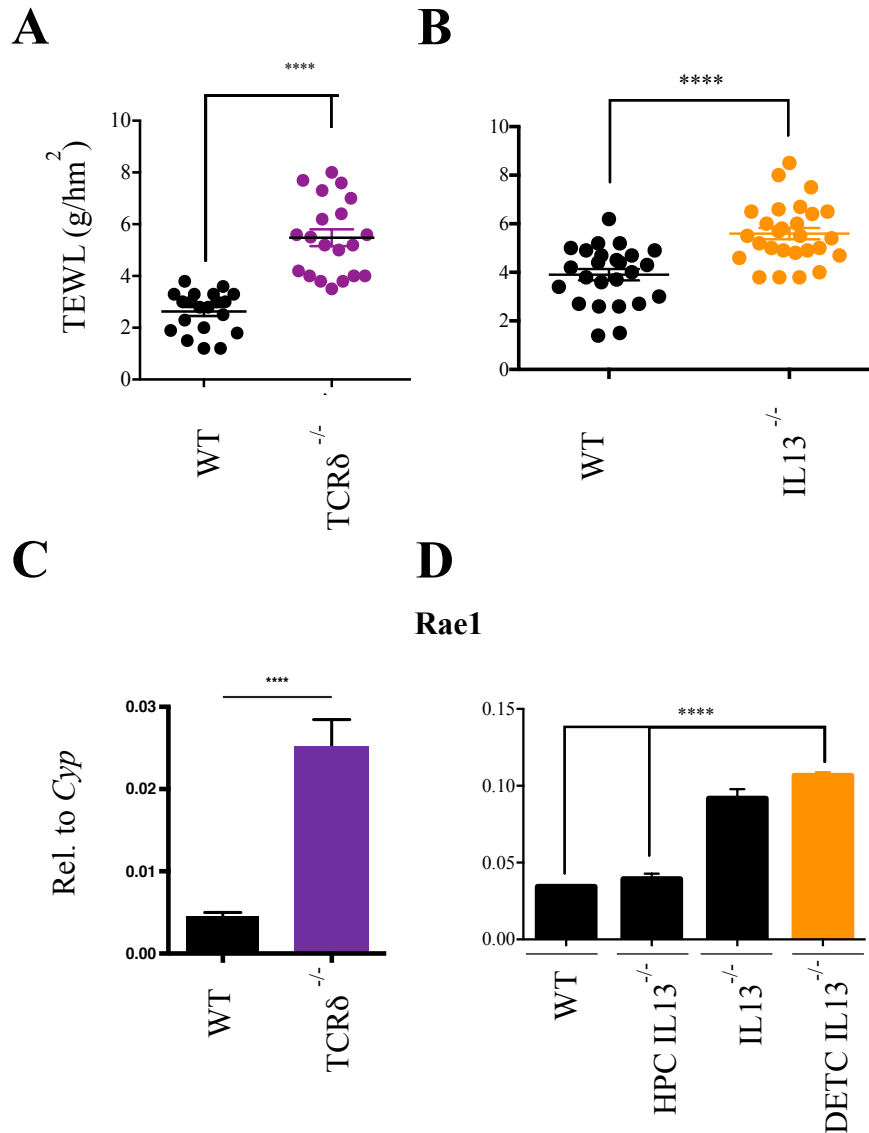


Figure 4.1 | Canonical DETC and IL13 maintains a healthy epithelium

TEWL measured at steady state on dorsal ear skin of (A) FVB WT (n = 20) and TCRδ^{-/-} (n = 20) mice and (B) BALB/c WT (n = 25) and IL13^{-/-} (n = 27). *Rael* expression in the epidermis at steady state measured *via* qRT-PCR in (C) FVB WT (n=6) and TCRδ^{-/-} mice (n=6) and (D) WT/IL13^{-/-} bone marrow chimeras (n=4 per group). The orange bar represents chimeras where the epidermal DETC are IL13^{-/-} but the systemic compartment is not. See Table 3.0 for key to bone marrow chimeras. HPC stains for haematopoietic compartment. Data are expressed as mean ± 1 SEM relative to the control gene cyclophilin (*Cyp*). Statistics *via* Students T test where here; * = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001, **** = P ≤ 0.0001

To interrogate this further, TEWL was measured following perturbation of the epidermal barrier by tape stripping. Immediately following this insult TEWL was significantly higher in $\text{TCR}\delta^{-/-}$ and $\text{IL13}^{-/-}$ animals than their respective WT controls (Figure 4.2 | A), suggesting animals deficient in canonical DETC or IL13 have poorer tolerance to damage. Importantly, to assess barrier repair over time, TEWL was measured daily following tape stripping in controls and mutant mice (Figure 4.2 | B) and determined the repair as % of the original TEWL. Notably the data showed that throughout the time-course mice deficient in canonical DETC (Figure 4.2 | B, left panel) as well as mice deficient in IL13 (Figure 4.2 | B, right panel) had a significantly higher % original TEWL compared to their controls. WT animals had mostly resolved the damage at 96 hours, whereas both $\text{TCR}\delta^{-/-}$ and $\text{IL13}^{-/-}$ still showed a significant TEWL. IL13-deficient ears in particular felt papery thin on gross examination. Following tape stripping the ears appeared to have more of a shine, which could be interpreted as more dermis being visible through a thinned epidermis. The ears of mice lacking the canonical DETC felt like their respective wildtype controls. This demonstrates that canonical DETC and IL13 are important for restoring barrier integrity following insult.

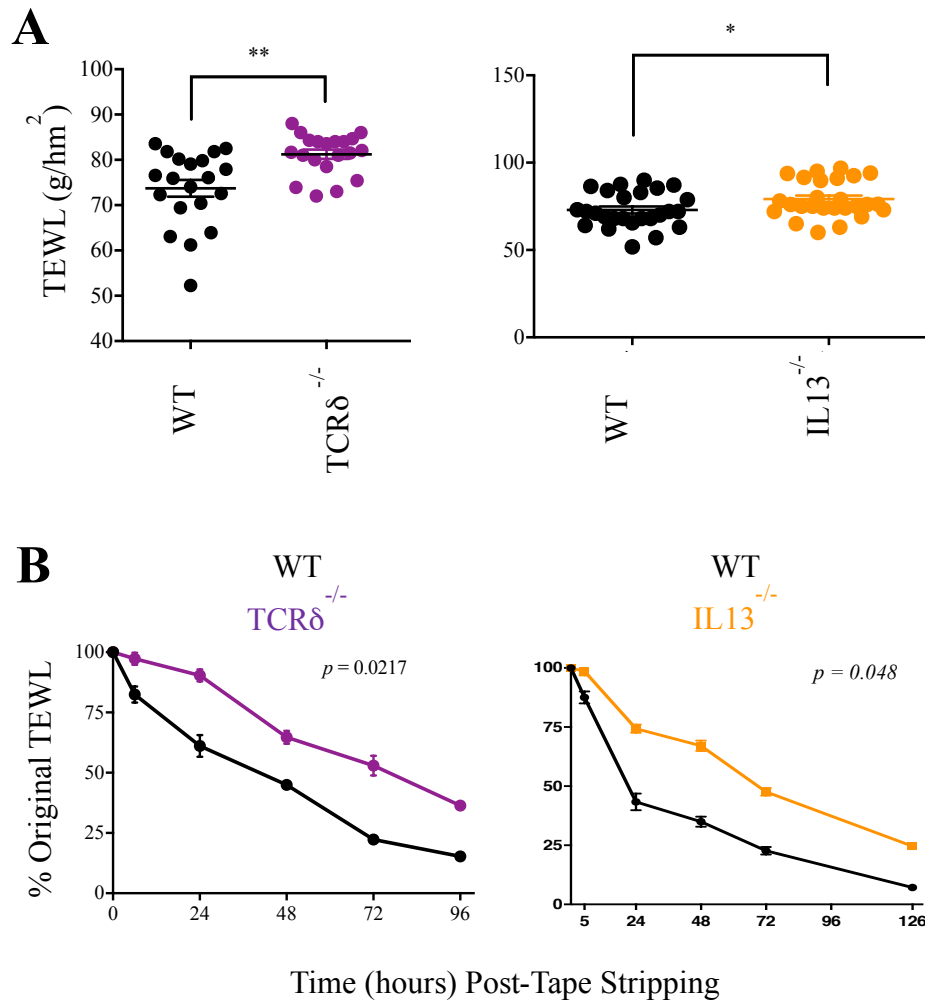


Figure 4.2 | Canonical DETC and IL13 promote barrier repair following epidermal insult

TEWL was measured (A) immediately after tape stripping on dorsal ear skin of WT FVB (n = 20) and TCRδ^{-/-} (n = 20), WT BALB/c (n = 25) and IL13^{-/-} (n = 27) and (B) daily, after tape stripping. Data in (B) is expressed as the mean % TEWL of the original TEWL immediately after tape stripping ± 1 SEM. (B) was repeated twice with similar results. For panel (A) statistics *via* Students T test where here; * = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001, **** = P ≤ 0.0001. For panel (B) statistics *via* linear regression.

Given the poorer barrier repair in mice lacking canonical DETC or IL13, several reconstitution experiments were performed, where 50 ng rIL13 was applied topically to TCRδ^{-/-}, IL13^{-/-} or WT ears following tape stripping. A comparable volume of vehicle (PBS) was applied to the other ‘control’ ear. Vehicle or cytokine was applied immediately following the post-tape stripping TEWL measurement, and again at 24 hours. Topical rIL13 was able to restore the pace of barrier repair in TCRδ^{-/-} animals completely to WT levels, to the extent

that the two repair curves no longer differed statistically (Figure 4.3 | A, top left panel). A similar barrier-enhancing effect was seen ‘adding-back’ rIL13 to IL13^{-/-} mice (top right panel). Strikingly, topical rIL13 also accelerated repair in WT animals (Figure 4.3 | B).

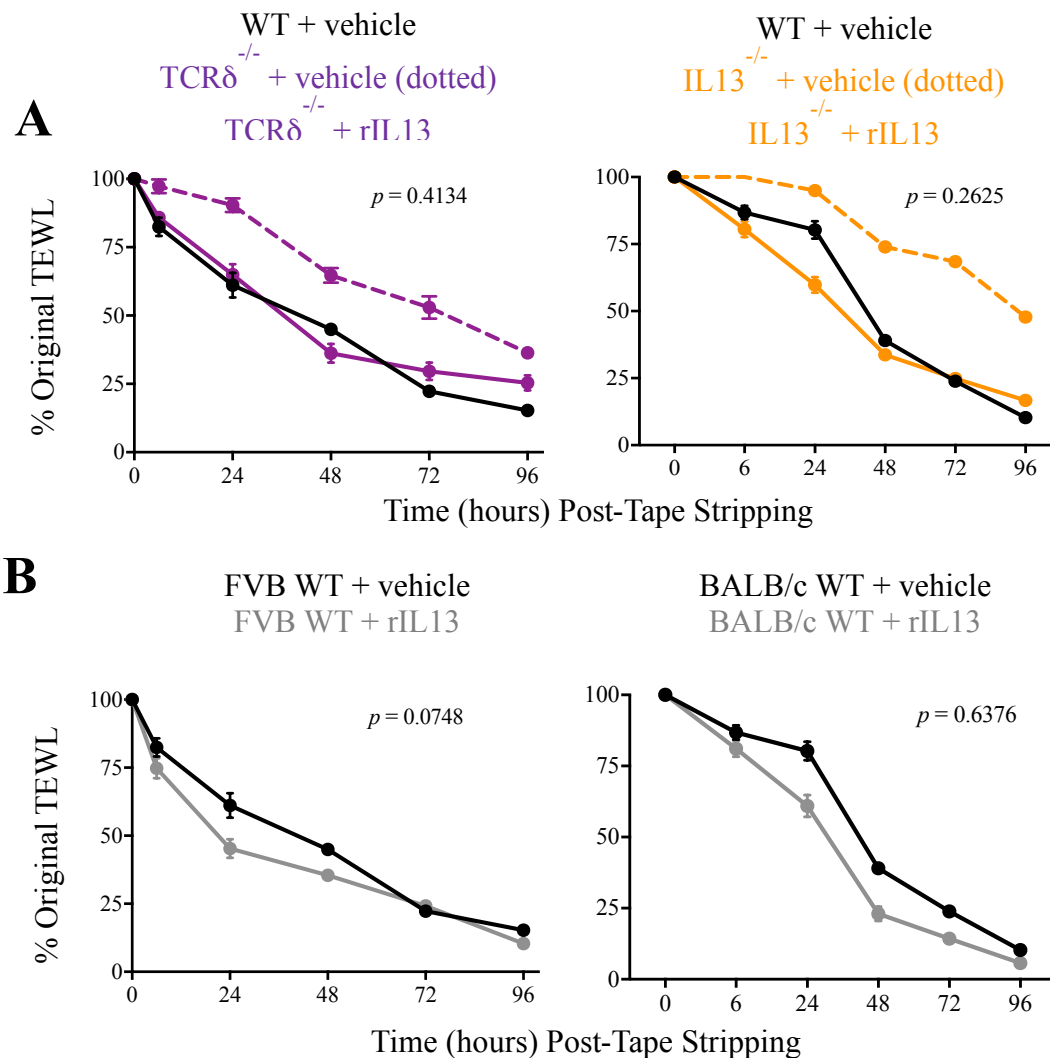


Figure 4.3 | Topical rIL13 application accelerates epithelial barrier repair after tape stripping

(A,B) TEWL was measured on the dorsal ear skin immediately after tape-stripping and at indicated time-points thereafter in (A) FVB WT + vehicle (n=10), $\text{TCR}\delta^{-/-}$ + vehicle (n=10), $\text{TCR}\delta^{-/-}$ + rIL13 (n=10) and BALB/c WT + vehicle (n=8) and IL13^{-/-} + vehicle (n=8) and IL13^{-/-} + rIL13 (n=8). Likewise in (B) FVB WT + vehicle (n=10), FVB WT + rIL13 (n=10), BALB/c WT + vehicle (n=8) and BALB/c WT + rIL13 (n=8). 50 ng rIL13 or PBS as vehicle control was applied topically immediately after tape stripping and again after 24 hours. Data is expressed as the mean % TEWL of the original TEWL immediately after tape stripping $\pm$ 1 SEM. Statistics *via* linear regression. P values in (A) compares the WT + vehicle v.s. mutant group + rIL13.

Given the role of IL13 in protecting against a superficial epithelial injury, the role of IL13 in repair of full thickness skin wounds was tested. To this end, a biopsy instrument was used to generate a single full thickness skin wounds on the lower back of WT and IL13^{-/-} mice. Wound size was measured daily on each animal using calipers and the results showed no difference in wound closure measured over time in the IL13^{-/-} compared to the WT (Figure 4.4). This data suggests, that IL13 has a key function in epithelial barrier repair but is redundant for full thickness wound healing.

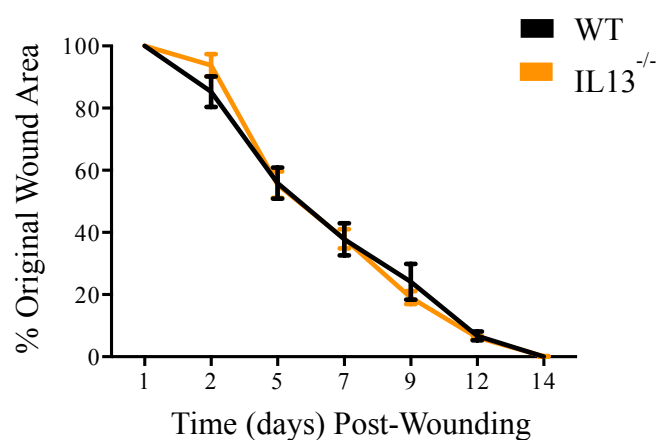


Figure 4.4 | Absence of IL13 does not affect full-thickness skin wound healing

Full skin thickness, 0.5 mm punch biopsy, wounds were made on the lower back of BALB/c WT (n = 8) and IL13^{-/-} (n=8) mice and wound sizes measured every 2 days with a microcalliper. Sagittal and transverse planes were applied to the ellipse area formula. The wound area is expressed as % original wound area $\pm$ 1 SEM.

To understand how IL13 may promote epidermal barrier repair, the regulation of genes of relevance to epidermal homeostasis was examined following tape stripping, to test if IL13-deficient mice demonstrated particular gene changes after a barrier disruption, which could possibly underlie the increase in TEWL observed. The data showed, that at 24h and 48h after tape stripping, KC intercellular junction genes *Cldn1* and *Ocln* (Claudin 1 and Occludin respectively) were upregulated in the WT controls (Figure 4.5), but not in the IL13^{-/-}. *Tgm1* (transglutaminase 1 – responsible for forming the CE) was likewise upregulated in the WT, especially at 48h, but this was significantly less increased in the IL13^{-/-}.

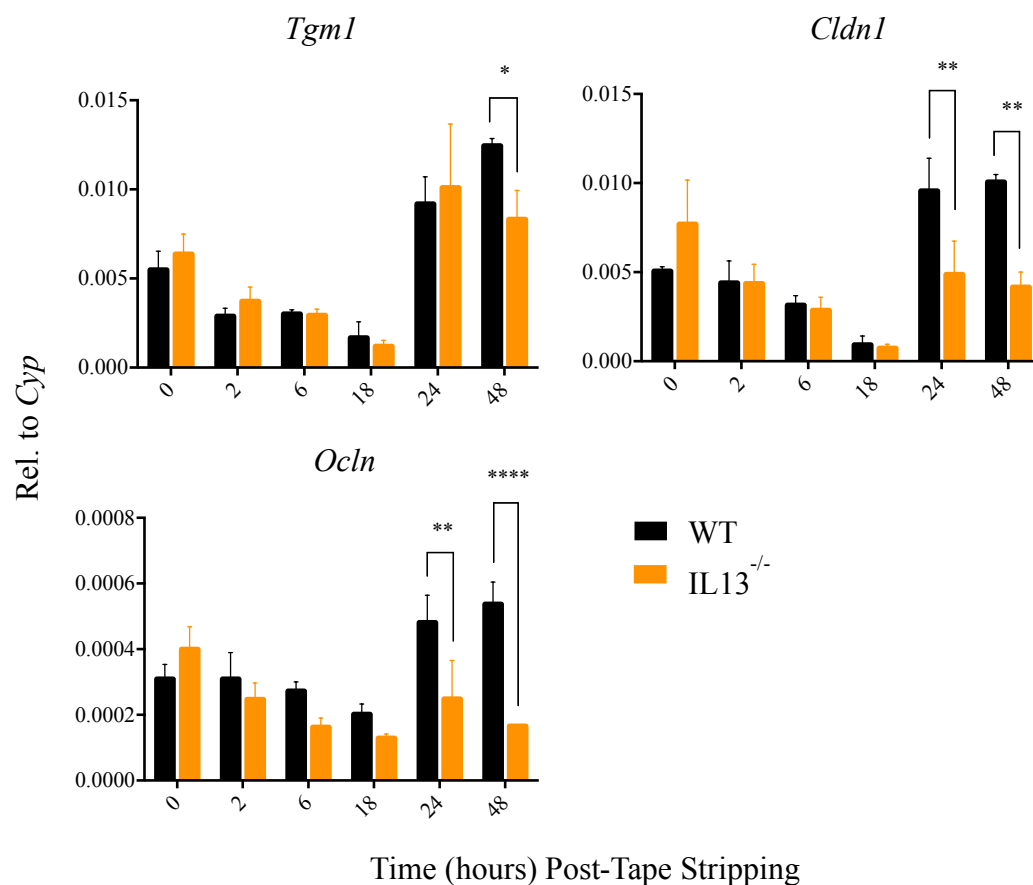


Figure 4.5 | IL13^{-/-} mice have reduced expression of epidermal barrier genes following insult

Dorsal ear skin of BALB/c WT and IL13^{-/-} mice were tape stripped 6x and the epidermis separated for qRT-PCR at the indicated time points (n=3 mice per time point). Data are expressed as mean $\pm$ 1 SEM relative to the control gene cyclophilin (Cyp). WT BALB/c shown in black bars, IL13^{-/-} mice shown in orange bars. Statistics *via* Students T test where here; * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, **** = $P \leq 0.0001$

Although the structural KC gene regulation differences picked up in the IL13^{-/-} mice *in vivo* were subtle, the data suggested there may be differences in KC maturation markers in the absence of IL13 in the tissue. To study this in a direct way, *in vitro* cultures of KC were set up and the question asked whether addition of rIL13 would alter expression of KC maturation/differentiation. This showed that addition of rIL13 to neonatal KC caused a strong reduction in *K5* and *K15* mRNA (Figure 4.6). No differences in proliferation or cell death were observed. *K5* is a basal KC cell marker, and *K15* is a marker of the basal, epidermal stem cell compartment. Differentiation markers were examined, known to be expressed in non-basal and granular KC as well, however, this data was very variable and difficult to interpret as these *in vitro* monolayer cultures clearly do not differentiate similar to *in vivo* KC in the tissue. The consistently strong downregulation of basal KC markers upon culture with rIL13, however, may imply that IL13 promotes KC maturation away from the basal state, supporting the defective barrier-gene expression *in vivo* in the absence of IL13 (Figure 4.5).

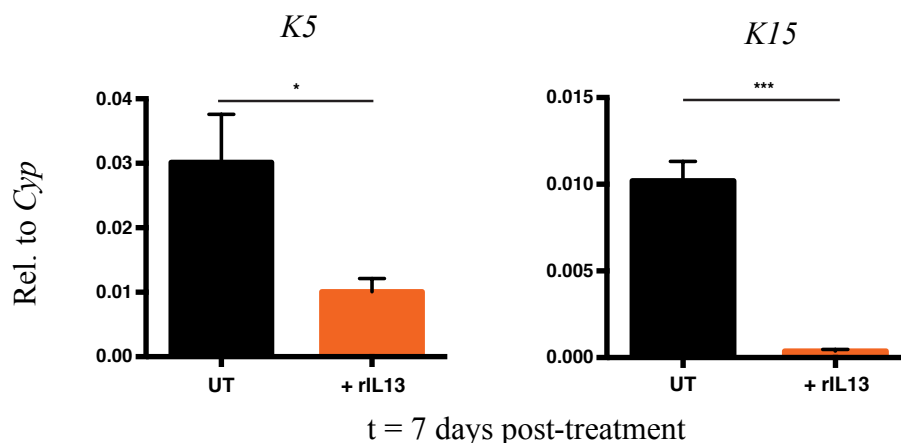


Figure 4.6 | rIL13 induces downregulation of basal keratin genes *in vitro*

Epidermal cell suspensions from BALB/c pups less than 7 days old were seeded into collagen-coated 12 well plates overnight. The following day, the medium was removed, the wells washed with PBS, and fresh medium with or without rIL13 (20 ng/mL) added. 7 days later the cells were processed for qRT-PCR. Untreated wells shown in black bars, treated wells shown in orange bars. n=6 pups per group. Data are expressed as mean $\pm$ 1 SEM relative to the control gene cyclophilin (Cyp). Statistics *via* Students T test where here; * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, **** = $P \leq 0.0001$

To further test the hypothesis that IL13 may promote ‘upwards maturation’ or *transit* of KC from the basal to outermost layers a model was devised where recently-proliferated KC were tagged *in situ*, and their movement from the basal to the suprabasal/granular layers of the epidermis monitored. Mice with untreated ear skin, or treated with 10nmol TPA for 24h (to

induce proliferation), were injected with BrdU intraperitoneally. Ear skin was fixed in formalin, embedded in paraffin and 5 micrometer sections prepared for immunohistochemical staining for BrdU and H&E. BrdU⁺ events were counted along the entire length of untreated and treated epidermis using a light microscope. Epidermal BrdU⁺ cells that were on the basal membrane were defined as 'basal'. BrdU⁺ epidermal cells not on the basal membrane were defined as 'granular'. Three 1 cm sections were counted per mouse, whilst blinded to the experimental group. At steady state, there were only a few proliferating cells per tissue section, and these were nearly all in the basal layer. There was no obvious difference between WT and IL13-deficient mice (Figure 4.7 | A; left panel). Following topical TPA, many more KC were proliferating and approximately half of these events had moved into the granular layer in the WT. However, in mice lacking IL13, although more cells were proliferating, this signal was confined almost entirely to the basal layer. (Figure 4.7 | A; right panel). This became particularly striking when comparing the basal:granular signal in WT and IL13-deficient mice (Figure 4.7 | B), indicating that KC in WT were migrating at a faster rate than in IL13-deficient mice. As a result, the epidermis in IL13-deficient mice was thinner than WT (Figure 4.7 | C), and lacked granular layers when compared to the WT. This is in support of the initial physical gross examinations of these animals' ears, which felt thinner.

Taken together, these data demonstrate that KC movement from the basal to suprabasal layers is under control of IL13, and hence this cytokine contributes to upwards maturation of skin EC and epithelial renewal.

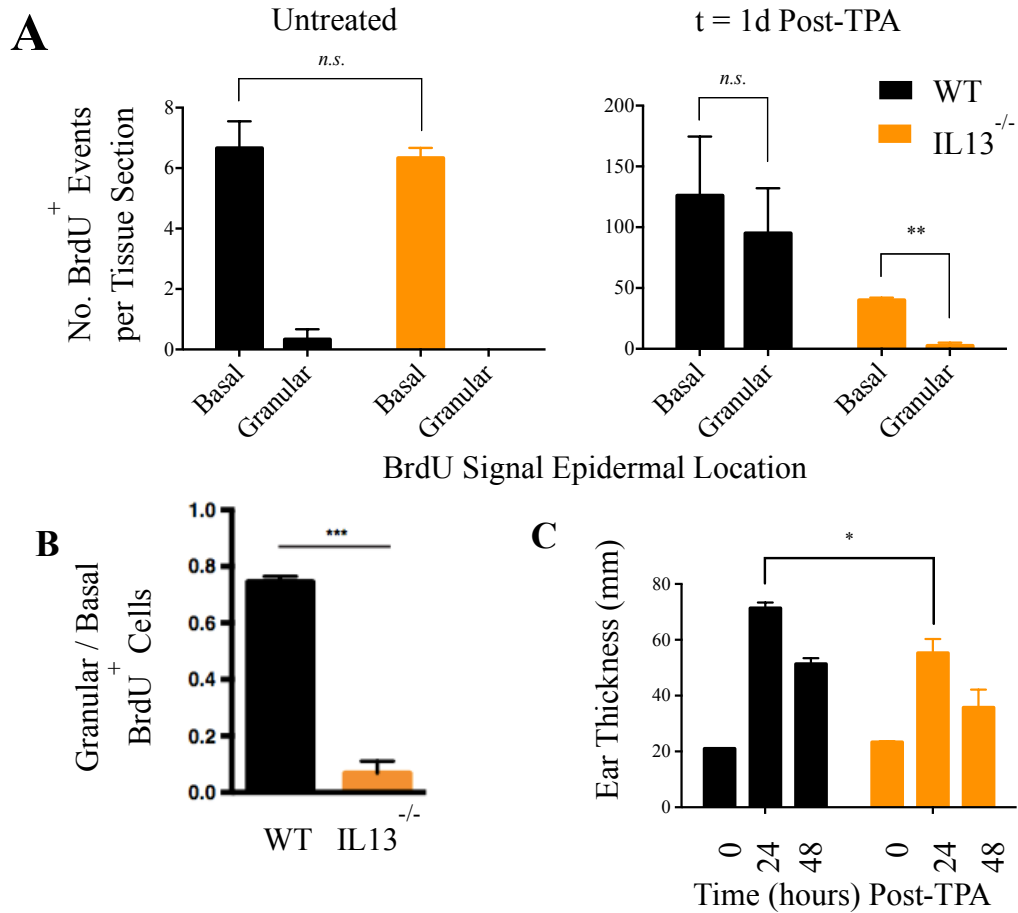


Figure 4.7 | IL13 promotes KC transit through the epidermis

(A) BALB/c WT ($n = 3$) and IL13^{-/-} ($n = 3$) were left untreated or treated topically with 10 nmol TPA on the dorsal ear skin. 21 hours later they were injected i.p. with BrdU, and culled 3 hours thereafter. Ears were collected and fixed in formalin for 24h and subsequently embedded in paraffin. BrdU and H&E staining were performed on 5 μ m sections. BrdU⁺ events were counted along the entire length of untreated and treated epidermis using a light microscope. Epidermal BrdU⁺ cells that were on the basal membrane were defined as ‘basal’. BrdU⁺ epidermal cells not on the basal membrane were defined as ‘granular’. Three 1 cm sections were counted per mouse and the mean BrdU⁺ events plotted (A) The data from (B) represented as a ratio of granular : basal BrdU⁺ cells in BALB/c v.s. IL13^{-/-} mice. (C) Ear thickness was measured with calipers every 24h after a single skin exposure to 10 nmol TPA. Statistics *via* Students T test where here; * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, **** = $P \leq 0.0001$. Experiment performed once with multiple mice per group.

Discussion

In Chapter 3, I described a molecular axis whereby DETC produce IL13 in response to tissue dysregulation and this regulates KC responses to stress, for example their production of TSLP and IL33. In this chapter, I have shown a biological role for this axis of immune-communication between DETC and KC *via* IL13. Notably, a potent effect of DETC-derived IL13 in promoting barrier repair following tape stripping and in regulating KC maturation and transit in the epidermis.

DETC-Derived IL13 Promotes Epidermal Integrity

I first examined the contribution of DETC and IL13 on skin homeostasis. I measured TEWL from the skin, a common physical metric of barrier integrity¹³⁷. Animals deficient in either the canonical $\gamma\delta\text{TCR}^+$ DETC, or IL13, showed greater epidermal water loss both before and after tape stripping – and a poorer repair of this barrier over time (Figure 4.1-4.2). Strikingly, topical application of rIL13 was able to completely reverse this phenotype (Figure 4.3). These observations are in agreement with reports that mice lacking the canonical DETC show greater TEWL¹³⁸. Interestingly, the repair curves for animals lacking the canonical DETC or IL13 were parallel to their wildtype controls (FVB and BALB/c respectively). This suggests that the effect of not having the canonical DETC or IL13 in this setting is established early on in the timecourse, and thereafter the rate of repair is the same. This is supported by the initial observations that steady state TEWL, and TEWL immediately post tape stripping is higher in the deficient mice. With regards to IL13, a protective role for type 1 IL13 receptor signaling has been previously shown outside of the skin in lung epithelial injury and homeostasis¹³¹, while neutralization of IL13 reduces lung epithelial repair following injury¹³⁹. Interestingly, mice lacking the type 2 IL13 receptor, IL13Ra2, have mildly increased ear TEWL at steady state and following epicutaneous allergen challenge¹⁴⁰. The type 2 IL13 receptor lacks intracellular signaling machinery and is thus commonly referred to as a ‘decoy receptor’¹⁴¹. Therefore, excess IL13 signaling due to removal of a regulatory mechanism or absence of the IL13 system entirely, as I have shown, both result in body barrier dysfunction.

In support of this, mice lacking the canonical DETC were also found to have higher steady-state expression of endogenous stress ligand *Rae1* in the epidermis (Figure 4.1 | C). *Rae1* expression was also examined in the bone marrow chimera animals, which lacked IL13 systemically or specifically either in the DETC or in the hematopoietic compartments. Mice

lacking IL13 only in the DETC showed significantly higher *Rae1* expression than the WT chimera, even at steady state. Indeed, I demonstrated in Chapter 3 that IL13 is constitutively expressed by the canonical DETC, and this expression increases after skin injury or DETC stimulation. These data are in agreement with previous reports of type 2 cytokine upregulation in acutely barrier disrupted skin^{142,143}. In a WT animal, *Rae1* is upregulated following injury such as skin abrasion⁹⁰. It and other NKG2D-ligands are regulated transcriptionally in response to pathophysiological states and perturbation of tissue homeostasis¹⁴⁴. Therefore, my data support the notion that canonical DETC and DETC-derived IL13 are in fact key modulators of healthy epithelium; driven by observations of physical barrier measurements (TEWL) and transcriptional indices of tissue stress (*Rae1*).

Beyond the epithelium, closure of full-thickness skin wounds is significantly delayed in $\gamma\delta$ T cell-deficient mice¹⁴⁵, at least in part due to loss of KGF production from such cells during cutaneous injury⁹⁹. Following a full-thickness skin wound, leukocytes infiltrate the area and phagocytose dead cells and microorganisms – while angiogenesis and collagen deposition re-forms the supportive structure of the tissue for re-epithelialization. KC at the wound edge infiltrate and seed the denuded dermis and express keratins 6, 16 and 17¹⁴⁶. When a bed of KC is formed *via* a burst of proliferation, they switch to K5/K14 expression, and the suprabasal, differentiated layers are re-formed and express K1 and K10¹⁴⁶. IL13 and IL4 have a particularly well defined role in fibrosis, and extracellular matrix deposition¹⁴⁷ – crucial components of the response to wounding. During repair of skin wounds in mice, an IL4-driven pathway promotes collagen fibril assembly and repair¹⁴⁸. In addition to the DETC there are cells in the dermis such as ILC2 that can and do produce IL13, particularly in response to epithelial products released during injury. IL13 in addition to its direct effects, promotes IL33 from KC (Chapter 3) – and this cytokine has a described role in promoting cutaneous wound healing by promoting tissue-restorative AAM.³¹, and healing in the gut mucosa¹⁴⁹. It therefore seemed logical to propose that IL13 might modulate the repair of full thickness skin wounds. However, in my hands, I did not observe an affect of IL13 on modulating full thickness skin-wound healing (Figure 4.4) - suggesting that the protective effect of IL13 is restricted to the epidermis.

IL13 Modulates the Epidermal Elevator

I next aimed to characterize the mechanism by which IL13 promotes epidermal integrity and protects against acute abrasion injury. The skin epithelium is a dynamic tissue in which KC move from the basal proliferative layers through to the suprabasal granular layers, before entering terminal differentiation and forming the cornified SC. To maintain this complex arrangement, KC express a number of structural and intercellular junction proteins. These are tightly regulated and underlie the physical arrangement of the epidermis. The importance of such structural proteins for barrier integrity is demonstrated in mouse models, such as claudin1-deficient mice which die shortly after birth from dehydration¹⁵⁰. In human, AD patients have reduced claudin-1 expression associated with poorer skin barrier integrity¹⁵¹, while abundant skin allergen Der p 1 cleaves tight junctions, aiding penetrance of allergens into the skin.¹⁵² Cytokine modulation of junction proteins is not particularly well described in the skin, although some reports suggest IL4 induces re-localisation of E-Cadherin away from intercellular junctions¹⁵³, and decreases SC cohesion by reducing corneodesmosome formation (a crucial SC junctional protein)¹⁵⁴. Therefore, type 2 cytokines can potently modulate the structural proteins of the skin and hence affect the epidermis' physical arrangement. I hypothesized such a modulation may form the mechanistic basis of the physical barrier defect observed in mice lacking IL13 (Figure 4.1-4.3)

To that end I examined expression of three crucial structural proteins in the epidermis during and following tape stripping. WT mice upregulated epidermal expression of claudin-1, occludin and transglutaminase 1 at 24-48 hours following tape stripping (Figure 4.5), in line with morphological repair of the tissue at these time points when examined histologically⁸⁸. This upregulation was significantly albeit mildly reduced in mice lacking IL13 (Figure 4.5). In addition, KC treated with rIL13 *in vitro* strongly downregulated basal KC genes K5 and K15 (Figure 4.6), while untreated KC maintained their expression. K5 is a marker of basal KC and K15 a marker of epidermal stem cells which are also basally located¹⁵⁵. These data suggests, that IL13 might promote maturation of KC away from the basal layer. The epidermis is essentially an 'escalator tissue', where KC's upward transit provides the cellular substrate to the granular layers of the epidermis – required for the reformation of a tape stripped barrier in a skin abrasion model. Poorer reformation of the superficial SC in the IL13-deficient mouse, due to slower 'transit' of KC, could conceivably result in higher water loss in this animal as observed; given the role of the hydrophobic CE in preventing water loss. This idea is supported by the reduced transglutaminase-1 expression in the IL13-deficient mice following tape stripping (Figure 4.5). This enzyme is crucial for crosslinking

protein and lipid components of the CE, and a deficiency therefore suggests a poorer reformation of the superficial SC following abrasion.

In order to examine this further, I studied the rate of KC transit through the epidermis using a BrdU pulse-chase experiment. BrdU-tagged KC were counted in the basal and suprabasal layers before and after skin insult with proinflammatory TPA. At steady state, there were only a few proliferating cells per tissue section, and IL13-deficient mice were not different from the WT (Figure 4.7 | A). 24 hours following cutaneous TPA exposure many more KC were proliferating (BrdU-tagged), and in the WT approximately half of the epidermal BrdU signal was localized to the granular, suprabasal epidermis, which I interpreted as KC transit. By contrast, IL13-deficient mice demonstrated not only a reduced number of BrdU-tagged KC following TPA exposure, but an almost entirely absent granular BrdU signal compared to WT (Figure 4.7). This was also reflected in ear thickness, where IL13-deficient mice had significantly thinner ears post-TPA (Figure 4.7 | D); a finding supported by a gross observation that their ears felt thinner and more ‘papery’ upon examination. This is consistent with previous reports that overexpression of IL13 in basal KC induces thickening of the epidermis¹²⁹. Taken together, these data support the idea that transit of KC from the basal layer is under the control of IL13.

The idea that cytokine and/or IEL might modulate epithelial homeostasis by affecting EC turnover – or the differentiation state of the tissue – is not entirely new. Komano *et al* demonstrated mice lacking canonical $\text{TCR}\gamma\delta^+$ IEL in the gut had a reduction in EC turnover and BrdU-labelled crypt and villus IEC in the small intestine¹⁵⁶. Furthermore, in a study germane to my ideas, it was shown that the rate of gut epithelial cell movement is under the control of IL13 specifically¹³⁵, and the authors speculated that this ‘elevator’ underlies the protective function of IL13 on gut parasite expulsion. Given the predominance of IL13 expression at barrier sites generally, this modulation of EC homeostasis by IL13 may represent a conserved mechanism of immune control of barrier integrity; and hence a fascinating, novel component of immune surveillance. In the skin, I postulate this mechanism accelerates delivery of cells to the granular epidermis in a manner conducive to barrier repair. As the skin faces the external world, damaged cells (with or without attached parasites/chemicals) can be shed to the environment, without the need for an extra clean-up mechanism to remove dead cells – such as the cilia elevator in the lung. Additionally, outside

of the skin IL4 and IL13 have been described to decrease resistance and increase permeability^{157,158} in the lung and gut; and it has been suggested that decreased EC adhesion and an increased barrier permeability could counter-intuitively promote tissue regeneration¹¹⁶ in part by aiding tissue turnover and promoting penetration of AMP.

The prevailing narrative nonetheless with type 2 cytokines and the skin is that they are pejorative for barrier function. This comes from two main strands of observation. Firstly, IL4/IL13 are often found to be expressed in acute and chronic atopic skin lesions, with the acute phase of AD particularly characterized by influx of inflammatory, Th2 cells⁵⁹. In the clinic, blockade of the IL4 receptor alpha subunit can be highly effective in treating moderate-to-severe AD¹⁵⁹ – firmly implicating the IL4/IL13 system in AD immunopathogenesis. Secondly, addition of recombinant IL4/IL13 cytokine to KC *in vitro* cultures is often reported to induce downregulation of a number of different genes important for epidermal homeostasis – particularly those present in the EDC locus; these gene changes are usually interpreted as pejorative for skin barrier homeostasis. Many of these studies, however, are overtly reductionist, and draw conclusions from gene expression changes in KC grown in 2D cultures, which do not faithfully reproduce the complex skin and epithelial environment. Nonetheless, numerous keratins, and differentiation products crucial for barrier integrity such as filaggrin, loricrin and involucrin are all reportedly downregulated by IL4/IL13 *in vitro*¹⁶⁰. Indeed, many different cytokines can affect the epidermal barrier components at multiple levels, as summarized by D Gutowska-Owsiak and G Ogg¹¹⁶. Given my data however, I suggest that the pervasive paradigm is too simplistic, and is underpinned by observations of a deleterious effect of skin IL4/IL13 in a dysregulated or excessive state (such as in AD), or artificial model systems (2D cultures). Indeed, central to immune surveillance is the understanding that there is likely a tipping point at which any immune mechanism when overtly zealous becomes immunopathological. With reference to AD, an inherent immune hypersensitivity combined with genetic barrier dysfunction (such as that caused by common *FLG* mutation) may tip the balance of a nominally protective type 2 tissue immunity to become destructive.

Conclusions

In this chapter I demonstrate that DETC-KC communication *via* IL13 has direct effects on epithelial biology and epidermal integrity. My main findings were:

1. DETC and IL13 promote barrier integrity and repair following insult.
2. IL13 promotes epithelial maturation and transit through the epidermis

Thus, here I challenge and update the canonical view of type 2 cytokines in the skin, and reveal a positive role of constitutive IL13 expression by the DETC – which has known host-protective properties in many settings – on maintenance of the epidermal barrier. I suggest that the function of IL13 as a modulator of the ‘epithelial escalator’ may underlie its role in barrier integrity (see Model in Figure 4.8). In the epidermis this mechanism could plausibly be protective in multiple other settings of tissue perturbation, such as promoting parasite shedding, expelling noxious xenobiotics or chemicals – or in removing DNA-damaged KC before they have an opportunity to develop into an epithelial tumour.

I therefore, in the following chapter, proceeded to investigate the effects of DETC and IL13 in another model of EC dysregulation – namely epithelial carcinogenesis.

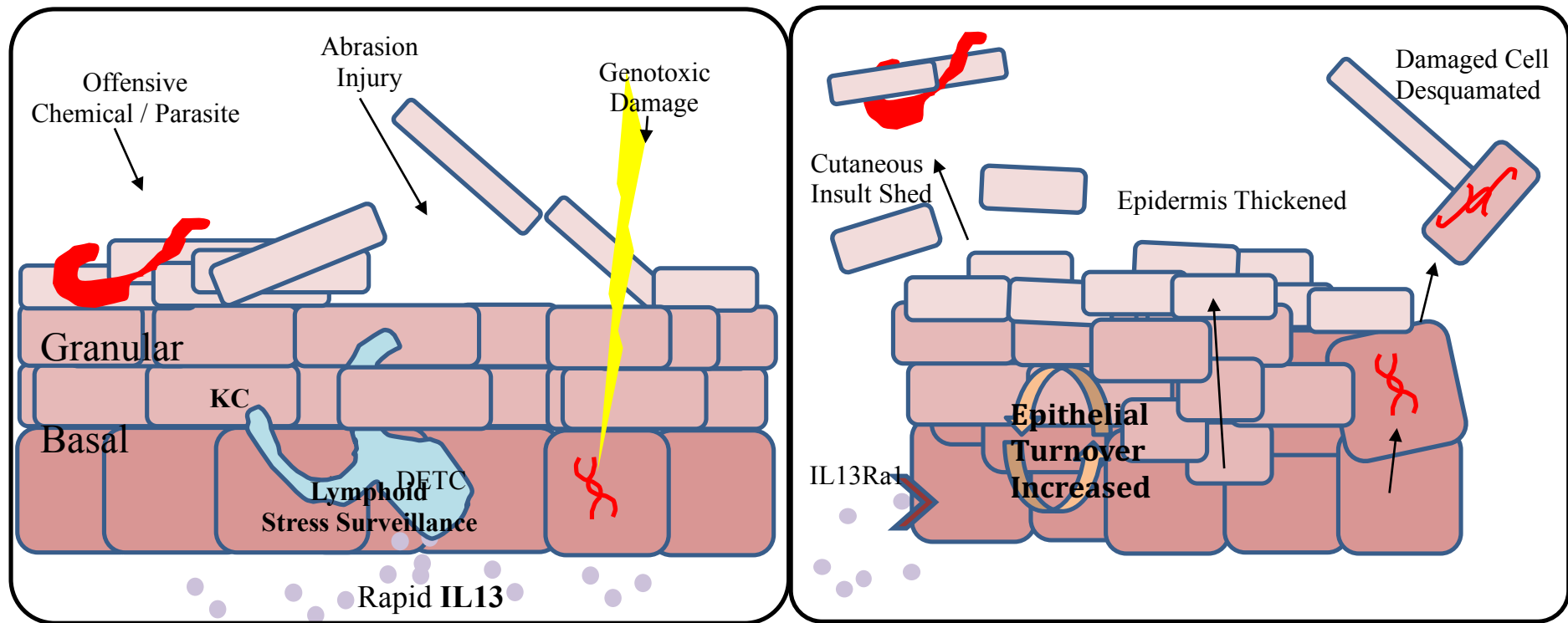


Figure 4.8 | IL13 modulates the epithelial elevator

The DETC impart immune surveillance in the epidermis, one function of which is rapid IL13 secretion. KC ligate IL13 *via* stress-modulated IL13 receptors. Basal keratins such as K5 are downregulated and KC transit upwards. This has the effect of increasing turnover of KC, accelerating the removal of damaged cells and cutaneous insults, and providing a cellular substrate to help repair abrasion injuries.

Chapter 5 | DETC and Carcinogenesis

Introduction

Skin-resident IEL can recognize and respond to alternations in EC stress antigens, such as NKG2D ligands, and thus survey the health of a particular cell. This mode of rapid afferent sensing, with induction of potent tissue immunity in synchrony with innate immune responses is termed lymphoid stress surveillance. Interestingly, NKG2D ligands are often upregulated early upon cellular dysregulation or transformation, while most epithelial tumours also express them¹⁴⁴. The NKG2D-pathway is strongly associated with anti-tumour responses in both humans and mice¹⁶¹ and Girardi and Hayday demonstrated the protective capacity of murine $\gamma\delta$ T cells in the skin to chemical cutaneous malignancy¹⁶². Further work by Strid *et al* characterised potent DETC sensing of the endogenous stress ligand Rae1 in KC *via* DETC-expression of NKG2D^{88,90}; sensing of which induced pleiotropic changes in the skin and systemic immune compartments and elicited potent type 2 immunity. These elegant studies therefore demonstrated a potent role of the DETC in immune surveillance of epithelial dysregulation. Despite the fact that recognition of a specific tumour-associated antigen (TAA) by a $\gamma\delta$ T cell has not been described, additional molecular interactions facilitating recognition of tumours cells by $\gamma\delta$ T cells are being discovered. Additional NKG2D ligands MICA, ULBP1 and ULBP4 have also been described⁸⁹. Additionally, the V γ 9V δ 2 TCR may be able to recognise phosphoantigens expressed on endogenous butyrophilin BTN3A1¹⁶³. These phosphoantigens can be upregulated due to metabolic stress, and hence this represents another mechanism whereby information pertaining to the health status of a cell is communicated to the IEL; a mechanism likely to be of critical relevance to carcinogenesis, in which cells are highly dysregulated.

The consequences of sensing of stress by the IEL, and the DETC in particular, is induction of rapid type 2 tissue immunity⁹⁰. My data of rapid IL13 production by DETC in response to heterologous topical tissue stressors (Chapters 3, 4) also support this notion. Type 2 immunity is conventionally considered deleterious to tumour

eradication and protective tumour immunity is usually associated with type 1 immunity and cytotoxicity. However, the association between tumour-protective DETC and the NKG2D pathway with induction of rapid type 2 immunity indicates that this type of immunity may also be protective in settings of acute immune surveillance. This chapter directly explores the role of type 2 immunity in tumour immune surveillance.

Cancer Immune Surveillance

Both positive and negative effects of type 2 immunity on tumour growth and carcinogenesis have been reported in the literature. Contrasting results are clearly in part due to the differing experimental approaches and models, but most likely also reflect the divergent roles that type 2 immunity may play at different stages of carcinogenesis and in different tissues. CD4⁺ T cell-derived IL4 has been reported to induce granulocyte infiltration, thereby promoting tumour clearance (an action enhanced by IL13), and conversely increase tumour cells' resistance to apoptosis by up-regulation of anti-apoptotic proteins¹⁶⁴. Many studies carried out in the 1990s and early 2000s demonstrated that tumours or tumour cell lines engineered to produce IL4 would exhibit increased rejection and retarded growth *in vivo*; curiously a phenotype often dependent on CD8⁺ cytotoxic T cells¹⁶⁵. IL10 has been reported to be both “pro-tumorigenic” by inhibiting tumour cell lysis by cytotoxic T cells and “antitumorigenic” by promoting NK-cell-mediated tumour clearance and inhibiting angiogenesis¹⁶⁶. The EC-derived cytokines IL33 and TSLP have been shown to enhance tumorigenesis by promoting epithelial-mesenchymal transition (EMT) in organotypic culture of *ex vivo* carcinoma-associated fibroblasts (CAFs) and squamous cell carcinoma cells¹⁶⁷ and by enhancing Th2 inflammation¹⁶⁸. On the contrary TSLP has convincingly been shown in mouse *in vivo* models to be critically important for resistance to skin carcinogenesis^{38,39} establishing TSLP as a tumour suppressor in the skin. Many epithelial cancers express receptors for type 2 mediators such as IL4 and IL13, allowing for a direct effect on tumour growth, death, and proliferation that is independent of their effect on immunocytes¹⁶⁹. This perhaps also explains the likely divergent effects of type 2 immunity in early cancer immune surveillance versus in established tumours. Human breast and renal cancer cell lines treated with exogenous IL13 *in vitro* demonstrate reduced proliferation¹⁷⁰ – although an ovarian cancer line

demonstrated enhanced invasive and enzyme (protease) activity¹⁷¹ suggesting IL13 aids primary tumour invasion and metastasis in this model. No doubt the heterogeneity of cancer cell lines and *ex vivo* tumours derived from patients as well as differing experimental animal models clouds a consistent description of how potent type 2 cytokines may affect growth and immune surveillance of tumours *in vivo*. Cellular type 2 players, such as eosinophils, have an extensive description in the cancer literature, and have been shown to play a protective role against chemically induced tumours *in vivo* – and directly kill chemically induced fibrosarcomas *in vitro*¹⁷², suggesting efficacious tissue immune surveillance. Mast cells frequently infiltrate the tumour microenvironment and are usually correlated with a poor prognosis in human cancers¹⁷³, although both tumour rejection and promotion has been attributed to them in the mouse. Interestingly, the ancient and highly conserved¹⁷⁴ immunoglobulin isotype IgE has been shown to play a significant role in immune surveillance of tumours. Since the 1990s IgE mAbs have been considered for cancer immunotherapy; particularly given IgE's extreme biological potency and presence of high and low affinity receptors on various effector cell types⁴. Indeed, associations between allergy history and cancer risk have been investigated in numerous epidemiological studies and their association is being defined in the nascent field of “AllergoOncology”. Recent overviews of the epidemiological literature demonstrate that both potent inverse and positive associations exist, which point to complex underlying interactions as well as reflecting the heterogeneity of these diseases. Accordingly, although the relationship between cancer and allergy has intrigued researchers for decades, the biological nature of this association remains unclear. Intriguingly, inverse associations are more common for tissues that interface with the external environment, such as the skin^{4,91}. Nonetheless, no molecular mechanism(s) for how atopy may translate into a distinct functional advantage against EC carcinogenesis has hitherto been described.

Therefore, LSS-induced type 2 immunity may represent a novel molecular pathway that directly links epithelial type 2 immunity and malignancy. I have demonstrated that these mechanisms are non-redundant for tissue health in response to various acute insults (Chapter 3 and 4); I will now examine their role in the setting of cutaneous malignancy.

Hypothesis

DETC has been described in the literature to be protective against inflammation-driven chemical cutaneous carcinogenesis¹⁶². In the previous chapters, I have described that DETC are potent producers of IL13 upon EC dysregulation and DETC-derived IL13 plays a role for canonical KC stress responses and in promoting tissue integrity.

I hypothesize:

That IL13 is protective against epithelial carcinogenesis in the skin

Aims

1. To examine the susceptibility of mice lacking canonical DETC or IL13 to epithelial cutaneous chemical carcinogenesis using two different models
2. To study the EC response in DMBA exposed skin and in tumours of mice with/without DETC or IL13

Results

To study spontaneous epithelial carcinogenesis in the skin, and the role of IL13 and DETC in this process, the 'DMBA complete' model of chemical cutaneous carcinogenesis was used. In this model, the skin is repeatedly, once weekly, treated with the carcinogen DMBA. The ensuing accumulation of EC mutations will eventually lead to the onset and outgrowth of cutaneous tumours. Using this model, there is no considerable inflammation in the skin, and the tumour onset is driven by mutational load. WT mice were compared to mice deficient in IL13 or $\gamma\delta$ T cells. Mice were examined closely, number of tumours on each animal counted, and tumour size measured weekly by an investigator blinded to the experimental groups.

The results clearly showed that mice lacking IL13 or canonical DETC were significantly more susceptible to DMBA complete cutaneous chemical carcinogenesis (Figure 5.1). IL13-deficient mice developed more tumours on average per mouse (Figure 5.1 | A) and the tumours were also bigger (Figure 5.1 | B) than their respective WT BALB/c controls. Likewise, $\gamma\delta$ T cell deficient mice lacking, canonical DETC, also developed more and bigger tumours than WT FVB controls (Figure 5.1 | C,D).

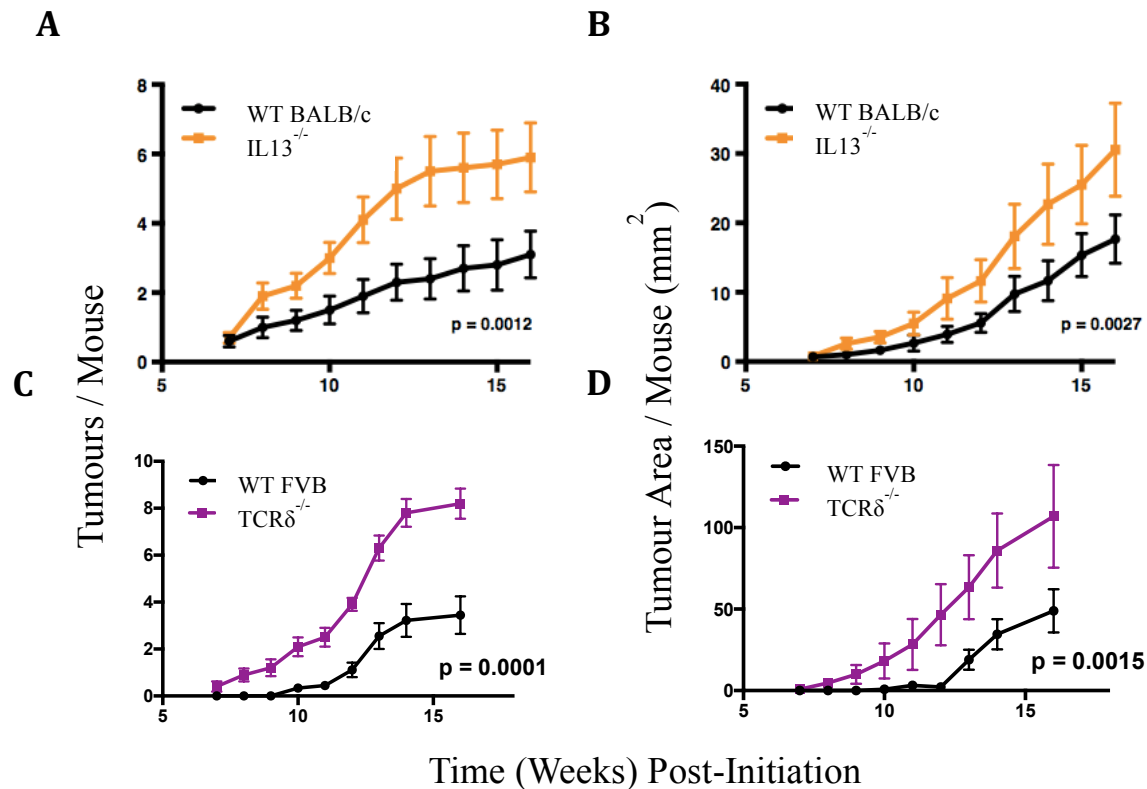


Figure 5.1 | IL13 and $\gamma\delta$ T cells protect against oncogene-driven cutaneous carcinogenesis

(A-D) The entire back skin was shaved and the mice rested for one week. Carcinogenesis was initiated by pipette application of 200nmol DMBA and the mice were subsequently treated similarly with DMBA once weekly. Animals were monitored closely and tumours counted and measured once weekly. Data are expressed as (A) average number of tumours +/- SEM per WT BALB/c and IL13^{-/-} mouse and (C) per WT FVB and TCRδ^{-/-} mouse and as (B) average tumour size +/- SEM per WT BALB/c and IL13^{-/-} mouse (n=10 mice per group) and (D) per FVB WT and TCRδ^{-/-} mouse (n=10-14 per group). Linear regression analysis was performed to compare WT and mutant groups over the course of the experiment.

Time of tumour onset was monitored closely on every mouse and this demonstrated that despite the increased susceptibility to tumours in mice lacking IL13 (Figure 5.1 | A), these mice did not in fact acquire tumours earlier than WT controls (Figure 5.2 | A). In contrast, TCRδ^{-/-} mice acquired tumours earlier and faster than WT FVBs (Figure 5.2 | B). All TCRδ^{-/-} mice had developed tumours by week 12, whereas some WT FVBs were still tumour-free by the end of the experiment.

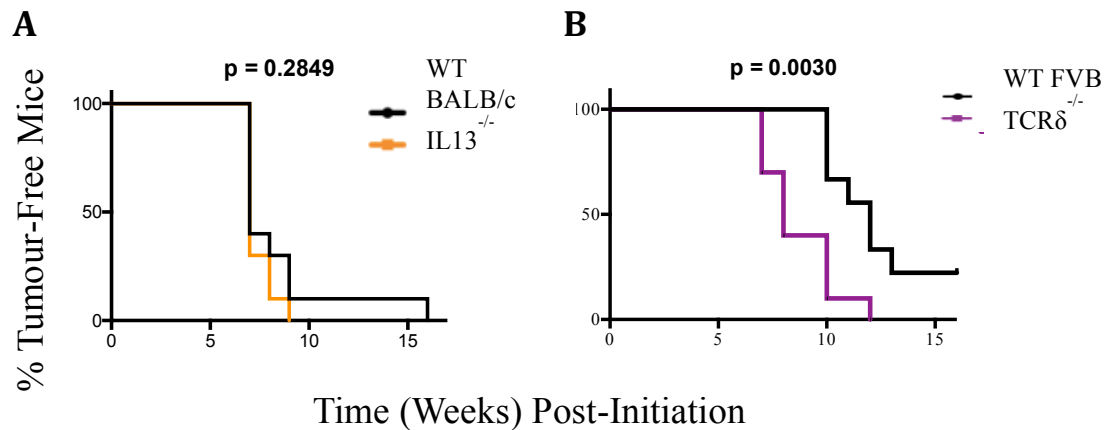


Figure 5.2 | TCR δ ^{-/-} mice develop epidermal tumours earlier than FVB WT

(A,B) Mice were exposed to DMBA complete carcinogenesis as described in Figure 5.1 and the time to first tumour appearance is expressed as % tumour-free mice over time in a Kaplan-Meier plot. Tumours were counted once weekly and the ‘onset of tumour’ defined as when the mouse carried one skin tumour of at least 1 *mm* in diameter.

The data shown in Figure 5.1 and 5.2 thus demonstrates that $\gamma\delta$ T cells and IL13 is strongly protective against chemical cutaneous carcinogenesis with DMBA. It was next determined whether this protective effect of IL13 was unique or whether IL4, which partly shares a similar receptor system, would also confer protection against DMBA complete carcinogenesis. IL4 and IL13 share receptor subunits and can activate common pathways¹⁷⁵. Additionally, *Il4* is adjacent to *Il13* on chromosome 11 in mice, chromosome 5 in humans, and shares a number of cis- and trans-regulatory elements. An effect of IL4, like IL13, has been described in numerous models of malignancy. DETC has been shown to also produce IL4 mRNA, although protein expression is not nearly as abundant as IL13. Therefore, the role of IL4 in DMBA complete carcinogenesis in WT FVB and IL4-deficient mice was examined.

The results clearly showed that mice lacking IL4 were significantly more susceptible to DMBA complete cutaneous chemical carcinogenesis (Figure 5.3). IL4-deficient mice developed more tumours on average per mouse (Figure 5.3 | A). Unlike the IL13-deficient mice, the IL4-deficient mice also developed tumours significantly earlier than the FVB WT (Figure 5.3 | B). This could suggest a divergent role of IL4/IL13 in early tumour immune surveillance, or simply a background difference

between the IL4^{-/-}, which are on FVB background, and the IL13^{-/-}, which are on BALB/c background.

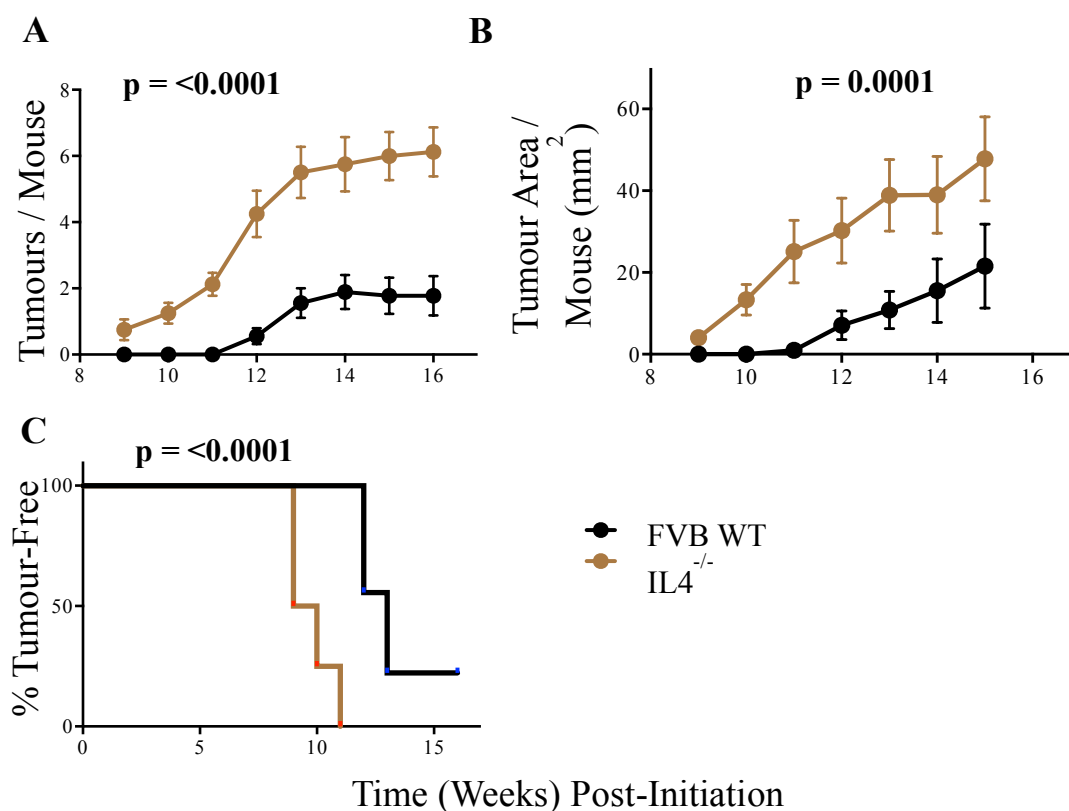


Figure 5.3 | IL4 protects against oncogene-driven cutaneous carcinogenesis

(A,B,C) The entire back skin was shaved and the mice rested for one week. Carcinogenesis was initiated by pipette application of 200nmol DMBA and the mice subsequently treated similarly with DMBA once weekly a further six times. Animals were monitored closely and tumours counted and measured once weekly. Data are expressed as (A) average number of tumours +/- SEM per WT FVB (n=9 mice) and IL4^{-/-} mouse (n=8), and (B) average tumour size +/- SEM per mouse, same numbers. Linear regression analysis was performed to compare WT and mutant groups over the course of the experiment. (C) Time to first tumour appearance is expressed as % tumour-free mice over time in a Kaplan-Meier plot. Tumours were counted once weekly and the 'onset of tumour' defined as when the mouse carried one skin tumour of at least 1 mm in diameter.

Given the increased susceptibility of mice lacking $\gamma\delta$ T cells or type 2 cytokines to DMBA complete cutaneous carcinogenesis, it was determined whether the tumours themselves were overtly different between mutant and WT mice. It was previously described that DETC-derived IL13 was key in promoting canonical epimmunome gene expression (Chapter 3) – particularly IL33 and TSLP – to acute skin stress including DMBA treatment. It was also demonstrated that in the absence of IL13, *Rae1* was significantly upregulated – a molecule with particular significance in carcinogenesis. It was speculated that IL13-deficient animals' tumours would have a gene expression signature more conducive to tumour growth, or may demonstrate a dysregulated epimmunome. To that end, analysis of gene expression of canonical KC genes *Il1a*, *Il6*, *Il33*, *Tslp* was prioritized in tumours and in skin adjacent to wild type tumours, and in naive normal skin from BALB/c and IL13-deficient mice. Tumours from both WT BALB/c and IL13-deficient mice expressed significantly higher levels of *Il1a*, *Il6*, *Il33* and *Tslp* than either resting skin or WT treated skin adjacent to the tumours (Figure 5.4).

This data demonstrates that the epimmunome genes, which were shown in Chapter 3 to be rapidly and transiently upregulated in response to acute DMBA insult, are constitutively over-expressed in established tumours. Interestingly, there was no difference in *Il1a*, *Il6*, *Il33*, *Tslp* expression between WT and IL13-deficient tumours (Figure 5.4). Although the acute EC stress response was shown to be regulated by IL13 (Chapter 3), the tumours transcript data suggest that once immune surveillance is breached and the tumour is established, IL13 no longer modulates canonical KC gene expression. To further examine this, a number of epidermal homeostasis genes in WT and IL13-deficient tumours were assayed. During acute stress, it was observed that IL13 mildly modulated expression of some EDC genes (Chapter 4). Despite this there was no difference in tumour expression of *K1*, *K10*, *K14*, *K15*, *Tgm1*, *loricrin*, *involucrin* between WT and IL13-deficient mice. In addition, expression of important leukocyte cytokines including *Il10* and *Ifng* was analysed. Expression of these genes likewise did not differ between WT and IL13-deficient tumours – or tumours and adjacent skin. Taken together the qRT-PCR data suggest that KC exhibit a canonical, type 2-biased stress response in established tumours. The nature of the tumour

response is similar to that seen following acute DMBA treatment, but the magnitude is greater. No differences were seen between WT and IL13-deficient tumours.

In addition to studying the cytokine expression in the tissue during carcinogenesis, immunoglobulin transcript expression was examined. The Strid laboratory has an ongoing interest in 'B cell surveillance' of skin tissue and tumours; immunoglobulins have been shown to play a role in carcinogenesis (unpublished data). In connection with this, DMBA-treated skin and DMBA-induced tumours were examined for mature, immunoglobulin transcripts of the IgE, IgG1, IgG2a/c and IgM isotype. It was speculated that the 'type 2' bias in cytokine gene expression would be evident in any B/plasma cell-derived antibody transcripts in the tumours.

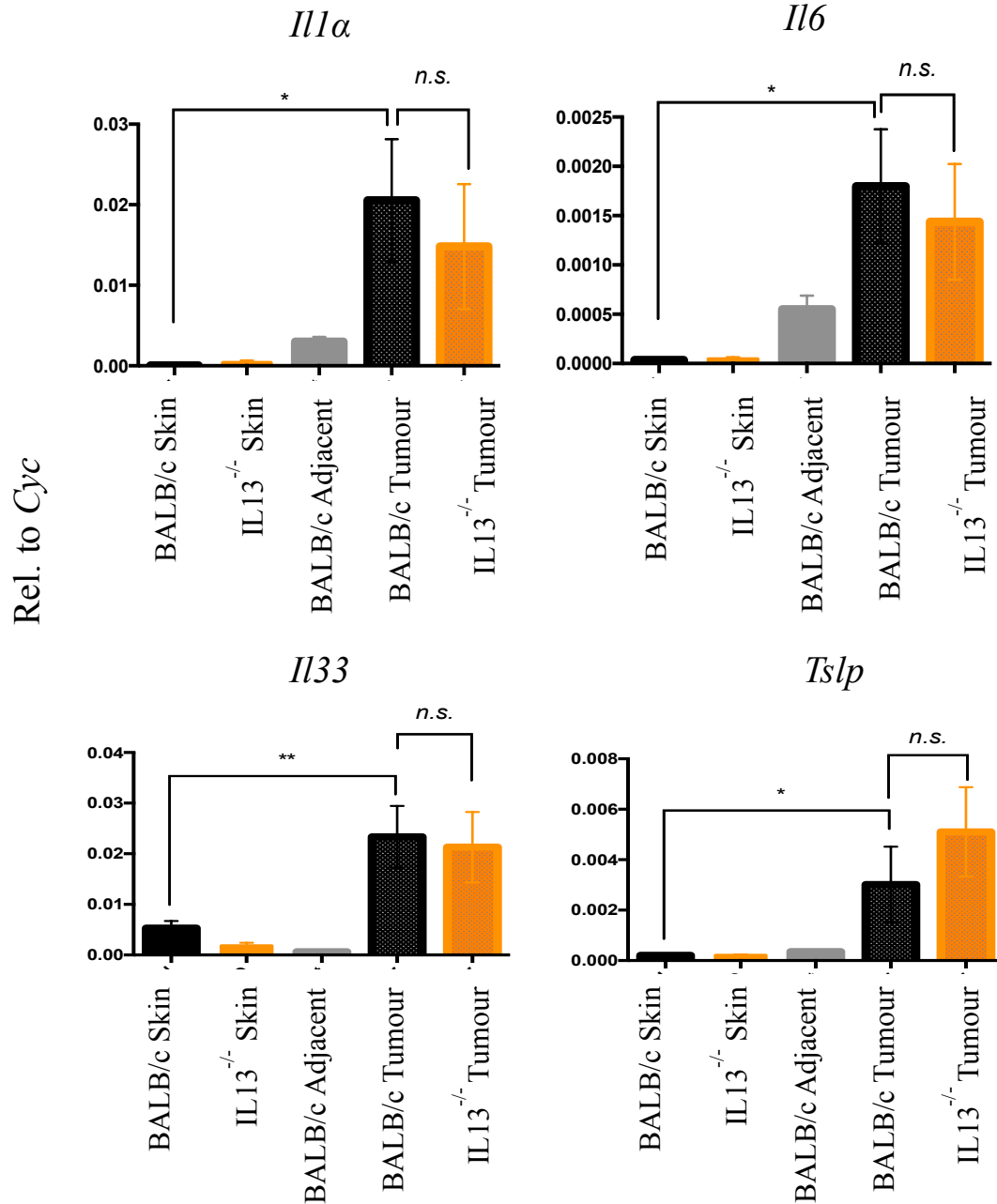


Figure 5.4 | BALB/c and IL13^{-/-} DMBA-induced tumours express canonical epimmunome cytokines

Healthy epidermis from BALB/c (at least n=5 different animals) and IL13^{-/-} mice (*Il1a*, *Il6* n=3, *Il33*, *Tslp* n=5), and excised tumours (n=8 tumours, each from a different animal) or whole skin adjacent to tumours (n=5), each from a different animal) was processed for qRT-PCR for the indicated genes. Data are expressed as mean $\pm$ 1 SEM relative to the control gene cyclophilin (Cyp). Statistics *via* Students T test where here; * = $P \leq 0.05$, ** = $P \leq 0.01$.

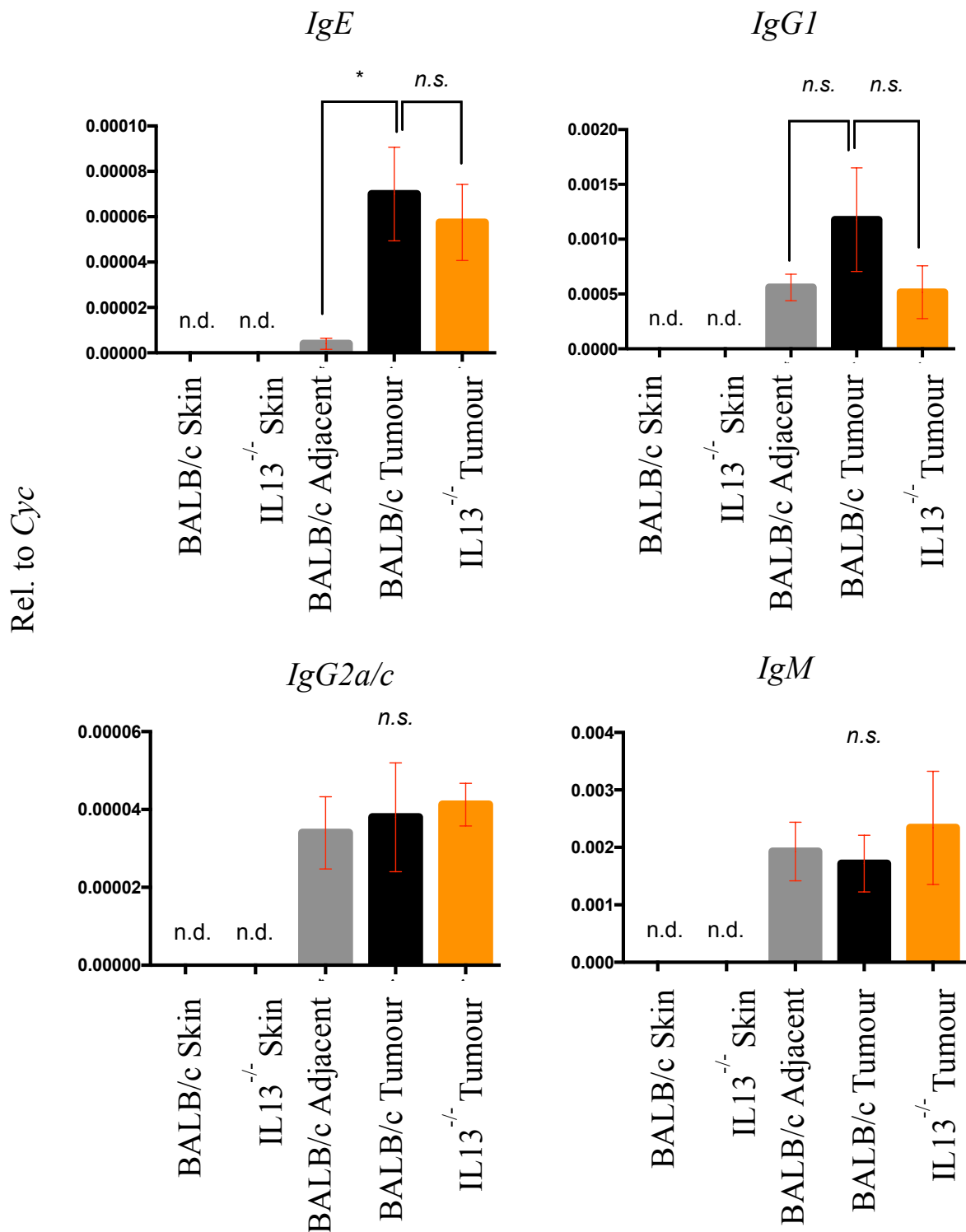


Figure 5.5 | Chronic DMBA treatment induces mature immunoglobulins in the skin and particularly drives IgE expression in tumours

Healthy skin from BALB/c or IL13^{-/-} animals (n=3), excised tumours (n=8 tumours, each from a different animal), whole skin adjacent to tumours (n=5, each from a different animal) was processed for qRT-PCR for the indicated genes. Data are expressed as mean $\pm$ 1 SEM relative to the control gene cyclophilin (Cyp). n.d. stands for not detected. Statistics *via* Students T test where here; * = $P \leq 0.05$.

Mature immunoglobulin transcripts were not detected at all in normal, untreated skin, (Figure 5.5) suggesting that antibody-producing B/plasma cells are not present in the skin at steady state. Conversely, mature IgE, IgG1, IgG2a/c and IgM transcripts are detectable in skin adjacent to tumours (grey bars). This suggests that chronic DMBA treatment is sufficient to drive an influx of antibody-producing cells into the dermis of broad isotype specificities. DMBA treatment was stopped several weeks prior to cull of these animals, demonstrating that these cells are maintained in the skin. When examining tumours, there was a significant and selective enrichment of IgE transcripts in tumours when compared to adjacent skin, suggesting that the tumours themselves are promoting IgE accumulation; above and beyond the effect seen from repeated DMBA treatment in adjacent skin. Similarly to other genes analysed, the WT BALB/c and IL13-deficient tumours did not differ significantly in IgE, IgG1, IgG2a/c or IgM expression. These data thus further support the notion of a particular role of IL13 in modulating acute epithelial responses, which becomes less consequential in outgrown tumours.

Given the accumulation of IgE uniquely in the tumour tissue, serum from these animals was assayed for total IgE protein by capture ELISA. IgE in particular may play a role in antagonizing noxious xenobiotics and chemicals at barrier surfaces, as part of the ‘prophylaxis hypothesis’ of immune surveillance. Therefore one could hypothesise that serum IgE might increase in response to chemical cutaneous insult. Strid *et al* observed robust IgE and IgG1 responses generated to topically-administered antigen encountered concurrently with epithelial stress⁹⁰ and indeed serum IgE also increases significantly following cutaneous carcinogen exposure. On the BALB/c background, total serum IgE increased 11-fold in BALB/c at 10 weeks post-initiation with DMBA compared to steady state (Figure 5.6). It increased further between 10 weeks (when DMBA treatment was stopped) and 16 weeks, at which point it was 16-fold steady state level. The level of IgE thus increased with increased tumour burden irrespective of cessation of DMBA treatment, suggesting that the dysregulated epithelium itself may drive IgE production. This was true also in IL13-deficient mice, which likewise showed a significant increase in total serum IgE over the course of the tumour experiment. However, compared to WT BALB/c, the IL13-deficient mice had significantly less serum IgE at week 10, suggesting that IL13 at this time supports IgE production. Nonetheless serum IgE continued to increase also

in IL13-deficient mice over the course of the tumour experiments and was 38-fold greater than at steady state at week 16. This large fold-increase at week 16 concurs with the significantly increased number of tumours in IL13-deficient mice at week 16 (Figure 5.1 | A). The role of IL4 in generating IgE during carcinogenesis was further examined by analysing total serum IgE in IL4-deficient animals undergoing DMBA complete carcinogenesis. The IL4-deficient mice were on FVB background, which are known to be prone to type 2 responses. WT FVB animals undergoing carcinogenesis had a 17-fold higher serum IgE at week 13 post-initiation compared to steady state, and a 12-fold higher IgE at week 16 (Figure 5.6 | right panel). The IL4-deficient mouse did not have significantly increased serum IgE at either time point. The serum IgE in WT FVB animals was generally a lot higher than WT BALB/c animals, supporting the notion that FVB mice are high IgE responders. In sum, these novel observations support the idea that IgE is increased in response to DMBA-induced epithelial dysregulation and increases with tumour load. The IgE response is dependent on IL4, which agrees with the described role of this cytokine in promoting Th2 class switch recombination¹⁷⁶ but is also partly controlled by IL13.

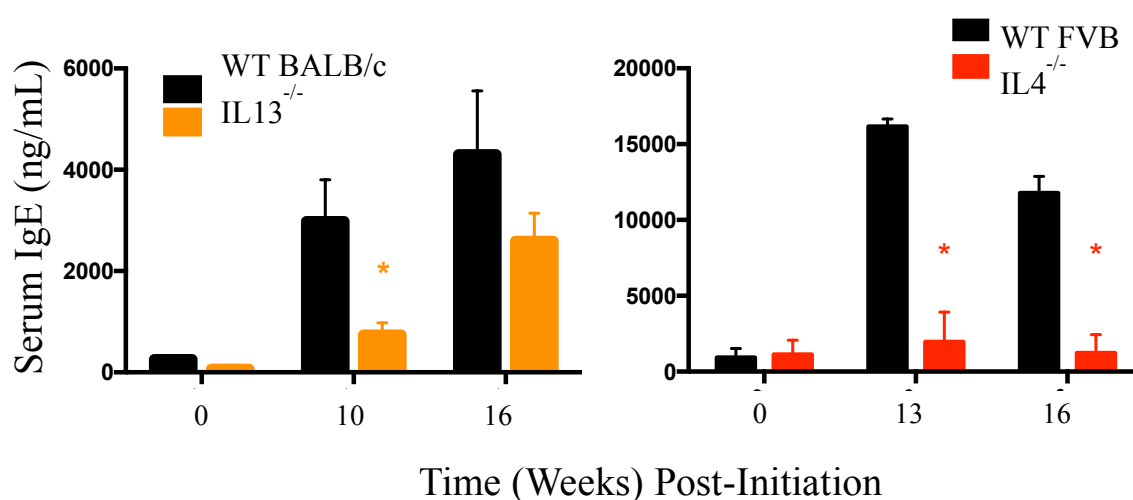


Figure 5.6 | Serum IgE is increased in response to DMBA-complete carcinogenesis and is dependent on IL4 and partly on IL13

Serum IgE was measured by capture ELISA at indicated time-points during DMBA-complete carcinogenesis in BALB/c WT (n=10), IL13^{-/-} (n=10), FVB WT (n=9 and IL4^{-/-} (n=9). Mice were bled from the tail vein before initiation of the experiment (t=0 weeks), 10 or 13 weeks after initiation, and at the end-point of the experiment (t=16 weeks). Statistics *via* Students T test where here; * = P ≤ 0.05.

To further examine the role of IL13, IL4 and the canonical DETC in epithelial carcinogenesis, the two-stage, DMBA-TPA, model of skin carcinogenesis was used. Two-stage chemically-induced skin carcinogenesis is one of the best-established *in vivo* models to study the multistage nature of tumour development and represents a classical inflammation-driven tumour model. This model was chosen as it recapitulates features of epithelial carcinogenesis in humans³⁹ and TCR $\delta^{-/-}$ mice are already known to be more susceptible to carcinogenesis in this model¹⁶². In the DMBA-TPA model, the skin is ‘initiated’ with a single sub-carcinogenic dose of DMBA, followed by twice weekly pipette application of TPA. The TPA creates a chronic low-grade inflammation in the skin, which allows for the few clones mutated by DMBA to grow out. In the absence of this inflammation, no tumours grow following the single DMBA dose.

IL13 was protective in a DMBA+TPA model. IL13-deficient mice acquired tumours earlier than WT and significantly more tumours, bigger tumours, faster than BALB/c WTs (Figure 5.7 | top three panels). In contrast to the DMBA complete model, using the inflammation-driven DMBA-TPA model of carcinogenesis, IL4 was shown to *promote* carcinogenesis (Figure 5.7). IL4-deficient mice acquired significantly fewer tumours than WT FVB mice, however they acquired tumours at the same rate (Figure 5.7, bottom right panel). Therefore this shows that the protective effect of IL13 in inflammation-driven carcinogenesis is unique to IL13 and not a general ‘type 2’ cytokine phenomenon and suggests that the closely related cytokines IL4 and IL13 have divergent functions in the inflamed tissue.

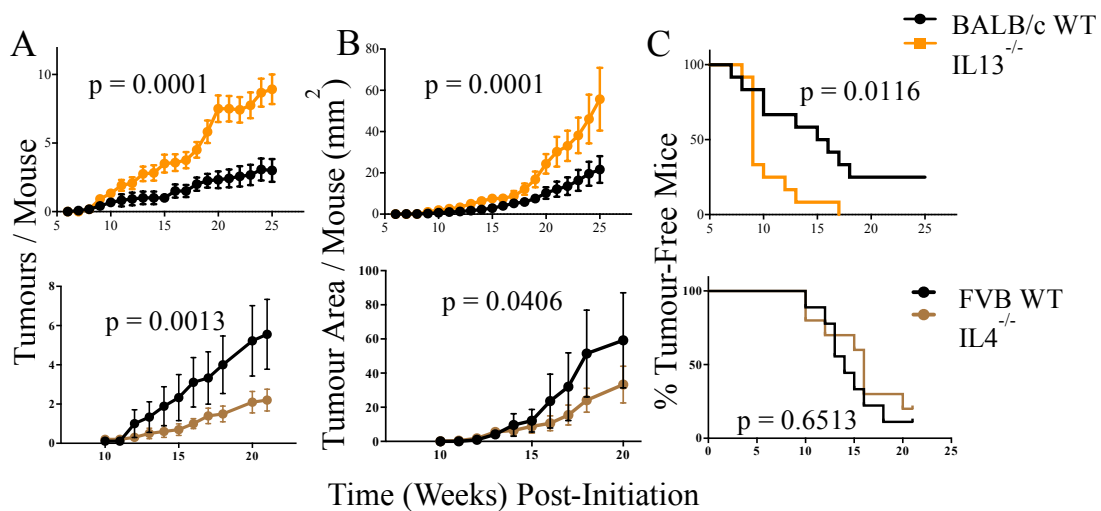


Figure 5.7 | IL13, but not IL4, protects against inflammation-driven cutaneous carcinogenesis

(A-C) Mice were shaved and rested for one week. Back skin of BALB/c strain mice were treated once with 600 nmol DMBA followed by 20 nmol TPA twice per week (BALB/c and IL13^{-/-}). FVB background mice were treated once with 200 nmol DMBA followed by 5 nmol TPA twice per week (FVB and IL4^{-/-}). Animals were scored, tumours counted and measured once weekly. Data are expressed as average number of tumours per (A) WT BALB/c and IL13^{-/-} mouse (top row) and WT FVB and IL4^{-/-} (bottom row) and average tumour size per (B) WT BALB/c and IL13^{-/-} mouse and WT FVB and IL4^{-/-}. (C) Time to onset of first tumour is shown as a survival curve for % tumour-free mice over time evaluated as per Figure 5.2. (n=12 BALB/c and n=12 IL13^{-/-} top row, n=10 FVB and n=10 IL4^{-/-}). Statistics *via* linear regression analysis between experimental groups.

Given the protective effects of IL13 against spontaneous epithelial carcinogenesis in the epidermis, in both the mutagen-driven and inflammation-driven model, it was assessed whether IL13 may play a general role in suppressing tumour growth. It was asked whether IL13 would also protect against tumour growth in a site other than the epidermis. To this end BALB/c WT and IL13^{-/-} mice were inoculated subcutaneously in the flank with the murine, BALB/c, colon carcinoma cell line CT26. Subcutaneous tumour growth was assessed daily and tumour mass measured with callipers. The results showed, that IL13 did *not protect* against subcutaneous tumour growth of CT26 cells (Figure 5.8). In fact, IL13 marginally increased tumour area suggesting that IL13 here promotes CT26 proliferation (Figure 5.8 | A). WT BALB/c and IL13-deficient mice acquired palpable tumours however at the same rate (Figure 5.8 | B).

Taken together, this data demonstrates that the tumour protective effect of IL13 is restricted to the epithelial skin surface. This finding echoes data from Chapter 4, where IL13 was protective against acute epithelial abrasion injury, but redundant for repair of injury beyond the epithelium.

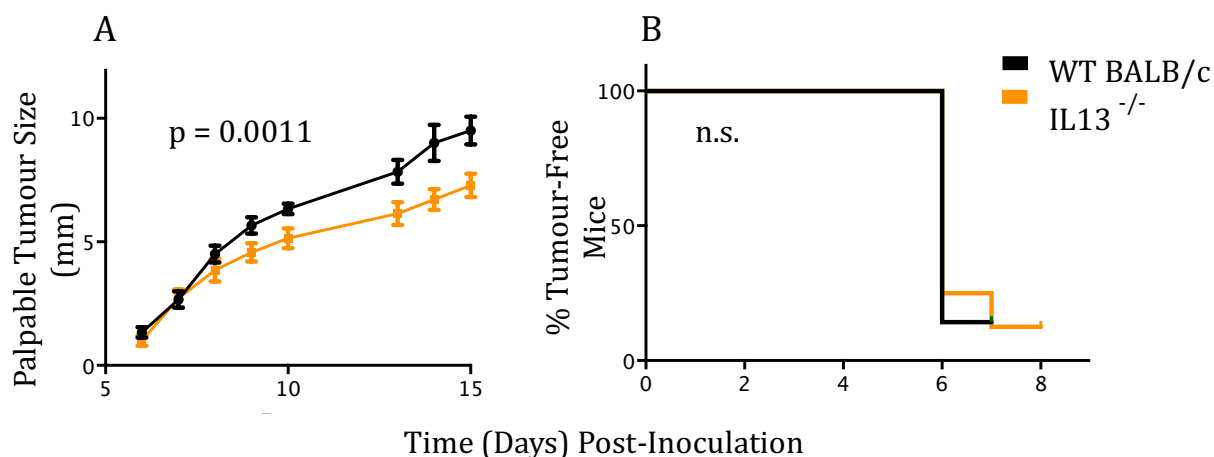


Figure 5.8 | IL13 does not suppress subcutaneous tumour growth

(A,B) BALB/c ($n = 6$) and IL13^{-/-} ($n = 7$) mice were inoculated subcutaneously in the flank with 1×10^5 CT26 cells. The flank of mice was palpated daily, and tumour sizes were measured with calipers. (A) Average tumour area per mouse is shown over time. Linear regression analysis was performed to compare WT and mutant groups over the course of the experiment. (B) Time to first tumour appearance is expressed as % tumour-free mice over time in a Kaplan-Meier plot. The onset of tumour was defined as when a tumour was palpable. Statistics *via* linear regression analysis between experimental groups.

Discussion

In Chapters 3 and 4, I showed that DETC and IL13 are vital for maintaining a healthy epidermis, and play an important role in restoring tissue integrity following insult. It is known that the canonical DETC is protective against inflammation-driven cutaneous carcinogenesis⁴⁷, while also being an elicitor of potent local and systemic type 2 immunity to tissue dysregulation^{88,90}. Here I examined the role of IL13, and related cytokine IL4, in two models of epithelial cutaneous carcinogenesis, namely mutagen- and inflammation-driven. The role of the canonical DETC was also examined in the complete carcinogenesis model using $\gamma\delta$ T cell deficient mice. I demonstrated that IL13 is uniquely protective against both mutagen-driven and inflammation-driven epithelial carcinogenesis, in contrast to IL4, which is only protective against mutagen-driven carcinogenesis. Therefore a protective effect of IL13 is expanded from acute settings of epithelial dysregulation, to also include more chronic perturbations. The mutagen-induced tumours in WT BALB/c and IL13-deficient mice showed upregulation of canonical epimmunome genes and of mature IgE transcripts. Further examination of this indicated that total IgE antibodies may be raised as a result of tumour burden. This supports the notion of a fundamental molecular link between atopy and malignancy.

The canonical DETC, IL4 and IL13 protect against mutagen-driven cutaneous carcinogenesis

DETC-KC crosstalk *via* IL13 is a regulator of epithelial responses to acute stress. Therefore, I examined the contribution of IL13 and the DETC to mutagen-driven carcinogenesis. Type 2 responses are classically considered deleterious to tumour eradication as potent type 2 cytokines such as IL4 and IL13 are thought to antagonise priming of tumouricidal Th1 immunity. Therefore, one may have expected that in the absence of IL4, or IL13, a more Th1, cytolytic environment conducive to tumour eradication may prevail. This did not seem to be the case, as mice lacking the canonical DETC, IL4 or IL13 were all more susceptible to mutagen-driven cutaneous carcinogenesis (Figures 5.1 to 5.3). I demonstrated in Chapter 4 that the rate of EC movement through the epidermis is partly under the control of IL13. I suggest that this mechanism, which was protective to acute skin abrasion, may also be protective in a model of chronic EC dysregulation; namely mutagen-driven carcinogenesis. In

support of this, an increased transit time of skin EC from the basal layers to the outer layers of the epidermis has previously been shown to be tumour-suppressive in skin carcinogenesis¹⁷⁷. Likewise, mice lacking the major structural protein keratin 10 have a strongly accelerated EC turnover, and are significantly protected against skin carcinogenesis¹⁴³. Additionally, mouse skin cells resistant to terminal differentiation have been shown to be associated with skin carcinogenesis¹⁷⁸. Therefore, I suggest that a slow-down in the ‘epithelial elevator’, as a consequence of lack of IL13 or canonical DETC, results in poorer shedding of initiated KC, resulting in more tumours. (See model in Chapter 4 conclusion). DETC are also potently cytolytic and capable of killing stress-ligand expressing cells. Therefore reduced killing of DNA-damaged KC early-on in tumour initiation, in the absence of DETC, could underlie DETC-deficient animals also acquiring tumours more rapidly (Figure 5.3). In support of this, DETC can kill stress-ligand expressing KC *in vitro*¹⁷⁹, and are known to express cytolytic granzymes and perforin¹⁰⁷. This is supported by observations from colleagues that DETC-deficient mice have significantly increased development of papillomas under chemical cutaneous carcinogenesis – but crucially their papilloma to carcinoma conversion remains unchanged in mutant mice⁸⁸. Thus DETC/IL13 are key to early immune-surveillance of dysregulated KC with protective consequences throughout carcinogenesis.

The acute tissue repair functions of type 2 immunity, and DETC-mediated immune surveillance, dovetail with a protective role in chronic epithelial dysregulation and carcinogenesis. Indeed, there is a significant link between wounding and tumour development¹⁸⁰, that led some to term tumours as ‘wounds that do not heal’¹⁸¹. Chronic wounds are known to be a permissive site for tumour formation, and epidermolysis bullosa patients with chronic skin damage have an increased risk of squamous cell carcinoma¹⁸². A reduced capability to repair the skin barrier in DETC or IL13-deficient mice as demonstrated in Chapter 4, may thus be permissive to enhanced tumour development.

The canonical DETC and IL13, but not IL4, protects against inflammation-driven cutaneous carcinogenesis

The DETC itself is known to be a crucial, non-redundant regulator of inflammation⁴⁷ and DETC-deficient animals are also more susceptible to inflammation-driven mutagenesis¹⁶². This suggests, that in an inflammation-driven tumour model, DETC modulate inflammation in a manner detrimental to tumour growth. The effect and modulation of inflammation in the TPA-driven model is likely the crucial determinant of the protective phenotype. Indeed, during the promotion phase, pro-inflammatory cytokines secreted by the KC itself, particularly TNF, are thought to contribute to a pro-tumoural milieu¹⁸³ – supported by observations that blockade of TNF has been shown to protect against TPA-driven tumour formation¹⁸⁴. In 1863 Virchow first hypothesised that the origin of cancer was at sites of chronic inflammation⁷⁶. It is now clear, that sustained cellular proliferation at sites rich in inflammatory cells with associated growth factors and activated stroma promotes neoplasia¹⁸³. The protective effect of IL13 in the DMBA-TPA model may thus relate to its anti-inflammatory properties. It has previously been shown, in the gut, that IL13 antagonises and ameliorates pro-inflammatory and harmful Th1 and Th17 responses¹⁸⁵. However, other data has demonstrated that IL13 can also promote angiogenesis, steroid hormone metabolism and infer a tissue environment conducive to growth¹⁸⁶. My data analysing the effect of IL13 on subcutaneous growth of the CT26 tumour cell line supports the notion that IL13 can promote proliferation as tumours in IL13-deficient mice grew slower than in WT (Figure 5.8). The precise mechanism of the potent protection from inflammation-driven epithelial carcinogenesis by IL13 was not determined here but it is clearly a pleiotropic cytokine and its effect will likely be defined by its spatial/temporal expression and the context of its production.

An anti-inflammatory, protective effect of IL13 in the inflammation-driven model may also be caused by the secondary mediators it induces. As demonstrated in Chapter 3, DETC-KC crosstalk drives potent expression of IL33 and particularly TSLP, to acute epithelial dysregulation. TSLP has long been implicated in the development of allergic disease in humans and the mouse³⁴. More recently it is emerging as a potent tumour suppressor¹⁸⁷, and interestingly it has been shown by several groups to mediate protection against carcinogenesis in the skin³⁹. The mechanism of protection by TSLP in models of inflammation-driven skin

carcinogenesis has been suggested to be *via* TSLP-mediated suppression of tumour-promoting tissue inflammation³⁸. Likewise, loss of TSLP signalling has been shown to lead to harmful inflammation in a *Trichuris* gut infection model¹⁸⁸. It is conceivable that the protective effect we see from IL13 could be related to its control of KC TSLP release, emphasizing the importance of an integrated tissue response to challenge. IL4, in contrast to IL13, was not protective in the inflammation-driven model. Interestingly, although IL4 and IL13 have partially shared receptors, their expression patterns often differ¹⁷⁵ and they have previously been shown to have divergent functions in allergy models¹⁸⁹. IL4 is also mainly produced by the inflammatory infiltrate and it is likely that the spatiotemporal setting of IL4/IL13 expression is a key determinant of whether they are protective or detrimental to tumour growth; a principle likely common to the effect of any cytokines in skin biology¹¹⁶. In support of this, others have shown a protective effect of IL13 in inflammation-driven mutagenesis, but not IL4¹⁹⁰, and the authors speculate divergent inflammatory programmes activated by these two cytokines underlie the phenotypic difference.

IgE is generated following DMBA induced KC dysregulation

I observed that total serum IgE was increased in WT animals of both BALB/c or FVB backgrounds in response to DMBA complete carcinogenesis, and this increase was dependent on IL4 (Figure 5.6) and partly on IL13. The importance of IL4 for IgE generation concurs with many studies showing a non-redundant role for IL4 in promoting IgE class switching¹⁷⁶. The potent induction of IgE (and IgG1) both locally in the skin and systemically suggests that class-switch recombination to type 2 antibodies is a response to chronic skin perturbation with DMBA. This observation would agree with the prophylaxis model of type 2 immunity-cancer associations. Prophylaxis was first proposed by Profet¹⁹¹, and suggests that the symptoms and mechanisms of allergic disease serve to repel and clear potentially mutagenic substances at the external body surfaces before mutagenesis can occur; a coopted function of type 2 immunity which also serves to expel parasites and helminths. Itch induced by type 2 mediators such as TSLP, goblet cell hypersecretion of mucus, sneezing, coughing, vomiting, and diarrhea all act as repulsive mechanisms and are particularly common allergy symptoms. In addition to physical expulsion, type 2

cellular players directly deactivate noxious xenobiotics. In mice at least, IgE raised to a conserved component (and allergen) of many venoms is protective against a repeat exposure⁶⁸. The unpleasantness of allergy symptoms also conditions the animal to avoid potentially (carcinogenic) triggers. Thus, the “prophylaxis” hypothesis predicts an inverse, causal relationship between allergic disease and cancer, particularly at the exposed body barrier surfaces¹²⁰. This hypothesis requires that moieties encountered at the body surfaces are directly carcinogenic, or are pro-carcinogens – as is the case with PAHs and DMBA specifically. Interestingly, serum IgE continued to increase even when DMBA treatment had stopped, suggesting that IgE was increasing with increasing tumour burden. Strid *et al* previously demonstrated that transgenic expression of stress ligand *Rae1* in the skin acted as an adjuvant on IgE and IgG1 antibody production to epicutaneous antigen (OVA)⁸⁸. EC dysregulation thus represents one mechanism whereby LSS modulates downstream systemic immunity and antibody production. The antigen to which these antibodies are raised, and the direct IgE-inducing mechanism following topical carcinogen exposure is currently being investigated in the Strid lab.

Conclusions

I show that $\gamma\delta$ T cells and IL13 are protective against both mutagen- and inflammation-driven epithelial skin carcinogenesis. I propose that IL13 is tumour protective in part by promoting EC turnover (Chapter 4) to shed putatively DNA-damaged EC, and in part by perturbing the balance of inflammation in the tissue, perhaps *via* promotion of EC-derived cytokines such as TSLP (Chapter 3).

These data in sum suggest a host-protective role of IL13 in epithelial cancer, which dovetails with its protective role in acute epithelial dysregulation. In the following chapter, I will examine directly the role of DETC and its type 2 mediators in early immune surveillance of acutely DNA-damaged KC.

Chapter 6 | DETC and Immune Surveillance

Introduction

Each of the human body's $\sim 10^{13}$ cells receives thousands of DNA lesions per day¹³. These lesions are induced by a plethora of sources including misincorporation of bases during replication, or DNA breaks caused by reactive oxygen species generated during normal cellular metabolism. The environment additionally provides a continuous assault from pervasive DNA-damaging agents such as ultraviolet (UV) light, and noxious environmental chemicals with a propensity to bind to and damage DNA. In order to combat this existential threat, complex metazoans have evolved a series of overlapping pathways for sensing DNA damage and repairing lesions, and ultimately allowing cells with lesions that cannot be repaired to die in an orderly fashion; a preemptive strategy to prevent malignancy.

While these strategies are traditionally thought of as being fundamentally cell-intrinsic, it is increasingly evident that they can be modulated and sculpted by leukocytes. A role for immune surveillance and 'killing' of cells by a litany of cytotoxic immune cells in order to combat pre-malignant and malignant cells is now fairly well accepted. In this chapter, I will explore a novel idea, which is the direct modulation of KC DNA repair processes by neighboring IEL (DETC); following challenge with the genotoxic chemical DMBA.

Studying Murine Cutaneous Chemical Carcinogenesis

Polyaromatic hydrocarbons (PAHs) are common, noxious environmental chemical generated by a large number of industrial processes such as combustion exhausts and the petrochemical industry. PAHs with mutagenic potential form bulky, stable, covalent adducts with genomic DNA, leading to double-stranded DNA breaks – the most toxic and difficult to repair DNA lesion¹⁹². Humans are therefore frequently exposed to a series of toxic, DNA-damaging chemicals as a result of industrial society. DMBA has been commonly used to study PAH-initiated carcinogenesis and DNA repair pathways to mutagenic insult. In the early to mid 1980's a flurry of work

described one of the key genomic targets of DMBA that underlies its carcinogenic potential; the cell cycle-regulator gene *hras*. An activating mutation of *hras* was found within a majority (> 90%) of DMBA-initiated squamous cell carcinomas in murine skin¹⁹³. One particular mutation being an A → T transversion within *hras* codon 61; a mutation detectable in both epithelial carcinomas as well as benign papillomas. Interestingly, the frequency of this mutation was dependent on the initiating agent used in skin chemical carcinogenesis – not the promoting chemical. Hence it seemed likely *hras* mutations accrue early on following DMBA insult¹⁹⁴ – indeed recent, more sensitive work has detected DMBA dose-dependent skin *hras* mutations *via* pyrosequencing as early as 1 day following insult¹⁹⁵. Early biochemical analysis revealed that DMBA has a particular propensity to bind to G and C residues within codons 12, 13 and 61 of *hras* – although codon 61 seems to be the major target due to either i) highest binding affinity, ii) postulated poorer repair of codon 61 lesions relative to 13 or 16, or iii) a neighbouring chromatin structure at codon 61 more amenable to adduct formation¹⁹⁶. An obvious question relating to murine skin DMBA carcinogenesis is whether other somatic mutations in addition to the well-defined *hras* are acquired – and whether they are required for epithelial transformation and carcinogenesis. Recent whole exome sequencing of murine skin following DMBA treatment confirmed the early work and demonstrated that the most frequently mutated gene was *hras* (67% of all murine SCCs) – with all tumours expressing the specific codon 61 A to T mutation¹⁹⁷. Interestingly, *kras* mutations were the second most common, also inducing activation of the *ras* critical series of pathways regulating pleiotropic aspects of cell proliferation¹⁹⁸. Inactivating mutations in the well characterized tumour suppressor gene *p53* were also detected – again reinforcing findings from the early 1990s that this gene is a common target in murine skin carcinogenesis. Early work showed a mutant *p53* signature particularly in late, progressing murine skin tumours¹⁹⁹, and that *p53* gene dosage was particularly crucial for malignant progression and not early initiation events²⁰⁰. In addition, whole exome sequencing revealed that *p53*-mutant tumours had severe chromosomal aberrations not found in pre-malignant papillomas¹⁹⁷ – also highlighting the ‘mutation march’ associated with cancer, whereby increasing mutational load and chromosomal instability accrues with time.

Skin Metabolism of PAH DMBA

Topically applied PAHs are clearly absorbed through the skin in mouse and man²⁰¹, and thus it seems likely that an IEL population in the skin which helps to maintain a healthy physical barrier (as shown in Chapter 4) may promote physical resistance to penetration of DNA-damaging agents and their bioavailability. With successful penetration of PAHs to the epidermal compartment, the next step is their metabolism in the skin and bioavailability of the DNA-damaging chemical. A (T cell-independent) non-redundant role for epidermal LCs has been shown in DMBA carcinogenesis in the murine skin. LCs are required for DMBA metabolism *in situ*, forming the more potent DNA-damaging metabolite DMBADE. LC-deficient animals are significantly protected from tumour formation with single-dose DMBA, and have fewer *hras* mutations following treatment²⁰². Modi *et al* further described that LCs internalise and metabolize DMBA in vesicles; and the p450 enzyme CYP1B1 mostly underlies the LC formation of DMBADE, along with CYP1A1 in neighboring KC²⁰³. Hence, the authors described a role of a skin-resident leukocyte population in modulating DNA-damage susceptibility to acute DMBA insult. This unfortunate consequence of LC-metabolism falls under the umbrella of ‘xenobiotic response’, whereby xenobiotic-activated receptors (XARs), such as the aromatic hydrocarbon receptor (AHR) orchestrates a nominally protective series of functions to chemical insults, ranging from sensing of chemicals and their metabolism, to inflammation and tissue repair. PAH engagement of XARs, including AHR, markedly upregulates xenobiotic-metabolizing enzymes, including the p450 family, and the pregnane X receptor (PXR) in LCs²⁰⁴. Interestingly the DETC is also AHR⁺, and AHR-deficient animals show a near-total lack of DETC²⁰⁵. Despite maturing in the thymus and trafficking to skin normally, they do not persist, demonstrating a role of AHR in maintaining the populations which use the receptor to sense chemical insult.

DNA Repair Pathways

The DNA damage response (DDR) is a complex series of sensors, signal transducers and effectors for responding to DNA damage (Figure 6.0). There are two major pathways for repairing double stranded DNA damage (DSBs) – non-homologous end-joining (NHEJ) and homologous recombination (HR); the latter is generally considered to be higher-fidelity repair as it uses a sister chromatid to drive template-driven repair. In order to mobilize the components of either of these pathways

effectively, initial DNA damage is detected by one of at least three sensors; the multi-protein (i) MRN complex (comprised of Mre11-Rad50-NBS1), (ii) Ku (Ku70-Ku80), and (iii) PARP²⁰⁶. Ku localizes within seconds to double-stranded breaks²⁰⁷, and initiates the NHEJ pathway of repair – PARP acts similarly, initiating the ‘alternative’ NHEJ pathway. The MRN complex senses DSBs and promotes the activation of ATM for HR-repair. ATM is a master regulator of a huge variety of cellular processes conducive towards DNA repair – including p53 activation – which itself causes cell cycle-withdrawal until DNA damage is repaired, by directly acting on the CHK1/2 cell cycle regulators. Interestingly, the gene *Rad50* is buried within the Th2 locus close to *IL4*, *IL5* and *IL13* – and is described in an immunological setting as a key regulator of Th2 cytokine expression²⁰⁸. Its product, RAD50, is also one of three components of the MRN complex – the DNA-binding, multi-protein sensor of DNA damage and crucially an inducer of ATM and thus RAD50 plays a central role for DSB repair²⁰⁹. The importance of which is highlighted in mouse MRN component-deficiency models which are embryonically lethal, and RAD50-deficient patients who suffer from chromosomal instability and defective ATM-mediated signaling²¹⁰.

In order to promote the large cascade of proteins and pathways that ensues a DNA-damaging event, a series of ‘warning flags’ are deposited at sites of double-stranded breaks, and at areas up to 1-2 megabases away from damage. These take the form of a histone protein H2AX, which in its active, phosphorylated form is termed γ H2AX. This scaffold of γ H2AX at sites proximal to double-stranded breaks serves to induce chromatin conformational changes, which are conducive to repair, and promotes the recruitment and retention of repair proteins²¹¹. γ H2AX is phosphorylated by ATM – although it has also been described to be phosphorylated by the second-messenger within the Ku pathway DNA-PKc – and hence may contribute generally to promoting DSB repair rather than acting on a specific pathway²¹².

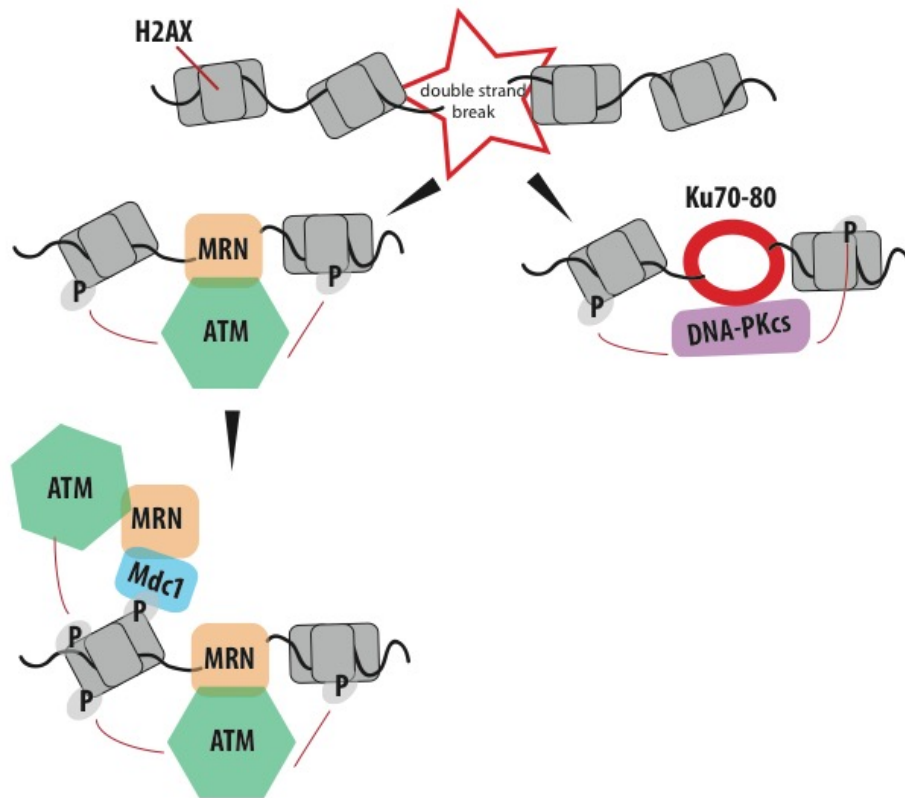


Figure 6.0 A | MRN and Ku DNA Damage Sensors Initiate the DNA Damage Response and induce HR and NEHJ repair respectively

MRN and Ku are highly conserved, abundant nuclear proteins that compete to detect DSBs and initiate the DDR. The MRN complex is rapidly recruited to sites of DSBs and recruits ATM. ATM phosphorylates H2AX forming γ H2AX, thereby inducing high-fidelity HR DSB repair with the availability of a sister chromatid. Binding of γ H2AX by the MRN subunit Mdc1 promotes accrual of further ATM. Ku70-80 recruits DNA-PKcs as a second messenger, which can also phosphorylate H2AX and induces the lower fidelity, NHEJ DSB repair. γ H2AX phosphorylation therefore acts as an inducible scaffold for DNA damage repair mediators. (Adapted from “Recruitment and activation of ATM kinase in the absence of DNA-damage sensors.” A J Hartlerode, D O Ferguson *et al.* Nat. Struc. Mol. Biol. (2015))

Although commonly interpreted solely as an indicator of the presence of DNA DSBs (particularly in Immunological journals), γ H2AX’s formation by phosphorylation by key proteins such as ATM, secondary to their recruitment to sites of damage, makes it rather an indicator of the repair process (see schematic of DDR in Figure 6.0 B). Additionally, γ H2AX is de-phosphorylated as part of the repair process, and prolonged γ H2AX signaling leads to over-active DSB repair and chromatin instability²¹³.

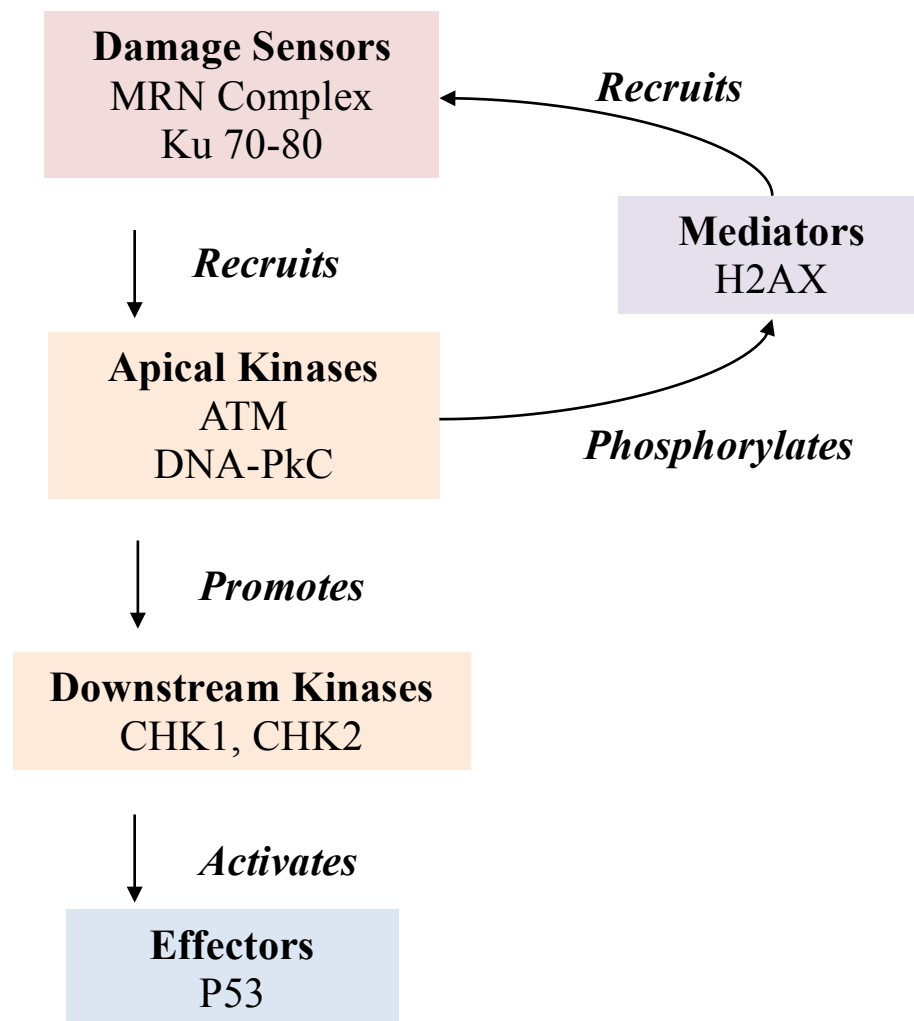


Figure 6.0 B | Summary Schematic of the DDR

Apical kinase ATM is recruited by DNA damage sensor MRN complex. Recruited ATM phosphorylates neighbouring H2AX, which promotes further recruitment of the MRN complex, hence amplifying the DDR. At the terminal end of the DDR, kinases such as CHK1 and CHK2 activate P53, which has pleiotropic effects on the cell conducive to DNA repair. Therefore, H2AX is an inducible mediator at the early stages of the DDR, while p53 represents a terminal effector.

Cytokine and Leukocyte Modulation of DNA Repair

In addition to PAH-induced DNA damage, a common method of investigating DNA damage responses in the skin is using UV irradiation; a highly relevant model given the pervasive nature of UV light. The majority of data thus far reporting on cytokine or leukocyte modulation of DNA repair comes from models of UV-induced DNA damage. The lesions induced by UV are typically cyclopurimidine dimers (CPD), of which a major repair pathway seems to be nucleotide excision repair (NER)²¹⁴. Cells

in the human skin that are so badly damaged they cannot be repaired are induced to enter apoptosis, at least in part by p53; these so-called ‘sunburn cells’ are detectable throughout UV-treated human epidermis²¹⁵. Interestingly, the cytokine IL12 has been shown to have a protective role against UV-induced apoptosis of KC – particularly by promoting expression of NER-components and hence enhancing repair of UV-induced lesions²¹⁶. Follow-up work in a UV carcinogenesis experiment demonstrated that IL12 deficient mice were significantly more susceptible to UV-induced cutaneous carcinogenesis and accruing more CPD and sunburn cells²¹⁷. The same group also demonstrated a similar effect of IL18 on reducing DNA-damaged epidermal cells following UV exposure – an effect dependent on the cells being NER-sufficient²¹⁸. In line with these studies, a direct role of the DETC in modulating DNA repair of UV treated skin was recently described by Wendy Havran’s group. They demonstrated that $\text{TCR}\delta^{-/-}$ have a significantly greater proportion of CPD^{+} cells following UV treatment than wild type animals, along with more γH2AX^{+} cells²¹⁹. Thus, at least with a UV model there is a small body of evidence demonstrating an effect of cytokines and/or lymphocytes in directly modulating components of a relevant DNA damage repair pathway. Crucially, these cytokines are produced exogenously from the target cell (KC), and act in a paracrine fashion to promote an otherwise cell-intrinsic repair process. Distal from the skin, mouse duodenal epithelial cell line MODE-K cells when treated with supernatant from activated, gut IEL induced a 30-fold upregulation of NHEJ components²²⁰, suggesting a soluble immunocyte product modulates DNA repair components. IL4 has additionally been shown *in vitro* to increase PARP-mediated end-joining in murine fibroblasts exposed to cisplatin, along with reducing the number of γH2AX^{+} following bleomycin treatment²²¹.

To summarise; the (i) expression, (ii) activation and phosphorylation, (iii) retention and (iv) regulation of key mediators of the DDR including γH2AX , P53 and ATM can be used as indicators of the magnitude and quality of the DNA damage repair process. These indices can be compared between wild type animals and animals deficient in DETC or cytokines in experimental models of DNA damage, to examine the contribution of specific cells, or cytokines, to DNA repair.

Hypothesis

I have described a role for DETC and its cytokine IL13 in regulating EC stress responses, epidermal barrier homeostasis and repair. In the previous Chapter I demonstrated that mice lacking canonical DETC, or IL13, are significantly more susceptible to inflammation-induced carcinogenesis suggesting that DETC regulate the balance of inflammation in the tissue in a protective manner. In addition, DETC and both its type 2 mediators – IL13 and IL4 – are strongly protective against DMBA complete-induced carcinogenesis. DETC were shown to delay the onset of tumours in this model, implying they act early during EC dysregulation, consistent with the tumour immune surveillance hypothesis. In the current chapter I explore this further and I hypothesise:

- DETC directly modulate DNA-damage/repair processes in dysregulated KC.

Aims

1. To examine the mobilization of DNA repair pathways following acute topical insult with DMBA and whether this is affected by DETC or its cytokine mediators.
2. To quantify the DNA damage sustained by KC following DMBA exposure and study effects of DETC on direct KC DNA-damage
3. To investigate the mutational burden in the epidermis following DMBA exposure and whether this is influenced by the presence of DETC

Results

In order to keep experiments as well controlled as possible, all have been performed on mice of a single background. Indeed, it is known that there are wide variations in DNA repair across mouse strains²²², making inter-strain comparisons difficult. FVB mice are known to be most susceptible to cutaneous carcinogenesis, and are the background on which we have TCR $\delta^{-/-}$ and IL4 $^{-/-}$ mice. Therefore, this background has been used for all experiments to examine the contribution of canonical DETC and IL4 on KC DNA repair.

For all experiments, mice were treated topically on the ears with DMBA. This treatment mimics the initiating dose used in a DMBA complete cutaneous carcinogenesis experiment; and is therefore appropriate to study the early initiating events and immune surveillance of skin carcinogenesis. Figure 6.1 outlines the investigations germane to DMBA-induced damage that can be performed in the skin, at various timepoints. From hours to several days following treatment (the initiation phase) there are no overt clinical signs in the animal, although DNA damage and its repair can be scrutinized. During the promotion phase, which lasts several weeks, the skin is treated repeatedly with either further DMBA, or TPA. Mice get small skin spots contiguous with skin during this period. During the outgrowth phase mice begin to develop skin tumours. When tumours manifest is of course dependent on mouse genetic background, chemical dose and frequency of treatments. During the promotion and outgrowth phases, mutation of the main DMBA-target *hras* can be detected *via* qRT-PCR.

Hence, at all crucial stages of a cutaneous carcinogenesis experiment, indices of genomic damage and its repair can be examined, and the effect of canonical DETC and/or IL4 inferred in TCR $\delta^{-/-}$ or IL4 $^{-/-}$ mice

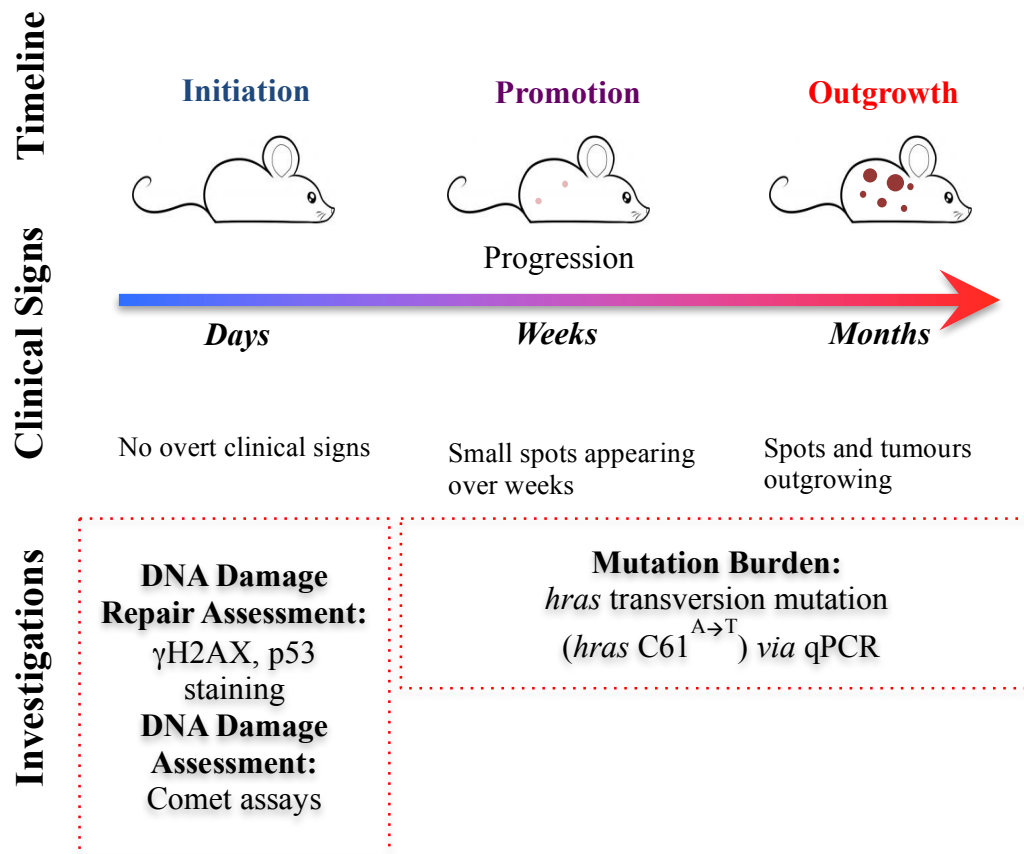


Figure 6.1 | Schematic of timeline following DMBA exposure and the analyses performed throughout

Mice were treated topically with a single dose DMBA and at various time-points thereafter the extent of DNA damage induced and the DNA double-stranded repair process was assessed *via* flow and IHC staining of γ H2AX and p53. In addition, direct visualisation of double-stranded breaks *via* the single cell electrophoresis comet assay was performed. Mice at this stage had no clinical signs of disease. Following the initial DMBA dose mice were either exposed repeatedly to an inflammatory tumour promoter (TPA), which is not itself tumorigenic, or once weekly to further DMBA. Small spots or raised tumours become apparent after several weeks and a progressive increase in tumour size and number occurs thereafter. The insulted skin without spots/tumours, and the tumours themselves were assayed for the presence of mutant *hras* – specifically the codon 61 A to T transversion mutation *via* probe hybridisation qPCR.

To analyse KC DSB following DMBA exposure, a flow cytometric assay was developed for the DNA damage marker, γ H2AX. γ H2AX⁺ cells are commonly assessed and enumerated *via* microscopy – however by developing a flow assay more cells can be analysed and positive cells quantified with ease and accuracy. An epithelial cell line, MB49, was used to test γ H2AX staining with and without carcinogen treatment *in vitro*. MB49 is a mouse urothelial carcinoma cell line that is widely used as a model of urothelial carcinoma. Untreated MB49 cells demonstrated uniform γ H2AX staining after 1 day in culture (Figure 6.2 | A), relative to the FMO control, suggesting there is a pre-existing fairly high level of DSB in these cells when grown *in vitro* even without further treatment. When MB49 cells were treated with the vehicle control acetone, γ H2AX staining was not increased relative to the untreated control. However, when treated with DMBA MB49 cells demonstrated a bimodal staining of γ H2AX, with 59.7% expressing higher γ H2AX than vehicle treated cells. This shows that in culture, DMBA induces DNA damage sufficient to induce a detectable increase in γ H2AX formation within 24 hours of exposure to the chemical – and demonstrates that γ H2AX can be quantified by FACS.

It was next assessed whether DMBA exposure *in vivo* would induce FACS-measurable increases in γ H2AX staining. A DMBA titration was performed to determine an *in vivo* dose of DMBA that would give a measurable response in FVB WT mice – the response defined as an acute increase in % γ H2AX⁺ and p53⁺ KC after treatment. At doses from 6 to 100 nmol DMBA, there were approximately 5% γ H2AX⁺ KC at 3 days following treatment (Figure 6.2 | B). With 200 nmol DMBA, this increases to 15% γ H2AX⁺ KC. Therefore, treating live animals topically with 200 nmol DMBA induces a small increase in KC γ H2AX at 1 day, which increases to approximately 1/6 of all KC γ H2AX⁺ at 3 days following treatment. Hence the *ex vivo* quantification of *in vivo* DMBA-induced DNA DSB in KC was possible *via* flow cytometry.

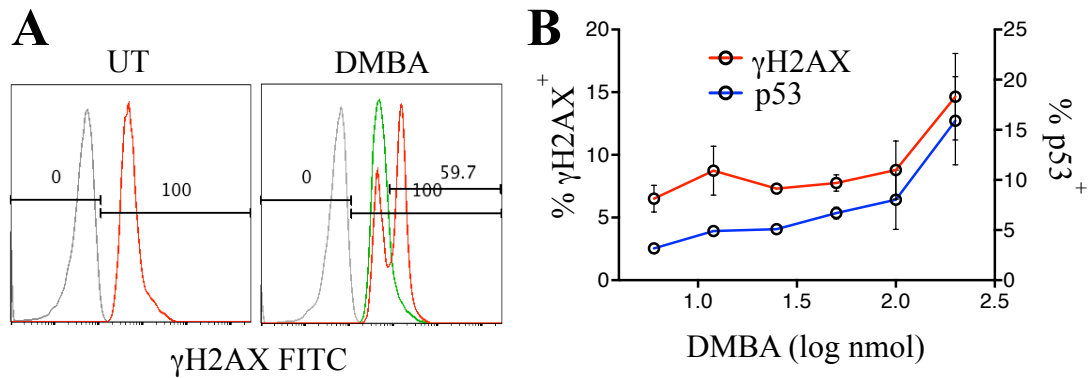


Figure 6.2 | Validation of γ H2AX flow cytometric assay *in vitro* and *in vivo*

(A) Near-confluent, adherent murine urothelial carcinoma cell line MB49 was treated with 20 nmol of DMBA or an equivalent volume of acetone. 24 hours later cells were recovered, washed and stained intranuclearly for γ H2AX. The first plot shows untreated, stained cells (red histogram) or untreated unstained cells (gray histogram). The second plot shows stained DMBA-treated cells (red histogram), stained vehicle-treated cells (green histogram) and untreated unstained cells (grey histogram). **(B)** FVB WT mice were treated topically on ear skin with DMBA ranging from 6 to 200 nmol, n=2 mice per dose. Epidermal cell suspensions were recovered 3 days later for intranuclear γ H2AX and p53 staining. Analysis was performed on live, singlet, CD45⁻ events.

With the flow assay for γ H2AX set-up, it was used to explore whether the acute DNA-damage response in KC following exposure to DMBA differed in mice lacking canonical DETC or IL4. A time course was performed (1, 3, 7, 10, 14 days) analyzing γ H2AX upregulation after single-dose DMBA exposure in WT FVB, TCR $\delta^{-/-}$ and IL4 $^{-/-}$ mice. At steady state, WT mice showed a mean 3.31% KC γ H2AX⁺ (Figure 6.3). This increased to 5.01% at 1 day, and increased further still to 11.23 % at 3 days. γ H2AX expression therefore peaks at 3-7 days in WT mice, and at 10 post-DMBA it decreases and has completely returned to baseline levels at 14 days (Figure 6.3 ; black bars). Interestingly, the TCR $\delta^{-/-}$ and IL4 $^{-/-}$ showed a complete lack of upregulation of γ H2AX at 3-7 days following DMBA exposure and γ H2AX levels were significantly lower than WT at this peak response time (Figure 6.3). This shows that the absence of DETC, or a DETC cytokine, in the epidermis alters the KC DNA damage response.

H2AX phosphorylation and p53 expression were examined by immunohistochemistry in freshly isolated whole epidermal sheets after exposure to DMBA. The addition of P53 to this analysis allowed for simultaneous analysis of the beginning component of DDR (phosphorylation of H2AX) and the terminal component of the DDR (P53) (See

Figure 6.0 in Introduction for DDR pathway schematic). FVB WT mice showed both a strong γ H2AX and p53 signal with many positive cells, which were homogenously distributed throughout the epidermis (Figure 6.4 top row). Recapitulating the flow cytometric data, far less γ H2AX staining was evident in the epidermis of $\text{TCR}\delta^{-/-}$ and $\text{IL4}^{-/-}$ mice. Additionally, p53 staining was also dramatically reduced in these animals and only small ‘islands’ of positive cells were found (Figure 6.4 middle and bottom row).

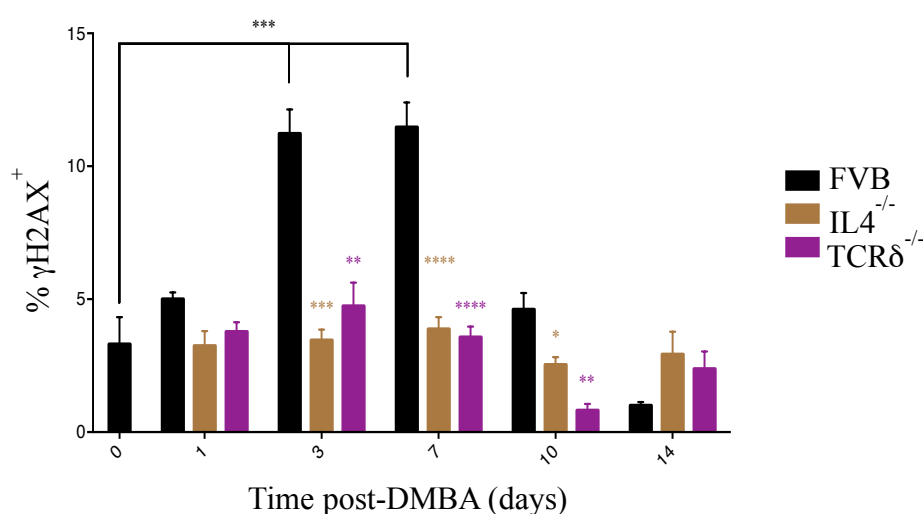


Figure 6.3 | $\text{IL4}^{-/-}$ and $\text{TCR}\delta^{-/-}$ mice have reduced γ H2AX phosphorylation following cutaneous DMBA exposure

200 nmol DMBA was applied topically to hair-trimmed back skin. At indicated time points epidermal cell suspensions were recovered and stained for intranuclear γ H2AX and assayed by flow cytometry (n=3-14 per time-point). Statistics *via* Students T test where here; * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, **** = $P \leq 0.0001$

Staining only for γ H2AX and p53 it is not possible to deduce whether this demonstrates less overall DNA-damage or a lack of repair of the DNA damage. However, since mice lacking the canonical DETC or IL4 both develop tumours sooner as well as more and bigger tumours (Chapter 5 data) it is tempting to speculate that the lack of peak upregulation of γ H2AX may suggest some defect in the DNA-repair machinery.

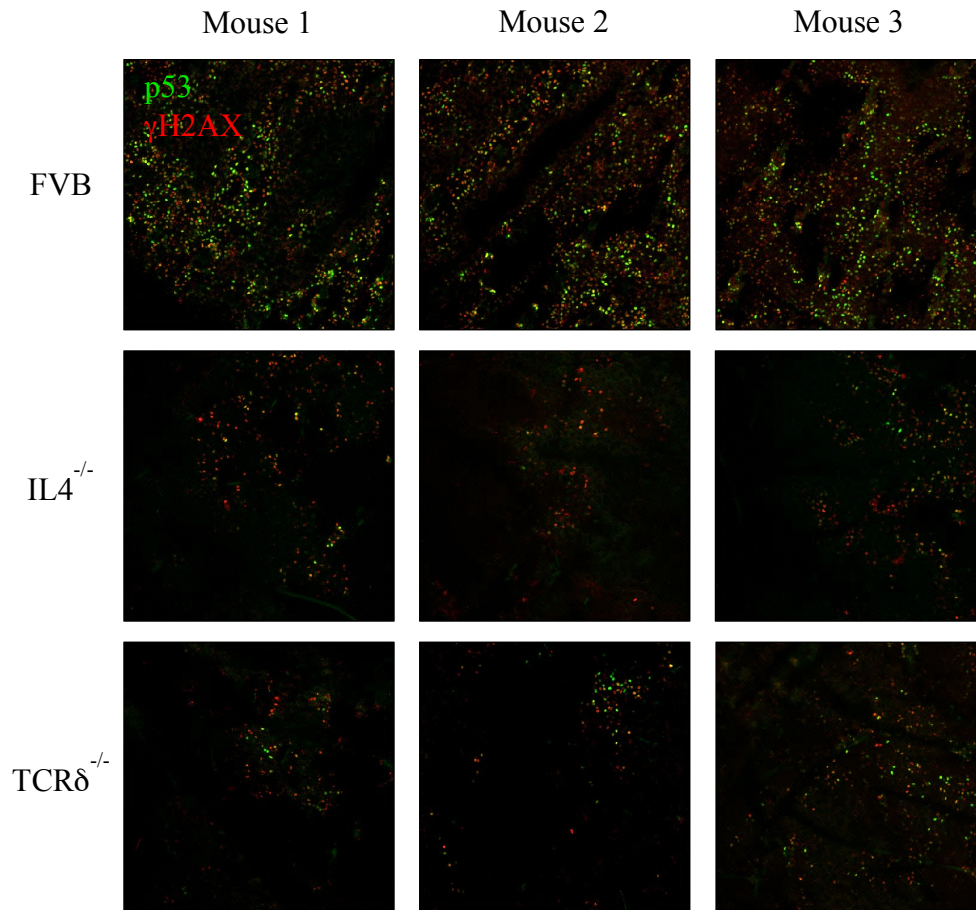


Figure 6.4 | $IL4^{-/-}$ and $TCR\delta^{-/-}$ mice have reduced activation of $\gamma H2AX$ and p53 following DMBA exposure

200 nmol DMBA was applied topically to the ears of FVB WT, $IL4^{-/-}$ and $TCR\delta^{-/-}$ mice (n=3 per group). 7 days later whole epidermal sheets were recovered and stained with purified anti- $\gamma H2AX$ (shown in red) or anti-p53 (shown in green), and an appropriate conjugated secondary layer antibody. Epidermal sheets were visualized by confocal microscopy and a representative field shown for each mouse. Original magnification x40. Secondary-only control stain did not yield any staining.

To test this hypothesis, an assay was optimized for directly quantifying DNA damage in KC. In order to do this, a series of ‘comet assays’ were performed. The comet assay is a horizontal gel electrophoresis assay, which directly measures the extent of DNA damage in single cells. A suspension of cells is immobilized in a liquid gel, which is then pipetted onto a slide. The slide is immersed in solutions to lyse the cells, and liberate the nucleoids onto the slide. Subsequently, the slide is immersed into a buffer suitable for electrophoresis – and a low voltage applied. DNA has an overall negative charge and hence migrates in the field. Following electrophoresis, the nucleoids are stained with a DNA-intercalating fluorescent dye, and visualized with a fluorescence

microscope. A nucleoid from a healthy cell with intact DNA, retains its overall superstructure well, and yields a spheroid shape when examined down the microscope (Figure 6.5 | A, bottom left panel). With increasing frequency of DNA lesions DNA strands are broken into smaller and smaller pieces, which migrate further in the electrophoretic field. This migration yields a tail shape, with the rest of the nucleoid remaining in the head – the overall shape is referred to as a comet. This assay was performed first on control cells provided by a company to validate the assay – these cells were untreated, or had been pre-treated with a low or high dose of bleomycin, to induce DNA damage. As can be seen, with increasing bleomycin treatment, most nucleoids yield longer comets with tails (Figure 6.5 | A). The most commonly reported unmanipulated metric of DNA damage by comet assay is % DNA in tail. This can be assayed automatically by specialist computer software in real-time, when the fluorescence microscope is connected to the computer. Untreated control cells yielded an excellent, low background level of tail DNA between 5 and 10%, which was detectably increased in the treated cells in a dose-dependent manner (Figure 6.5 | B).

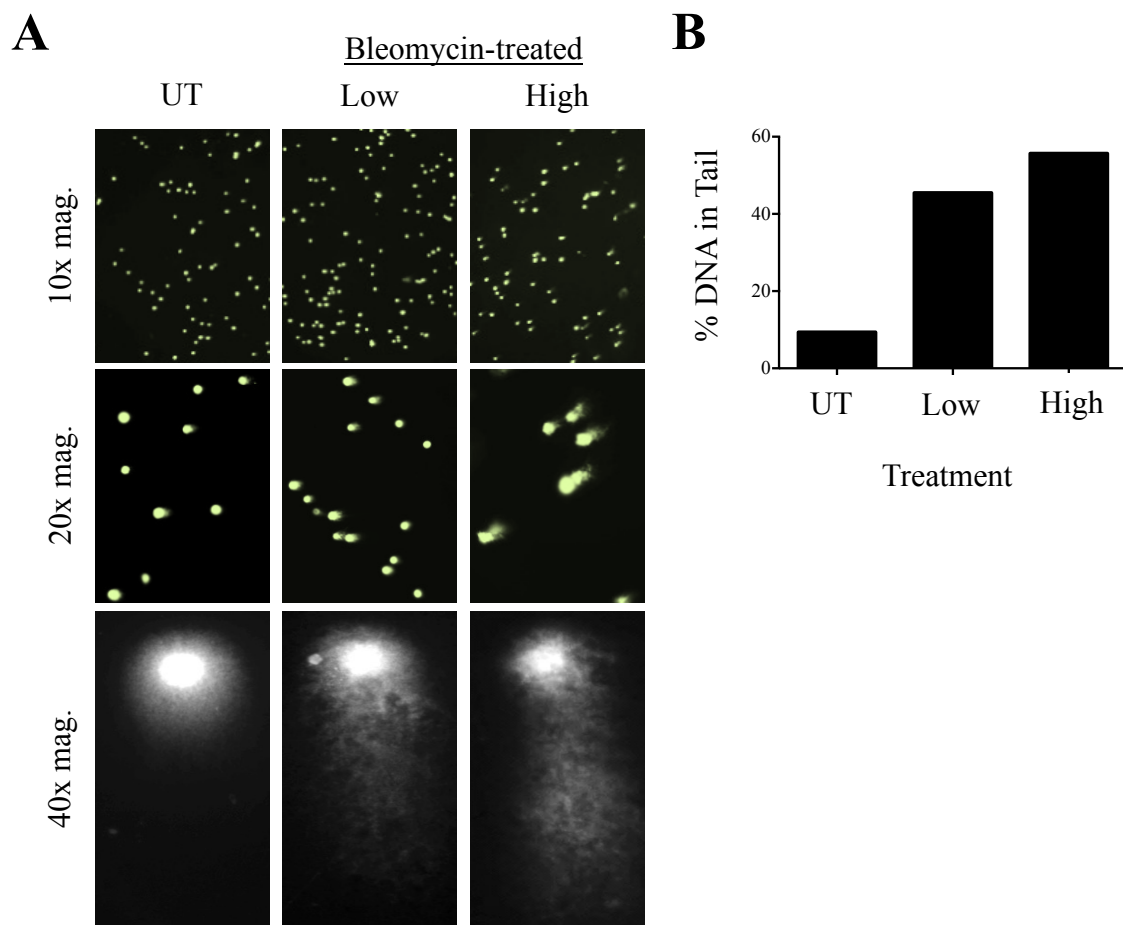


Figure 6.5 | Validation of the comet assay for direct detection of DNA damage

(A) Untreated (UT) or bleomycin-treated ‘control cells’ were used to validate the comet assay. Information pertaining to the bleomycin dose and nature of the control cells was not provided by the manufacturer. DNA was stained with SYBR gold and visualised with a standard fluorescence microscope set-up with the appropriate filter. Mag. denotes magnification. The orientation of the comets under high magnification is altered to fit the images into the figure. (B) Comet Assay IV software quantified the % DNA in the tail.

With the comet assay validated, questions were asked i) whether the actual DNA *damage* accrued by the IL4 or TCR δ -deficient mice was different than the WT control considering the highly differential phosphorylation of H2AX and their high susceptibility to carcinogenesis, and ii) whether any differences in DNA *damage repair* could be inferred by a reduction in comet tail DNA over time.

To that end, comet assays were performed on epidermal cell suspensions isolated from age-matched FVB WT, IL4 or TCR δ -deficient animals at resting state or

following topically exposure to a single dose DMBA. A time-course of DNA-damage was analysed at day 3, 7, 10 or 14 post-topical DMBA. The results from the comet assay showed that the % DNA in tail from untreated FVB control mice was at an acceptable low level (approximately 5 to 10%) and comparable to untreated test cells in spite of the epidermal isolation procedure. Interestingly, already at steady state mice lacking IL4 had a slight but statistically significant increase in tail DNA, suggesting higher level of basal KC DNA breaks in the absence of IL4 (Figure 6.6). Tail DNA breaks in the $\text{TCR}\delta^{-/-}$ were not significantly increased at steady state. Following a single exposure to DMBA, all mice demonstrated a large increase in % tail DNA to around 30%, showing a substantial and measurable increase in KC DNA damage following *in vivo* topical exposure to the carcinogen DMBA. This peaked, like the γH2AX expression, at 3-7 days after DMBA exposure. The level of DNA damage subsequently fell dramatically between day 7 and day 10, suggesting that lesions were being repaired during this time – or alternatively that DNA-damaged cells were disposed of. There was no major difference in peak levels of DSB between WT and $\text{TCR}\delta^{-/-}$ or IL4-deficient mice following this single-dose DMBA, although $\text{TCR}\delta^{-/-}$ animals showed a small but significant increase in % tail DNA 7 days after the DMBA exposure.

Overall this *ex vivo* comet assay demonstrated that the direct damage to KC DNA in $\text{TCR}\delta^{-/-}$ or $\text{IL4}^{-/-}$ mice following a single exposure to DMBA was comparable or slightly higher than in FVB WT. The % DNA in tail decreases between 7 and 10 days on all backgrounds– and this may demonstrate a repair of these lesions at these time points. Therefore it would be informative to quantify tail DNA at day 8 and 9. Due to time constraints during the PhD unfortunately this was not possible, however it is a high priority experiment for future work by the group.

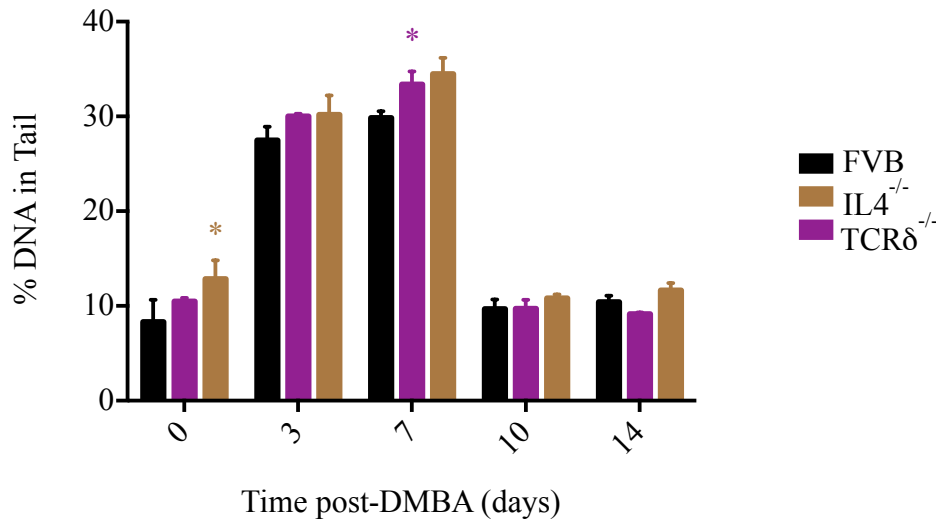


Figure 6.6 | IL4^{-/-} and TCRδ^{-/-} mice have marginally more KC DNA damage at steady-state and following cutaneous DMBA exposure

200 nmol DMBA was applied topically to the ears of FVB WT, IL4^{-/-} and TCRδ^{-/-} mice, and at the indicated time points epidermal cell suspensions were recovered for comet assay. 100 comets were counted per mouse at each time point (n=3 per genotype per timepoint). Data is expressed as the average % DNA in the comet tail per animal $\pm$ 1 SEM. Statistics *via* Students T test where here; * = $P \leq 0.05$.

To further investigate the role of DETC and IL4 in direct immune-surveillance of DNA-damaged KC, the frequency of *hras* mutations was analysed in epidermal KC following DMBA exposure. To this end, a probe hybridisation qPCR assay was performed on epidermal DNA for the specific *hras* codon 61 A to T transversion mutation (hereafter termed mutant *hras*). As a proof-of-principle of this qPCR assay, it was first assayed in DMBA + TPA-driven, established epidermal tumours; in which mutant *hras* is known to be selectively expanded. BALB/c or FVB tumours induced under the DMBA+TPA protocol all demonstrated detectable mutant *hras* (Figure 6.7). This mutation was found selectively in the tumours and was never detectable in the DMBA-treated, but un-transformed, skin adjacent to the tumour. The accumulation of this specific *hras* mutation varied between tumours and between individual animals – as shown by the spread of the data. There was no difference in mutant *hras* levels between BALB/c and FVB background in the DMBA+TPA induced tumours.

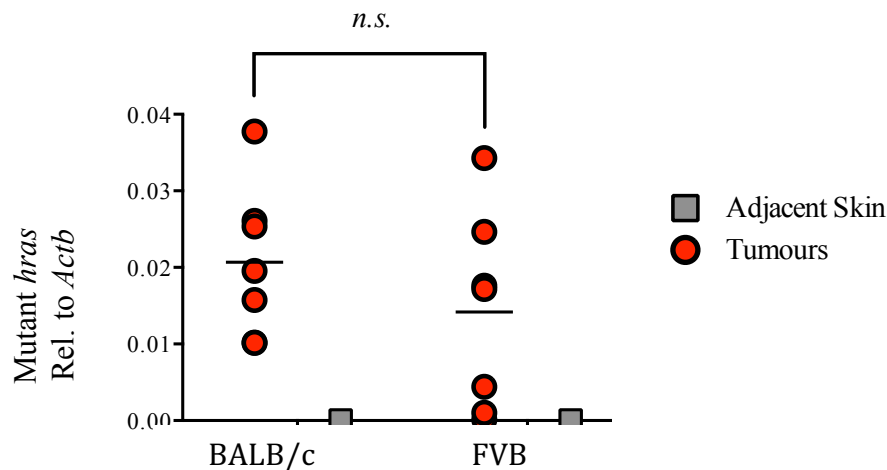


Figure 6.7 | Mutant *hras* is detectable in epidermal tumours and not in skin adjacent to tumours

Whole epidermal tumours (n=7 BALB/c, n=7 FVB) or whole skin adjacent to tumours was excised at the end of a DMBA+TPA tumour experiment, and processed for total DNA. Mutant *hras* was quantified *via* probe hybridisation qPCR. The Taqman probe used was specific for the *hras* codon 61 A to T transversion mutation. Primers for mutant *hras* and *Actb* were designed to detect genomic targets. Data are expressed as mean $\pm$ 1 SEM relative to the control gene *Actb*. A zero-height ‘bar’ was added for adjacent skin data, as no mutant *hras* signal was detected outside of tumours. Statistics *via* Students T test where n.s. = not significant.

Next, the extent of mutant *hras* in the epidermis of FVB, IL4 or TCR δ -deficient animals after five once-weekly doses of DMBA was scrutinized. At this time the animals were still ‘sub-clinical’ for tumours – meaning the skin appeared grossly normal and no ‘spots’ or tumours were yet evident. However, mutant *hras* was already detectable at this stage and remarkably the levels of this mutation was significantly higher in IL4 or TCR δ -deficient animals (Figure 6.8 ; left panel). Mutant *hras* was not detected in untreated skin - neither was it detectable when examining skin after only 3 weeks of carcinogenesis (data not shown). This data again suggests that mice lacking canonical DETC or IL4 have a defective regulation of DNA-repair mechanisms. In contrast, mice given just a single sub-carcinogenic dose of DMBA followed by inflammatory promotion, using the DMBA+TPA protocol, showed no difference in levels of mutant *hras* between FVB WT and IL4 or TCR δ -deficient animals (Figure 6.8 ; right panel).

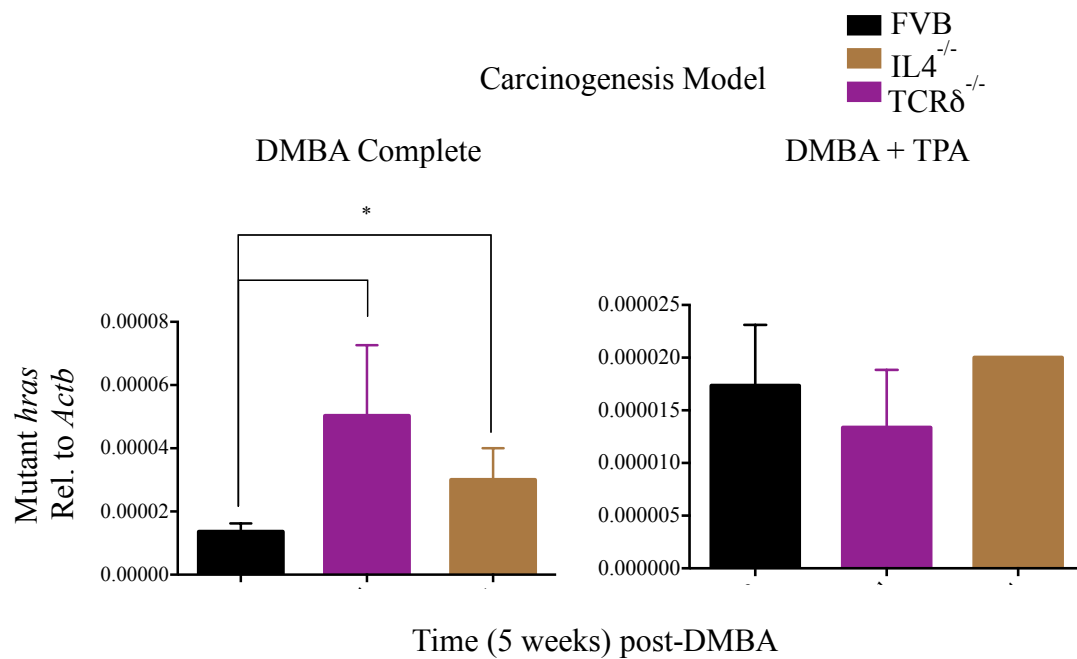


Figure 6.8 | IL4^{-/-} and TCRδ^{-/-} mice have increased epidermal mutant *hras* following repeated DMBA exposure

FVB WT, TCRδ^{-/-} and IL4^{-/-} mice were treated under the DMBA complete or DMBA + TPA carcinogenesis protocols for 5 weeks on back skin (n=5 mice per group). Epidermal cell suspensions were recovered and total DNA extracted. Mutant *hras* was quantified *via* probe hybridisation qPCR. The Taqman probe used was specific for the *hras* codon 61 A to T transversion mutation. Primers for mutant *hras* and *Actb* were designed to detect genomic targets. Data are expressed as mean ± 1 SEM relative to the control gene *Actb*. Statistics *via* Students T test where here; * = P ≤ 0.05.

Discussion

Virtually nothing is known of how T cells or cytokines may modulate the very cell-intrinsic process of DNA repair in tissue EC, however such a mechanism would represent a fascinating and hitherto unappreciated aspect of immune surveillance. In this chapter, I used DMBA-induced KC DNA-damage to examine the contribution of the canonical DETC and IL4 to protection against mutagenesis. I found that mice lacking the canonical DETC or IL4, exhibited comparable DNA damage following acute carcinogen treatment. However, the mutant mice demonstrated a failure to properly upregulate DNA repair machinery following acute DMBA exposure, and had a greater mutational burden in chronically treated skin.

Canonical DETC and IL4 modulate KC DNA repair

γ H2AX foci are induced due to treatment with ionizing radiation (IR), DNA-damaging drugs and chemicals, or mutations in DNA-damage repair (DDR) proteins resulting in genomic instability. Additionally, a variety of normal cellular processes, which induce DSBs promote γ H2AX foci formation. These include replication stress and telomere shortening, VDJ-recombination, class-switch recombination or meiosis²²³. In response to DSBs, H2AX is phosphorylated by one of three kinases; ATM, ATR or DNA-PKc. Formation of γ H2AX is therefore a regulated process, and represents the second-step of the DDR following recruitment of the initial kinases. The complex mesh of chromatin with nucleic acid represents a difficult environment for the recognition of DNA lesions and recruitment of protein mediators for repair. γ H2AX foci promote a chromatin environment conducive to repair, along with recruitment and retention of repair mediators, culminating in p53 activation (Figure 6.0). H2AX importance for modulating DNA repair is particularly well demonstrated in H2AX-deficient mice, which demonstrate radiation-sensitivity, retarded growth, immune deficiency, and a variety of genomic and chromosomal abnormalities²²⁴.

γ H2AX formation in response to ionizing radiation (IR) has been particularly well studied, however less is reported in the literature regarding the kinetics of formation of γ H2AX foci following DMBA exposure. Therefore I first aimed to examine the formation of γ H2AX in KC following topical DMBA in mice. I observed that 5% of live KC in FVB epidermis were γ H2AX⁺ at 24 hours following treatment (Figure 6.3). I had previously performed a series of rapid time-course experiments, examining

KC γ H2AX at 4, 6, 18 hours post-treatment (not shown) and it was not appreciably increased above untreated epidermis at these time-points. This is in contrast to IR exposure which is known to induce γ H2AX in mice within minutes of exposure²²⁵. Following topical DMBA, reports have indicated a small upregulation of γ H2AX foci at 1 day in FVB²⁰², BALB/c mice²⁰², or 2 days in C57BL/6²⁰⁴; supportive of my data. Furthering the time-course out to 3 days and beyond revealed that γ H2AX is in fact maximally upregulated at 3 to 7 days following DMBA treatment; and these foci are maintained up to 10 days. In response to IR treatment, it is known that there is a rapid ‘fast-phase’ of γ H2AX accumulation, followed by a ‘slow phase’ in which γ H2AX foci can persist for months following treatment. This persistence is thought to represent unrepaired DSBs which are in the process of slow, ongoing repair, or lesions that are simply too complex to repair²²⁶. It seems likely that the 3 day delay in maximal γ H2AX formation in the WT animals is due to the requirement of DMBA metabolism by the LC compartment in the skin. In support of this, γ H2AX formation following topical DMBA is known to be almost entirely absent in LC-deficient mice²²⁷, and those lacking the xenobiotic response receptors AhR and PXR²⁰⁴. In sum, I observed robust, γ H2AX formation in WT animals in response to DMBA treatment, which peaked between 3-7 days and then decreased over 2 weeks, likely in accordance with DSB repair.

I further examined γ H2AX responses in mice lacking the canonical DETC, or IL4. I had initially expected to see more γ H2AX foci in the canonical DETC-deficient animals. As γ H2AX is commonly interpreted as a marker of DNA *damage* and these animals have a defective barrier (Chapter 4), one may have expected to see more γ H2AX foci in the mutant mice. Surprisingly, this was not the case, as the mutant animals demonstrated a total failure to maximally upregulate γ H2AX at 3 or 7 days following DMBA treatment (Figures 6.3, 6.4). Additionally, p53 was also almost entirely absent at 7 days following treatment when compared to WT animals (Figure 6.4). As previously elaborated, the formation of γ H2AX and p53 are regulated processes that are part of the DDR mechanism. The absence of γ H2AX (second step in the DDR) and p53 (a terminal component of DDR) suggest that the DDR pathway is suboptimally activated in the mutant mice – and not simply that the mice have less DNA damage induced *per se*.

To examine this further, I performed the comet assay to quantify KC DNA damage following DMBA treatment. An advantage of this assay is that it allows one to directly visualize DNA lesions, and hence DNA damage; and infer repair of lesions by monitoring comet tails over time. I observed that WT and mutant mice showed greatest tail DNA (DNA damage) at 3 and 7 days (Figure 6.6), concurrent with and therefore supporting the γ H2AX data. The canonical DETC- and IL4-deficient mice did not show a large difference in tail DNA from WT. However, the IL4 had slightly higher tail DNA at steady state, while the canonical DETC-deficient mouse had slightly higher tail DNA at 7 days following DMBA compared to WT (Figure 6.6). The epidermis is a particularly mitotically active tissue, and it seems reasonable that a modulation of DNA repair by DETC/IL4 would be detectable at steady state. Despite this, I believe the tail DNA difference in the mutant mice is too small to come to that conclusion. Therefore, the comet data supports the notation that KC in mutant mice are DNA-damaged to a comparable extent as WT animals following acute DMBA exposure, while the γ H2AX and p53 deficiency in mutant mice suggests a defect in the DNA repair process. In support of this, WT and TCR δ -deficient animals have been shown to acquire a comparable number of DNA CPD lesions rapidly following UV exposure, although the repair of these lesions over time was reduced in TCR δ -deficient animals²¹⁹. While IL4 itself been shown to promote PARP-mediated DNA repair *in vitro*²²¹. The exact mechanism by which DETC/IL4 modulates DNA repair is currently unknown and further studies are clearly warranted. H2AX is phosphorylated by ATM, which itself is recruited rapidly by the DSB-sensing MRN complex. Therefore, deficiency in ATM or the MRN complex could explain the lack of γ H2AX in the mutant mice. RAD50 is a major, non-redundant component of the DSB MRN sensor²²⁸, and the *Rad50* gene is conspicuously buried within the Th2 locus where it regulates expression of *Il4*, *Il5* and *Il13*¹⁹². One possible although highly speculative mechanism would be that a positive, reciprocal regulation of *Rad50* by type 2 cytokines promotes expression of the MRN complex; and hence ATM recruitment and H2AX phosphorylation in response to DNA damage. DETC are known to produce IL4 and IL13 and hence the TCR $\delta^{-/-}$ would also demonstrate this phenotype. This reciprocal regulation of *Rad50* by IL4 has not been described in the literature, but it is being investigated in the laboratory in my follow-up work.

DETC/IL4-modulated DNA repair protects against KC mutagenesis

Comet analysis demonstrated that the KC tail DNA was comparable at 10 and 14 days after topical DMBA exposure in the WT and mutant mice (Figure 6.6); suggesting that by 2 weeks, DSBs were sufficiently repaired in all mice after a single dose of DMBA; despite the fact that the H2AX and p53-deficiency in the mutant mice indicated a defect in DDR. Alternatively, the damaged cells were sloughed off the epidermis or died comparably in all mice. I postulated that the DDR defect would be more consequential in mutant mice that had been treated several times with DMBA, and thus would accrue more mutations as a result. Therefore I proceeded to examine repair of DMBA-induced lesions in a different way – by studying a commonly induced mutation, namely Q61L *hras*. A proof-of-principle experiment established the sensitivity of a Taqman-based qRT-PCR assay for detecting genomic mutant *hras*. I detected *hras* with the DMBA-induced codon 61 mutation (often termed Q61L) in all DMBA-induced, established tumours on a FVB and BALB/c background (Figure 6.7). This is in agreement with reports showing $\geq 90\%$ of DMBA-induced murine squamous cell carcinomas are driven by activating mutations in *Ras* genes – of which the most common are *hras* missense mutations, and particularly the Q61L mutation¹⁹⁷. *Hras* and even Q61L mutations are also highly prevalent in human cutaneous squamous cell carcinomas²²⁹, which most commonly may not be initiated by DMBA. The skin adjacent to tumours on FVB and BALB/c mice did not yield detectable mutant *hras* (Figure 6.7) – despite the fact that the entire back skin area had been DMBA-treated. These observations agree with the notion that mutant *hras* is a crucial driver of cutaneous tumours across species.

After 5 weeks of once-weekly DMBA treatment, mutant *hras* was detected *via* Taqman qRT-PCR in 100% of WT FVB mice (Figure 6.8 | Left Panel). At this time-point the skin appears overtly normal - but the presence of *hras* mutation foretells the eventual tumour phenotype in these animals (Chapter 5). Strikingly, mutant *hras* was significantly increased in TCR $\delta^{-/-}$ and IL4 $^{-/-}$ mice after 5 weeks of DMBA treatment (Figure 6.8 | Left Panel), supporting the notion that canonical DETC and its mediators positively modulates DNA repair. Under a DMBA+TPA protocol, mutant *hras* was detectable in WT FVB animals, in agreement with previous reports that Q61L is detectable as early as 2 weeks following initiation of DMBA+TPA protocol in FVB

animals²⁰². Unlike the DMBA complete experiment, mutant *hras* was not significantly different in mice lacking the canonical DETC or IL4 (Figure 6.8 | Right Panel). I suggest that this comparable level of mutant *hras* in all mice indicates that DNA repair-modulation by DETC/IL4 is not as consequential when only a single dose of genotoxic chemical is applied to the animal. TCR δ -deficient mice are however known to be more susceptible to carcinogenesis also in the DMBA-TPA model. Despite a comparable level of mutant *hras* in this model, the canonical DETC-deficient mouse may go on to accrue more tumours due to its non-redundant role in regulating cutaneous inflammation⁴⁷; as inflammation drives clonal expansion and tumor growth in this model.

One caveat to these interpretations is that the introduction of the Q61L missense mutation must be induced by the repair process itself. Therefore, DNA repair cannot be absent but rather defective or less accurate in the TCR δ and IL4-deficient animals. I speculate there is a fine-tuning of the magnitude of repair by DETC and/or their products. Alternatively, perhaps the choice of repair pathway is altered in TCR δ and IL4-deficient animals – rather than the magnitude of the response. As stated in the introduction there are two main pathways for repairing DSBs; HR and NHEJ. The choice of which pathway to use to repair DSBs is dictated in part by competition between the two initiating DSB sensors in the respective pathways; MRN (HR) or Ku (NHEJ)²³⁰. NHEJ is typically thought of as lower fidelity than HR, which uses a sister chromatid to catalyse template-driven repair. When NHEJ does not repair in a timely fashion, it switches to an even lower fidelity ‘mode’ termed alternative-end joining (aEJ)²³¹. Therefore, if a lower fidelity repair mode is used to try and repair mutant *hras*, then missense mutations could accrue more readily. In other words, modulation of repair by DETC/IL4 may not be an ‘all-or-nothing’ phenomenon, but rather tip the balance of repair in favour of one modality over another – ultimately affecting the quality of the repair job, in addition to its rapidity. This possibility is being examined in future planned work.

The putative target cells for the *hras* mutation are mostly the proliferating, highly active bulge region KC²³². Likewise, in cutaneous tumours induced by transgenic expression of mutant *kras*, the hair follicle bulge stem cells, but not the TA or further

matrix cells led to papilloma formation²³³. Interestingly, the higher-fidelity DNA repair pathway, HR, can only be used when cells are in an active phase of the cell cycle – due to the requirement of a sister chromatid for template-driven repair. I have previously shown that a DETC-derived IL13 regulates the balance of epithelial turnover in the epidermis (Chapter 4), and it has been reported that mice lacking the canonical DETC have fewer KC-supportive growth factors and cytokines²³⁴. Therefore I suggest that if KC in the mutant mice proliferate less, then they would be less able to use the higher-fidelity HR mechanism to accurately repair DMBA-induced lesions, and they would conceivably get more mutation. This would represent a different mechanism by which TCR δ and IL4-deficient animals get more mutations – one in which it is not a direct modulation of repair processes that is responsible – but rather a state of proliferation in the tissue more amenable to accrual of mutations. Differences in KC homeostasis and proliferation to DMBA treatment between FVB WT and TCR δ and IL4-deficient animals are currently being examined.

Conclusions

Mice lacking canonical DETC or IL4 have a significant deficiency in KC DNA repair factors, and have a higher mutation burden after repeated genotoxic insult. This modulation of DNA repair processes by DETC and/or their effectors may in part underlie the increased susceptibility of TCR $\delta^{-/-}$ and IL4 $^{-/-}$ animals to DMBA-driven cutaneous carcinogenesis, thus reinforcing the contribution of the DETC to maintaining tissue homeostasis in multiple settings and by multiple mechanisms. Indeed, some of the most pervasive and common insults to the epithelium are DNA-damaging; UV and IR, genotoxic chemicals such as ubiquitous PAHs in the environment as a result of industrial processes, and viral infection. Therefore, the DETC may protect against a variety of genotoxic insults commonly encountered at body barrier surfaces, by directly regulating DNA repair. These mechanisms may dovetail nicely with the DETC-IL13 system described in Chapters 3 and 4, whereby an increased epithelial turnover would presumably aid shedding of DNA-damaged irreparable cells, along with increasing the physical barrier *via* epidermis thickening, reducing topical insult to ubiquitous PAHs. Finally, the presence of DNA damage sensor gene *Rad50* within the type 2 cytokine locus, and the regulation of these cytokines by RAD50, remains highly conspicuous. Perhaps this arrangement further supports the notion of a fundamental molecular link between atopic and allergic immunity, and malignancy. Such a relationship is beginning to be explored in the nascent field of Allergo-Oncology.

Chapter 7 | Final Discussion, Conclusions and Future Work

The intraepithelial lymphocytes comprise a conspicuously large pool of immunocytes constitutively resident at body barrier surfaces. The monoclonality and restriction of the DETC TCR, along with its sequestration in the skin, are characteristics incompatible with the traditional activation modality of naïve lymphocytes. Therefore, new thinking was required to understand how the IEL, and DETC in this case, might become activated and function, and how such functions affect tissue health. Innate-like $\gamma\delta$ T cells show very strict localization within sub-compartments of tissues. The DETC are localized within a separate anatomical niche to the dermal-resident, IL17-producing $\gamma\delta$ T cells. Likewise in the gut, IFN γ -producing IEL are segregated from IL17-producing $\gamma\delta$ T cells in the lamina propria²³⁵. This strict hierarchy, along with TCR sensitivity tuning and programming during ontogeny, permits these cells to respond rapidly to predictable insults at the body barrier surfaces.

The attractive notion that tissue-resident DETC may have an immune surveillance role was first supported by observations that they express potent cytolytic granzymes and perforin¹⁰⁷; suggestive of a direct killing capacity. Subsequently, they were shown to be capable of killing melanoma cells²³⁶ *in vitro* and *in vivo*, and reject transplanted tumours²³⁷. The idea that DETC responded to acute epidermal damage, and played a role in restoring homeostasis, was first explored in the early 2000s. $\gamma\delta$ T cells were shown to have a role in promoting full-thickness skin wound repair¹⁴⁵, and be important for skin homeostasis²³⁸. Subsequently, skin $\gamma\delta$ T cells were shown to be non-redundant regulators of cutaneous inflammation⁴⁷, and such potent immunomodulatory functions were shown to be consequential for cutaneous tumour development¹⁶². It was later shown that this tumour protective effect of skin $\gamma\delta$ T cells was specifically carried out by the DETC⁸⁸. The mechanism however, by which DETC discriminate between healthy and disordered cells remained enigmatic; although TCR-restriction and expression of innate-like immunoactivatory receptors

suggested that the DETC surveilled for predictable moieties indicative of tissue stress. Further elegant studies by Strid and others described the molecular axis known as lymphoid stress surveillance, and demonstrated that DETC-mediated sensing of KC endogenous stress ligands was sufficient to induce pleiotropic changes in the immune compartment in the skin⁸⁸; and modulate downstream immunity⁹⁰. In sum, this model of DETC:KC interactions updated the classic immune surveillance framework, and constructed a picture of the DETC playing a crucial role in modulating various aspects of epithelial biology; and responding to heterogeneous insults. Also revealed by Strid and others was the particular bias of type 2 immunity elicited and modulated by the DETC; dovetailing with a role of type 2 immunity being particularly important for immune surveillance at body barrier surfaces. In support of this, I have herein presented data that IL13 is produced by DETC at steady state and following injury, and this was crucial for a canonical KC stress response and epithelial health in general. Observations by others show that the ILC in the dermis is also capable of producing IL13 (along with other type 2 cytokines), and is activated by epimicrobiome products. Therefore, crosstalk between epithelial and stromal cells, with resident immunocytes, likely represents a series of ongoing molecular ‘conversations’, which are crucial to homeostasis of the tissue; both at steady-state and following insult.

Immune Surveillance as a Homeostasis Mechanism

Somatic cells are normally subject to rigorous quality control mechanisms, whereby endogenous stress (such as DNA damage) or exogenous stress (such as UV irradiation, nutrient deprivation) triggers cell-intrinsic programs for restoring homeostasis. An example of such a program would be the DDR, whereby the response of a cell to DNA damage is an attempt to repair that lesion. Should these cell-intrinsic mechanisms fail, the cell can display numerous molecular signals to induce accumulation and activation of immunocytes. Recruitment of phagocytes helps to clear-up dead and dying cells, while cytotoxic CTLs and NK cells kill cells with altered or missing self or induced-self. The ultimate display is achieved by cell necrosis and immunogenic cell death, in which ligands normally hidden from the immune system are revealed and further amplify immune surveillance. I reveal in this thesis that an immunocyte constitutively resident at the body barrier has a fundamental role in regulating the biology and homeostasis of the skin – not only in

response to acute trauma but also at steady state. This is supported particularly by the finding that mice lacking the canonical DETC or IL13 show a poorer skin barrier at steady state (Chapter 4) and upregulated stress ligands (Chapter 3) – a classic example of a stressed cell signal. DETC, and IEL more generally, by virtue of their location, and non-classical activation mechanisms, are well placed to carry out continual immune surveillance functions; and I reveal that such ongoing stress surveillance functions fundamentally affect the skin barriers' ability to handle physical, chemical and genotoxic insult.

The main tool for investigating DETC function in this thesis has been to examine the pathological consequences of their absence. I demonstrated that IL13 produced rapidly by the DETC fine-tunes the canonical epimmunome response; representing a mechanism of immunocyte-EC crosstalk not previously described. KC are known to produce a number of cytokines in response to perturbation including IL25, IL33 and TSLP; a repertoire termed the 'epimmunome'¹¹. These cytokines have a number of powerful effects on epithelial biology, and therefore modulation of the epimmunome represents a potent way of tuning epithelial responses to injury. Consistently in my work one of the genes most strongly modulated by IL13 was TSLP. This cytokine seems to have multiple roles at body barrier surfaces including promoting barrier defense by inducing itch²³⁹ and AMP²⁴⁰, regulation of inflammation in the gut¹⁸⁸ and skin in tumour models³⁸, tuning of DC maturation and promoting Th2 priming, involvement in AD³⁷ and asthma in the lung³⁵. Such functional pleiotropy has led to some terming TSLP a master regulator of barrier immunity³⁴. Likewise I showed, that IL33 was also prominently regulated by the DETC-KC cross-talk. This cytokine, unlike many conventional DAMP, in healthy cells has transcriptional functions including inhibition of cell death-induced inflammation²⁴¹. This is in agreement with the described role of the DETC in modulating cutaneous inflammation – and also my work demonstrating that IL13-deficient mice (which would by inference have less KC-IL33) respond weakly to cutaneous TPA (Chapter 4). DETC tuning of the KC epimmunome could thus be consequential in multiple settings.

A role of immune surveillance in helping to restore tissue homeostasis is likely not limited to purely pathophysiological settings. I have shown that absence of the canonical DETC or IL13 yields mice with a poorer physical skin barrier when

measured by TEWL – even at steady state – and poorer repair of topical abrasions. I observed that IL13 enhanced the transit of KC through the epithelium. Such an increase of KC transit would conceivably replenish KC in the superficial layer, which have been removed by tape stripping, more quickly. This mechanism could also aid the expulsion of attached parasites/chemicals – augmented by TSLP-driven itch (Chapter 4 Model). Tuning of the ‘epithelial elevator’ may represent a conserved mechanism of IEL-control of epithelial health, as IEL/IL13 modulation of gut enterocyte transit has also been shown. The importance of this ‘upwards push’ of the immune system has been shown in a wide range of settings, including carcinogenesis in the skin¹⁷⁷, and parasite expulsion in the gut¹³⁵. Therefore, DETC elicited immunity is likely consequential to both acute and chronic perturbations of homeostasis.

Immune Surveillance and Type 2 Immunity

Epithelial cancers are products of a series of events starting with dysregulated and stressed EC. It is now clear that challenges to EC can trigger discrete pathways promoting the release of specific cytokines, chemokines, and expression of stress antigens on the EC surface. Together this can powerfully drive immune responses, initially from cells resident in the epithelial and sub-epithelial compartment. The initial response to EC challenge and damage is often a very rapid type 2 immune response – as I demonstrated in Chapter 3.

In line with the ‘prophylaxis’ hypothesis of allergy/cancer relations, type 2 immune mechanisms at the body barrier may serve to remove or neutralize noxious challenging substances, clear waste, repair the tissue, dampen inflammation, and re-establish tissue homeostasis. It may also directly contribute to the elimination of damaged cells together with cytolytic mechanisms from resident IEL and other immunocytes. Rapid type 2 immune responses at body surfaces thus prominently contribute to immune surveillance of dysregulated EC. Since epithelial dysregulation contributes notably to a multitude of inflammatory diseases, this may not only be important in control of (pre-)malignancy but could be important at disease-initiating stages in a variety of diseases.

Interestingly I made the highly novel observation that tumour-burdened mice had

increased serum IgE; this was wholly-dependent on IL4 and partially dependent on IL13. Although the mechanism by which this antibody is raised lay outside the scope of this thesis, it is an observation highly germane to the emerging field on allergy-oncology, which aims to examine the relationship between allergy and cancer. In spite of many speculations and associations there is little evidence for a strong association between allergy and *overall* cancer risk. However, given that allergic disease occurs primarily at outer epithelial surfaces it is logical to examine the incidence of cancer at specific tissue sites, particularly those at which allergic disease is prevalent; such as the skin, respiratory, and gastrointestinal tracts. A large meta-analysis of more than 400 studies of relationships between allergy and cancer reported a preponderance of inverse allergy–cancer associations, and interestingly this was particularly strong for cancers of tissues that interface with the external environment, such as skin, mouth, throat, colon, and cervix⁹¹. The large number of published association studies nevertheless often paints a conflicting picture, some of which may be due to methodological constraints. In addition, the genetic and phenotypic heterogeneity of tumors, and the complex inflammatory niche in which they reside, may also confound epidemiological association studies¹²⁰.

Immune Surveillance and regulation of DNA repair

As argued above, the process of immune surveillance, whereby the immune system eliminates pre-malignant cells, or positively modulates tissue health, exerts a major homeostatic function. I finished this thesis with an exploratory chapter, examining how the canonical DETC modulates the otherwise very cell-intrinsic process of DNA repair in KC. This contextualized the acute mechanisms of DETC immune surveillance described in Chapters 3 and 4, with the consequence of their absence on carcinogenesis in Chapter 5. One mechanism by which the DETC can respond to acute genotoxic stress is *via* the immunoactivating NKG2D receptor. NKG2D-ligands are known to be induced by a huge number of stress pathways including DNA damage and DDR, heat shock, proliferative stress, wounding, cell senescence, and UV irradiation²⁴². Therefore stress-ligand expression, such as *Rae1* in murine KC, allows for rapid, tissue-based stress surveillance and elimination of pre-malignant cells. Outside of the skin, CD4⁺ T cell-mediated immune surveillance of pre-malignant hepatocytes has been shown to limit early liver cancer development²⁴³, while the DDR program functions generally as an anti-cancer barrier in early

tumorigenesis²⁴⁴. Notwithstanding the efficacy of these mechanisms, an affect of a cell extrinsic mechanism (from an immunocyte as for example from DETC) on modulating the cell intrinsic mechanisms (such as DNA repair) – as part of the immune surveillance toolkit has been little described. I demonstrated that animals lacking the canonical DETC, or IL4, failed to maximally upregulate important parts of the DNA repair machinery, while demonstrating comparable DNA damage. This demonstrates in a similar vein to Chapter 3, the requirement of IEL-EC crosstalk in maintaining the fundamental biology of the epithelial barrier. I examined the consequence of this failure by quantifying the extent of mutant *hras* in the epidermis after several weeks of treatment. Mutant mice had significantly greater mutant *hras*. I suggested these findings may also underlie the mutant mice increased tumour burden (Chapter 5). A role of IL4 on modulating PARP-mediated DNA repair within human and murine cells²²¹, and a role for DETC on modulating repair of UV-induced lesions within murine KC²¹⁹, has been described previously. However, a direct effect of DETC, or immunocyte-derived cytokines, on modulating KC DNA repair *in vivo* has not been previously shown. I suggest that potent cytokine released by DETC as a consequence of genotoxic stress promotes genomic stability of the tissue – and is another weapon in the immune surveillance armamentarium.

A problem with the immune surveillance idea in general is demonstrating that it actually occurs *in vivo*. I believe that by examining the consequences of a lack of DETC in a stepwise fashion, as I have here, I have partly addressed this problem. Specifically, mice lacking the canonical DETC have a poorer barrier and a reduced DDR response to acute genotoxic stress. This results in greater mutational burden in the intermediate term, and increased tumour acquisition longitudinally. Additionally, the effect of IL13 on promoting epithelial turnover (Chapters 3, 4) would also likely promote shedding of DNA-damaged cells, thus reduced mutational load in the epidermis. Likewise, desquamation and shedding would presumably aid clearance of genotoxic chemicals on the skin – as would itch which is promoted by (IL13-regulated) TSLP²³⁹. Therefore, it seems likely that the immune surveillance mechanisms described do not function in isolation, but rather in an integrated manner conducive to maintaining tissue health.

Immune Surveillance and Type 2 Immunity in Human Skin

There is no direct equivalent of the DETC in human skin, and it is clear that there is considerable evolutionary divergence between the mouse and human TCR γ and TCR δ loci²⁴⁵. Despite this, mouse and human $\gamma\delta$ T cells share a number of functional and developmental properties – particularly tumour cell recognition *via* natural killer cell receptors (NKR) and potent cytokine secretion. Like the mouse, tissue site-specific $\gamma\delta$ T cells also exhibit particular TCR gene segment usage, and a large number of activating ligands have been described for different human $\gamma\delta$ T cell populations. The V δ 1 T cell has been shown to be activated by the MHC class I homologue MICA – comparable to Rae1 in the mouse²⁴⁶. Various human $\gamma\delta$ T cells are activated by phycoerythrin comparably to mouse $\gamma\delta$ T cells²³⁵. Indeed, it is clear that human $\gamma\delta$ T cells, like those in the mouse, can detect transformed cells *via* NKG2D to promote cytolysis and mobilize anti-tumour potential. Indeed, the anti-tumour potential of human $\gamma\delta$ T cells is beginning to be exploited clinically, such as with the use of aminobisphosphonate drugs that upregulate $\gamma\delta$ T cell-activating phosphoantigens⁸⁹. These observations suggest that $\gamma\delta$ T cell-mediated stress surveillance is comparable in man and mouse – even if not by the exact same cell type. In the human skin, both epidermis and dermis contains a population of V δ 1 $\gamma\delta$ T cells, which express the NKG2D receptor. Stimulation of dermal $\gamma\delta$ T cells *ex vivo* induces large amounts of TNF, IFN γ , and they express receptors for homing to non-inflamed skin²⁴⁷. Likewise, human epidermal $\gamma\delta$ T cells have been shown to be high producers of type 2 cytokines – including IL13²⁴⁸. The $\gamma\delta$ T cell, and immunocyte compartment in human skin in general is thus quite heterogeneous; and it seems likely that human skin contains cells capable of performing a DETC-equivalent function. Indeed, recent work demonstrates that human ILC2s promote cutaneous wound healing in an IL33-dependent manner²⁴⁹. Therefore I suggest that the human skin has a different albeit functionally similar stress surveillance system to the mouse.

The role of type 2 cytokines – and IL13 in particular - in human skin remains hotly debated, particularly in the context of skin disease such as atopic dermatitis and psoriasis. Several studies have shown a correlation between skin barrier dysfunction and type 2 cytokines in human skin, resulting in the consensus that IL4/IL13 is deleterious for skin barrier function. Genetic association studies also show a link

between polymorphisms in IL13, and AD^{250,251}. Likewise, IL13 is known to be expressed in atopic skin lesions⁵⁹, and recently it has been shown that antibody-mediated blockade of the IL4R α 1 subunit can be highly effective in treatment established moderate-to-severe AD¹⁵⁹. Therefore, the IL4/IL13 system is firmly implicated in AD immunopathogenesis. Despite these associations in dysregulated and diseased skin, less thought has been given to *why* resident immunocytes in the skin produce IL13, some constitutively, and what the effect of homeostatic IL13 is. Is there for example ‘a correct’ amount’ of cytokine, ‘a right time’ and ‘right place’ (epidermis/dermis), and is it important which cell produces IL13? The strict hierarchy of which cells seed which subcompartment within the skin, and the divergent cytokines they produce, seem to agree with this notion. I demonstrated a particular effect of IL13 on promoting repair of epidermal abrasion injury, but not full skin thickness wounding (Chapter 4), and a less consequential effect of IL13 on subcutaneous tumours than epidermal tumours (Chapter 5). It is possible that the answer to these questions may also determine when IL13 may indeed be beneficial (a ‘goldilocks effect’), as I believe I have shown in mouse models, versus detrimental. Outside of the skin IL13 is known to have a beneficial tissue repair function in the lung^{131,139}. While some *in vitro* reports suggest that IL13 negatively affects EDC gene expression and therefore skin biology¹⁶⁰. Notwithstanding the limitations of *in vitro* cultures, I believe that the canonical view of a detrimental effect of IL13 on epithelial function is too simplistic. In particular, studies usually do not take into account the spatiotemporally-coordinated regulation of these cytokines in the skin. I revealed a potent steady-state production of IL13 by DETC, and inducible production following injury; along with stress-modulated IL13 receptors on KC. While this mechanism may be detrimental to skin health in established skin disease, I suggest it aids homeostasis in a non-immunopathological context. Indeed, immune surveillance by its nature is mainly important in the initiation phase of tissue damage and tumor control – for maintenance of tissue homeostasis. The same effector molecules and mechanisms may play a very different role during the progression phase of tissue damage and tumor growth. Failure to eliminate the original damaging substance, damaged cells, or to repair the tissue may lead to a continual stress response with excessive release of DAMP, persistent stress-ligand expression, inflammation, and a chronic wound-healing response and this may determine the transition point between

beneficial and detrimental functions of type 2 immunity in the course of disease. Similarly, continual exposure to noxious environmental substances may eventually overwhelm the immune surveillance mechanisms keeping the damage in check and result in pathology or tumor growth.

Clearly, we have much more to learn about DETC, IL4/IL13 and their complex receptor arrangement before we can truly understand their role in health and disease. Various skin-resident immunocytes, in different tissues, produce IL13 in response to different conditions. This consistency of observation strongly supports the notion that the IL13 system has a fundamental role for body-barrier maintenance. This is in agreement with the expanding role of allergic immunity from simple parasite defense, to an integrated mechanism helping to maintain tissue homeostasis.

Conclusions

I believe my data overall provides a novel perspective on the role of type 2 immunity in tissue homeostasis and tumour immune surveillance; connecting atopy and cancer *via* molecular communication between intraepithelial lymphocytes and epithelial cells. Given the considerable and increasing burden of atopic and malignant disease, these data have exciting translational appeal to human medicine.

Future Work

DETC and KC Crosstalk and Lymphoid Stress Surveillance

Further work is needed to characterize the afferent mechanisms of stress surveillance in the epidermis, along with how DETC-mediated stress surveillance modulates tissue homeostasis. The DETC TCR ligand, although believed to be SKINT1, is not yet known; and convincing crystallography binding data has proven elusive. I have demonstrated an effect of DETC-derived IL13 on promoting the canonical epithelial stress response; the readout of which was expression of epimune cytokines IL33 and TSLP (Chapter 3). The actual mechanistic basis for this response is unknown – for instance, whether IL13 *via* the type 1 IL13 receptor directly induces IL33/TSLP expression in KC. Analysis of cell signaling pathways in KC exposed to recombinant IL13 would prove insightful and could be carried out in a mechanistic fashion *in vitro* to avoid confounding stimuli.

The canonical DETC and its products had clear, measurable effects on the physical barrier integrity of the skin, and the tolerance of the epidermis in particular to abrasion injury (Chapter 4). One possible explanation of this phenotype was the promotion of epithelial turnover by IL13, revealed by my BrdU experiments. It would be highly beneficial to examine the reformation of the SC components in tape stripped BALB/c and IL13-deficient mice. It seems logical that a reduced delivery of cells to the granular layers of the epidermis in the IL13-deficient mice, would also result in a poorer replenishment of the acellular components of the CE crucial for preventing water loss; the extracellular ‘mesh’ of extruded lamellar body components and lipids. A simple experimental approach to this would be to tape strip mice at different intervals, and stain the strip itself for CE components. I hypothesized that modulation of the epithelial turnover by IL13/DETC was likely to be highly consequential for not just repair of acute injury, but chronic epithelial dysregulation seen in carcinogenesis.

It would be interesting to examine whether artificial acceleration of turnover would also suppress carcinogenesis, perhaps with additional of recombinant IL13, in a similar vein to studies demonstrating differentiation-induced suppression of skin carcinogenesis.

DETC-modulation of DNA repair

I demonstrated a role of DETC/IL4 in promoting accumulation of DDR components to acute genotoxic insult in the epidermis. Absence of these responses led to greater mutation burden in the epidermis. I am currently investigating whether there is a direct effect of DETC cytokines on expression or phosphorylation of key DDR components, including ATM, p53 and the DNA damage sensor RAD50. The H2AX-deficiency in mutant mice could conceivably be explained by poorer mobilization of ATM, which forms H2AX in response to DNA damage. Also, a disparate level of cell death or proliferation in mutant mice could underlie the H2AX-deficiency seen, as stated in Chapter 4. The state of proliferation, apoptosis and cell death in the epidermis in response to DMBA is currently being assayed in wildtype FVB and mutant mice, by multiparametric flow cytometric analysis of markers of cell death and proliferation.

IL13 and homeostasis of human skin

The role of type 2 mediators, and IL13 in particular, on human skin homeostasis should be investigated. Particularly, the effect of IL13 on steady-state human skin, and after acute injury. Human skin explant cultures make an excellent model in which the effect of rIL13 could be examined in a reductionist fashion. A central finding in this thesis, that IL13 promotes murine KC epimune expression, could be easily investigated in human keratinocyte cultures, perhaps derived from foreskin samples. Certain variants of IL13 have been shown to correlate with atopic disease²⁵². It could be investigated whether mutations of IL13, the IL13 receptor or signaling components correlate with the skin barrier function and TEWL directly.

Publications associated with this Thesis

T Dalessandri, J Strid. **Beneficial autoimmunity at body surfaces – immune surveillance and rapid type 2 immunity regulate tissue homeostasis and cancer.** *Front. Immunol.* (2014). doi: 10.3389/fimmu.2014.00347.

T Dalessandri, G Crawford, M Hayes, RC Seoane and J Strid. **IL-13 is key to intraepithelial lymphocyte regulation of tissue homeostasis and protection against carcinogenesis in the skin.** *Submitted*

Introductions were supplemented in part with material from the published review, and my PhD Early Stage/Upgrade assessment report (unpublished).

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