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Identification and functional analysis of type 2 innate lymphoid cells in the skin and in lesional skin biopsies of patients with atopic dermatitis

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Abstract

Over the past four years, a previously unrecognised family of innate effector cells has been identified. Their comprehensive functional capabilities range from lymphoid organogenesis, tissue remodelling, wound healing, immune protection and homeostasis to contribution to inflammation and allergic responses. Here we investigate the presence and function of type 2 innate lymphoid cells (ILC2) in the skin. We show that human ILC2 are resident in human skin and express RORA and GATA3, and skin homing receptors. ILC2 further infiltrate the skin after allergen challenge, where they produce the type 2 cytokines IL-5 and IL-13. Skin-derived ILC2 express the IL-33 receptor ST2, which is up-regulated during activation. Signalling via IL-33 induces type 2 cytokine and amphiregulin expression, and increases ILC2 migration.

Atopic dermatitis (AD) is a chronic inflammatory skin disorder. Current evidence suggests that both skin barrier dysfunction and immune system abnormalities, particularly those of a type 2 phenotype, contribute to disease pathogenesis. We demonstrated that ILC2 are enriched in lesional skin biopsies from atopic patients and show higher expression of cytokine receptors, reflecting an activated phenotype. Down-regulation of E-cadherin is characteristic of filaggrin insufficiency, a cardinal feature of AD. Interestingly, E-cadherin binding to KLRG1 on human ILC2 dramatically inhibits IL-5 and IL-13 production. ILC2 may contribute to increases in type 2 cytokine production in the absence of the inhibitory E-cadherin ligation through this novel mechanism of barrier sensing.

CRTH2, a receptor for prostaglandin D_2 (PGD_2), is expressed by human ILC2. However, the function of CRTH2 in these cells is unclear. We sought to determine the role of PGD_2 and CRTH2 in human ILC2 and compare it with that of the established ILC2 activators IL-25 and IL-33. PGD_2 binding to CRTH2 induced ILC2 migration and production of type 2 cytokines IL-4, IL-5, IL-13 and release of other pro-inflammatory cytokines IL-3, IL-8, IL-9, IL-21, GM-CSF, and CSF-1 in a dose-dependent manner. ILC2 activation through CRTH2 also upregulated the expression of IL-33 and IL-25 receptor subunits (ST2 and IL-17RA) suggesting a synergistic role. The effects of PGD_2 on ILC2 could be mimicked by the supernatant from activated human mast cells and inhibited by a CRTH2 antagonist. Therefore, PGD_2 can be considered as an important and potent activator of ILC2 through CRTH2 mediating strong inflammatory responses.

Cell surface interaction mechanisms that activate ILC2 function are unknown. We observed the expression of NKp30 on ILC2 ex vivo and after culture. Using quantitative PCR we confirmed that ILCs express NKp30c splice variant, an immune-modulatory isoform. Incubation of ILC2 with the NKp30 ligand B7H6 and tumour cell lines expressing this protein induced production of type 2 cytokines. This interaction can be inhibited by NKp30 blocking antibodies. We further established that activation of NKp30 induces the canonical pathway of NFκB signalling.

Overall the work in thesis shows for the first time that ILC2 are resident in human skin and infiltrate rapidly after allergen challenge and in AD lesional skin. We have defined cytokine and lipid mediators that contribute to migration and activation of ILC2 and shown that KLRG1 and NKp30 act as inhibitory and activatory receptors respectively. The work defines novel pathways for barrier sensing and cutaneous inflammation, and identifies potential new targets for therapeutic intervention.

Abbreviations

AD	Atopic dermatitis
AMP	Anti-microbial peptide
APC	Antigen presenting cell
AREG	Amphiregulin
ATP	Adenosine triphopshate
BCR	B cell receptor
CARD	Caspase recruitment domain-containing protein
CASP1	Caspase-1
CLA	Cutaneous lymphocyte-associated antigen
CRTH2	Chemoattractant receptor expressed on Th2 cells (DP2)
CSF	Colony stimulating factor
CTLA	Cytotoxic T lymphocyte associated
DC	Dendritic cell
Der p	Dermatophagoides pteronyssinus
DMSO	Dimethyl sulphoxide
DP1	PGD2 receptor 1
EAD	Extrinsic atopic dermatitis
EC	Epicutaneous
ECP	Eosinophil cationic protein
EDC	Epidermal differentiation complex
EDN	Eosinophil-derived neurotoxin
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immunospot
Fox	Forkhead box protein
GM-CSF	Granulocyte-macrophage colony stimulating factor
GPCR	G-protein coupled receptor
HBD	Human β defensin
HDM	House dust mite
HEV	High endothelial venules
HC	Healthy control donor
HLA	Human leukocyte antigen
HPV	Human papillomavirus
HSV	Herpes simplex virus
IAD	Intrinsic atopic dermatitis
ICAM	Intracellular adhesion molecule
ICS	Intracellular staining
iDc	Immature dendritic cell
IFN	Interferon
IFN-γR	Interferon-γ receptor
Ig	Immunoglobulin

IL	Interleukin
IL-17RB	Interleukin 17 receptor B (IL-25R)
IL-7Rα	Interleukin-7 receptor subunit alpha (IL7R-α) also known as CD127
ILC	Innate Lymphoid cells
iNOS	Induced nitric oxide synthetase
IPEX	Immune dysregulation polyendocrinopathy enteropathy X-linked
IRF	Interferon regulatory factor
ISAAC	International Study of Asthma and Allergies in Childhood
JAK	Janus kinase
KLRG1	killer-cell lectin like receptor G1
LC	Langerhans cell
LFA	Lymphocyte function-associated antigen
LPS	Lipopolysaccharide
MBP	Major basic protein
MCP	Macrophage chemoattractant protein
MCV	Molluscum contagiosum virus
mDC	Myeloid dendritic cell
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MMR	Measles, mumps, rubella
NF-AT	Nuclear transcription factor of activated T cells
NF-κB	Nuclear factor κB
NK	Natural killer
NLR	Nucleotide-binding domain leucine rich repeat containing
NOD	Nucleotide-binding oligomerization domain-containing proteins
ORF	Open reading frame
OVA	Ovalbumin
PAF	Platelet activating factor
PAMP	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
pDC	Plasmacytoid dendritic cells
PGD2	Prostaglandin D2
PHA	Phytohaemagglutinin
PI3K	Phosphatidylinositol-3'-OH kinase
PMA	Phorbol-12-myristate-13-acetate
PRR	Pattern recognition receptor
Q-PCR	Quantitative polymerase chain reaction
RAG	Recombination activating gene
RANTES	Regulated on activation normally T cell expressed and secreted
SB	Stratum basale
SC	Stratum corneum
SCORAD	SCORing atopic dermatitis

SE	Staphylococcal enterotoxin
SFU	Spot forming units
SG	Stratum granulosum
SPINK	Serine protease inhibitor, Kazal type
SS	Stratum sphinosum
ST-2	a receptor for IL33, also known as IL-33R and IL1RL1
STAT	Signal transducers and activators of transcription
TCM	Central memory T cells
TCR	T cell receptor
TEM	Effector memory T cells
TEWL	Transepidermal water loss
TGF	Transforming growth factor
TH	T helper cells
TLR	Toll-like receptor
TMB	Tetramethyl benzidine
TNF	Tumour necrosis factor
TRAIL	Tumour necrosis factor-related apoptosis-inducing ligand
Treg	Regulatory T cell
TSLP	Thymic stromal lymphopoietin
TSST	Toxic shock syndrome toxin
VCAM	Vascular cell adhesion molecule
VLA	Very late antigen
VV	Vaccinia virus
VZV	Varicella zoster virus
WAO	World Allergy Organisation

List of publications

A role for IL-25 and IL-33-driven type-2 innate lymphoid cells in atopic dermatitis. Maryam Salimi, Jillian L. Barlow, Sean P. Saunders, L. Xue, D. Gutowska-Owsiak, X. Wang, D. Johnson, Seth C. Scanlon, Andrew N.J. McKenzie, Padraic G. Fallon and Graham S. Ogg; *J Exp Med*; 2013 Dec 16;210(13):2939-50

This paper has been chosen by editors of *The Journal of Allergy and Clinical Immunology* to be highlighted in the March edition of 'News Beyond Our Pages'.

The role of CRTH2 in group 2 innate lymphoid cells. Luzheng Xue[†], Maryam Salimi[†], Isabel Panse, Jenny M. Mjösberg, Andrew NJ. McKenzie, Hergen Spits, Paul Klenerman, Graham Ogg; *J Allergy Clin Immunol*; 2014 Apr;133(4):1184-94 †Authors contributed equally

Innate Lymphoid cells and the skin. Maryam Salimi and Graham Ogg. BMC Dermatology, Accepted

Enhanced isolation of lymphoid cells from human skin. Maryam Salimi , Sumithra Subramanian , Tharini Selvakumar , Xinwen Wang , Sophie Zemenides , David Johnson and Graham Ogg. Journal of Investigative dermatology, submitted

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Spontaneous atopic dermatitis is mediated by innate immunity with the secondary lung inflammation of the atopic march requiring adaptive immunity. S. P. Saunders, T. Moran, F. Wurlod, A. Kaszlikowska, M. Salimi, E. M. Quinn, G. Núñez, R. McManus, E. Hams, A. N. J. McKenzie, G. S. Ogg, P. G. Fallon. Science Translational Medicine, submitted.

Chapter 1. Introduction

1.1 Atopic Dermatitis

1.1.1 Definition of Atopy, Allergy and Atopic Dermatitis

Atopy is derived from the Greek word 'Atopia' meaning 'out of place or different' [1]. The *World Allergy Organisation (WAO) Nomenclature Review Committee-IAACI* has defined atopy as a personal and/or familial tendency to respond to ordinary exposure to common environmental allergens by producing allergen-specific immunoglobulin-(Ig)-E antibodies either in childhood or adolescence, and develop symptoms of asthma, rhino-conjunctivitis or eczema. The term atopy can only be used upon detection of allergen specific IgE or positive skin prick testing [2]. It was first used to describe asthma and allergic rhinitis but atopic dermatitis was added to the list in 1933 [1]. This was potentially confusing as some individuals with atopic dermatitis do not have atopy.

Allergy is a hypersensitivity reaction initiated by specific immunological mechanisms that can be antibody or cell mediated. The antibodies are mainly thought to be IgE isotypes and specific to an allergen.

The term atopic dermatitis (AD) or atopic eczema was first introduced by Fred Wise and Marion Sulzberger to describe a chronic eczema in the flexures. AD is a chronic

relapsing inflammatory skin disorder that is associated with hypersensitivity reactions to innocuous environmental allergens [3]. It is one of the most common disorders seen in early childhood so that 15% of the population experience symptoms of AD at some stage of their childhood. The prevalence of AD has increased 2 to 3 fold over the past few decades especially in developed countries [4]. Around 15-30% of children and 2-10% of adults are affected by AD. In 45% of children the disease manifests in the first 6 months of life, 60% are affected by the first year and 85% by 5 years of age [1, 5]. The term eczema was later proposed to replace atopic dermatitis for all disorders with definite clinical features involving compromised skin barrier [6].

1.1.2 Clinical features

Although features compatible with AD were described in the first text book of dermatology *De morbis cutaneis* by Girolamo Mercurialis in 1572, there is no single laboratory diagnostic test and the diagnosis is now typically based on clinical features of the disease that have been first proposed by Hanifin and Rajka and divided into major and minor criteria (Table 1.1) [7]. The clinical presentation of AD varies with age and chronicity of the disease. It includes three main stages; the infantile, childhood and the adult phase [1, 8, 9].

Table 1.1 Diagnostic features of atopic dermatitis as defined by Hanifin and Rajka

Major features (3 of 4 present)
pruritus
typical morphology and distribution of skin lesions
chronic or chronically relapsing dermatitis
personal or family history of atopy

Minor features (3 of 23 present)
xerosis
ichthyosis/palmar hyperlinearity/keratosis pilaris
immediate (type I) skin test reactivity
elevated serum IgE
early age of onset
tendency towards cutaneous infections/impaired cell-mediated immunity
tendency towards non-specific hand or foot dermatitis
nipple eczema
cheilitis
recurrent conjunctivitis
Dennie-Morgan infraorbital fold
keratoconus
anterior subcapsular cataracts
orbital darkening
facial pallor/erythema
pityriasis alba
anterior neck folds
itch when sweating
intolerance to wool and lipid solvents
perifollicular accentuation
food intolerance
course influenced by environmental/emotional factors
white dermographism/delayed blanc

Adapted from reference [10]

1.1.2.1 The infantile phase

The infantile phase is from birth to 2 years of age and often has an acute eczematous appearance. Extremely pruritic scaly erythematous patches and vesicles appear on the cheeks, scalp and forehead. The trunk and extensors might be involved. The lesions at this stage are usually oedematous with oozing and crusting without secondary bacterial infection. The affected areas are ill defined and symmetrical. The diaper area is usually unaffected probably due to over hydration, inaccessibility to scratching and protection from allergic triggers [1, 9, 11].

1.1.2.2 The childhood phase

During childhood till puberty, hands, feet, wrists, ankles and flexors are often involved. The lesions are less exudative and characterised by more chronic disease such as lichenified papules and plaques. Around 60% of eczema resolves but post inflammatory changes may persist [1, 11, 12] (Table 1.2 and 1.3)

TABLE 1.2

Clinical criteria for AD in pediatric patients

Essential features

Pruritus

Eczematous changes

Chronic or relapsing course

Typical and age-specific patterns: face, neck and extensor involvement in infants and children; flexural lesions especially in older children and adolescents; sparing of the groin and axillae

Important features (support the diagnosis but do not occur in all patients)

Early age of onset

Xerosis

Atopy (IgE reactivity)

Exclusions: The diagnosis of AD depends on the exclusion of conditions such as scabies, allergic contact dermatitis, seborrheic dermatitis, psoriasis and ichthyosis.

Table 1.3 Diagnostic criteria for atopic dermatitis

Criteria list	Requirements (number of criteria)
ISAAC, International Study of Asthma and Allergies in Childhood.	
Hanifin and Rajka diagnostic criteria, 1980	3 major + 3 minor
Kang & Tian diagnostic criteria, 1989	1 basic + 3 minor
Schultz-Larsen criteria, 1992	≥ 50 points
Lillehammer criteria, 1994	Visible eczema + 4 minor
U.K. diagnostic criteria, 1994	Pruritus + 3 minor
ISAAC questionnaire, 1995	Score ≥ 3
Japanese Dermatology Association criteria, 1995	All 3 features
Criteria of Diepgen, 1996	≥ 10 points
Millennium diagnostic criteria, 1998	Allergen-specific IgE + 2 principal
Danish Allergy Research Centre (DARC), 2005	3 features

Adapted from Reference [7]

1.1.2.3 The adult phase

The adult phase starts at puberty and often persists into adulthood. The antecubital and popliteal regions, face, neck, hands and back are often involved. Lesions are dry, scaly erythematous papules and lichenified plaques, but acute exacerbations can frequently occur.

In clinical trials the severity of atopic dermatitis should be defined using severity scores such as SCORAD (The Score in Atopic Dermatitis) [13], Eczema Area and Severity Index (EASI) [14] and the Patient Oriented Eczema Measure (POEM) [15, 16].

In chronic stages, the skin becomes thicker; nodules can be seen within plaques. In the case of superimposed infection the lesions may resemble impetigo, with weeping and crusting. Post inflammatory changes may cause hyper or hypo-pigmentation [17]

1.1.3 Histo-pathological changes

The histology and feature of atopic dermatitis lesions differ greatly depending on the stage of disease, chronicity, frequency of secondary superimposed bacterial infection and inflammation. Acute lesions present as papules and vesicles on erythematous background due to spongiosis in the epidermal layer. Widening of intercellular spaces and disruption of desmosomes occur. As lesions develop, scales and lichenification appear, and spongiosis and exocytosis of inflammatory cells can be observed. Acanthosis (epidermal hyperplasia especially in stratum basale and

stratum spinosum) and parakeratosis (retention of nuclei in the stratum corneum) can be present. Xerosis is another feature of AD lesions due to loss of moisture [17]. Projections into the underlying dermis (rete ridges) are elongated [18].

1.1.4 Pathophysiology in the light of systemic involvement

Several studies suggest that AD is the cutaneous manifestation of a systemic disease that includes allergic rhinoconjunctivitis and asthma. Eosinophilia and increased IgE are observed in these conditions. AD is usually the first manifestation of the so called 'atopic march'. Following subsidence of cutaneous signs, atopic features become prominent in other organs [19, 20].

It had originally been proposed that epithelial barrier dysfunction in atopic dermatitis is a secondary consequence of an intrinsic immunological defect known as the inside/outside hypothesis. Alternatively, the outside/inside hypothesis suggests that increased permeability of the skin barrier leads to inflammatory responses and increased cytokine production [21, 22] – this will be discussed in more detail below.

Epicutaneous sensitisation can lead to a systemic allergic response. *Spergel et. al.* showed that epicutaneous delivery of ovalbumin using tape-stripping can increase IgE production and induce dermatitis-like lesions in mice. Sensitised mice were subsequently challenged with exposure to inhaled ovalbumin. BAL fluid of challenged mice showed increased eosinophilia [23]. Epicutaneous sensitisation through barrier intact skin induces expression of TH1 (IL-2 and IFN-γ) and TH2 (IL-4) associated

responses in the local lymph nodes. Whereas epicutaneous sensitisation of environmental allergens through compromised skin reduces TH1 responses and up-regulates IL-4, IgE and IgG1 expression [1, 24]. A similar phenomenon was also observed in human studies as 91% of children treated with peanut containing emollient developed peanut allergy in contrast to 51% of atopic patients without exposure to peanut containing moisturiser [25].

1.1.5 Skin barrier defects in atopic dermatitis

There is an accumulating body of evidence that epithelial barrier dysfunction plays a major role in the pathogenesis of atopic dermatitis. Skin is not only a mechanical barrier but it also provides an anti-microbial and a biochemical barrier. Moreover, the innate and adaptive immune responses retain homeostasis in the skin. Stratum corneum (SC, outer most layer of the epidermis) consists of multiple layers of corneocytes (fully differentiated keratinocytes) embedded in a layers of insoluble proteins such as involucrin, loricrin and filaggrin [26]. The model of 'bricks and mortar' was proposed with corneocytes as bricks and the cell envelope is the mortar [27]. Corneocytes are a result of a highly organised differentiation process through which keratinocytes from the basal layer develop to form spinal and granular layers and finally the cellular contents become replaced with keratin, the cells flatten and enucleated and form an effective barrier [28, 29]. Cellular matrix in SC consists of bilayers of cross-linked corneodesmosomes and lipids [29-31]. Lipid contents of SC

include hydrophobic ceramides, cholesterol and fatty acids that prevent evaporation and water loss and contribute to barrier function.

Proteases and protease inhibitors that assist in shedding of corneoytes by digesting corneodesmosomes are delivered by lamellar bodies. Dysregulation of protease such as dipeptidyl peptidase 10, transglutaminase and SC chymotryptic enzyme were reported in AD lesions [32]. In Nethertons syndrome, a dysregulated protease inhibitor function due to SPINK5 deficiency leads to AD like lesions in the skin [33].

AD lesions are characterised by increased trans epidermal water loss (TEWL) and dry skin which is thought to contribute to the higher antigen uptake and subsequent hypersensitivity reactions [9]. Ceramide content of the skin inversely correlates with TEWL and is reduced in lesional and non-lesional skin of patients with AD [34, 35].

1.1.6 Filaggrin

Filaggrin is a protein expressed by keratinocytes at the final stages of differentiation. Loss of function mutations of filaggrin gene (FLG) are among the most important gene defects that contribute to the pathogenesis of atopic dermatitis with an odd ratio of 3.12 - 4.78 [36]. There is a stronger association with moderate to severe disease. The filaggrin gene is located in chromosome 1q21 along with 60 other genes (e.g. filaggrin-2, hornerin, trichohyalin, trichohyalin-like 1, cornulin, and repetin) involved in epidermal differentiation and is termed the epidermal differentiation complex [26]. Around 9% of Europeans carry one of the two common mutations of

FLG, R510X and 2282del4, that are highly associated with AD [37]. Another 22 loss of function mutations have been identified in patients with AD. Heterozygous carriers of FLG null mutations express 50% less protein. Around 40-60% of patients with moderate-severe AD have FLG null mutations [38]. The disease in patients with FLG mutations is more persistent [39] with higher possibility of asthma [40] and infection with Herpes Simplex Virus (HSV) [41].

In stratified and cornified epithelia, filaggrin is stored in dense granules as profilaggrin. Patients with homozygous mutations lack these granules [42]. Profilaggrin along with loricrin are essential for maturation and squame formation of keratinocytes [26, 36]. In the upper-most granular layer, dephosphorylation of profilaggrin accompanied by derepression of the protease cascade, release active filaggrin monomers. The cells become flattened, packed, anuclear and cross-linked which constitutes the stratum corneum [26]. Transglutaminase covalently cross links filaggrin monomers [43]. The arginine residues are post-translationally modulated to citrulline to simplify further break down by proteases like caspase 14 into short peptides and amino acids identifies as natural moisturising factors (NMF) [44, 45].

1.1.7 Anti-microbial Peptides

The chemical component of the skin barrier is anti-microbial peptides (AMP) and proteases. AMP are produced by keratinocytes and stored in lamellar bodies. They can be induced by pro-inflammatory cytokines or bacterial infection. Some AMPs can

be produced under homeostatic conditions in response to endogenous and/or exogenous non-inflammatory stimuli [46, 47].

Lyzozyme, is a key anti-microbial peptide found in the cytoplasm of granular layers and malpighian cells in healthy skin. It is active against Gram-positive bacteria and to a lesser extent Gram-negative bacterium suggesting a defensive role in bacterial overgrowth.

Dermcidin and Cathelicidin LL-37 have antimicrobial properties and are found in the sweat. They are produced by eccrine glands and ducts [47]. The level of Dermcidin is reduced in patients with atopic dermatitis. Cathelicidin LL-37 is also produced by keratinocytes, and is effective against HSV1, HSV2, and VV [48]. RNase 7 is a major AMP of healthy skin with anti-Gram-positive (*Propionibacterium acnes, S. aureus*) and Gram-negative (*E. coli and P. aeruginosa*) activity. Psoriasin (S100A7) has antimicrobial activity against E.coli and is mainly expressed in uppermost parts of hair follicles [49].

Human β defensins are another AMP group produced by epithelial cells. They are small cationic proteins (3-5kDa) with 3 disulphide bonds. HBD-1 and HBD-2 are monomers while HBD-3 is a dimer [50]. Defensins have broad microbicidal activity against Gram-positive and negatives, yeasts and enveloped viruses [51]. HBD-3 has specific anti *S-aureus* activity at physiological salt concentrations. It has been suggested that type 2 cytokines IL-13, IL-4 and IL-10 contribute to lower gene levels of HBD-2 and HBD-3 in AD lesions [52].

Several chemokines such as MIP-3α (CCL20), CXCL9, CXCL-10 and CXCL11 produced by keratinocytes have microbicidal activities [53]. Sphingosine is another AMP closely related to ceramide metabolism. Defective ceramide metabolism in AD causes a reduction in sphingosine levels and increased *S.aureus* colonisation [54].

1.1.8 Environmental factors affecting Atopic Dermatitis

The prevalence of atopic disease has changed dramatically in the last 20-30 years which suggests that environmental factors play a critical role in determining expression of AD. Occurrence of AD in monozygotic twins is 0.72 confirming an important role for environmental factors [55, 56].

1.1.8.1 Hygiene hypothesis

Over the past 4 decades the prevalence of allergic asthma, atopic dermatitis and hay fever increased markedly in western countries. It has been suggested that levels of air pollution, exposure to indoor allergens and increase in living standards all contributed to the effect. However, further studies failed to strongly support any of the suggested causes as sole explanations [57, 58]. Furthermore, the inverse relationship between family size and risk of allergic disorders was the basis of the hygiene hypothesis [59] which argues that the prevalence of allergic disorders inversely correlates with the frequency of childhood infections. The hypothesis was

developed around the TH1/TH2 paradigm of adaptive immune responses in childhood.

Type 2 immune responses are required for protection against helminth infections. Aberrant type 2 responses can trigger allergic reactions. On the other hand, type 1 immune responses predominantly provide defence against bacterial and viral infections. In the absence of childhood infections, the immune system is believed to be skewed towards type 2 immune responses and development of allergic reactions. In developing countries the overwhelming burden of infections helps in expansion of type 1 arm of the immune system and suppression of type 2 responses. Although hygiene theory was generally accepted, it was largely contradicted by the increase in prevalence of type 1 driven conditions such as autoimmune disorders in western countries. Therefore, counter regulation hypothesis was proposed based on production of IL-10 by regulatory T cells. Frequent infections could increase IL-10 production, in turn reducing allergic reactions and autoimmune disorders [57].

There has been a recent resurgence of interest in the hygiene hypothesis with the increased understanding of the role of the microbiome in modulating inflammatory responses. Early life exposure to household pets and having siblings influence composition and diversity of gut microbiota and associates with development of atopic reactions [60]. Recently a 'biodiversity hypothesis' has been proposed implying that restricted exposure to diverse environmental microbiota leads to incomplete development of the immune system. The limited diversity of skin

Gammaproteobacteria microbiota in atopic patients associates with lower environmental biodiversity [61].

1.1.8.2 Allergen exposure

Exposure to allergens during early life increases the risk of sensitisation and development of allergic reactions. In one study (n=1901) the prevalence of asthma, hay fever and eczema was higher in those who migrated to a developed country before 2 years of age [62]. Moreover, regression analysis has shown that the hypersensitivity to birch pollen is associated with the exposure to birch flower during early infancy, which could be reduced by 28% if avoided [63]. The risk of house dust mite (HDM) allergy is higher in children born in autumn when HDM is more prevalent [64, 65].

The risk of sensitisation depends on the type and dose of allergen and duration of exposure. 40% of children with AD can show flare up after exposure to food allergens. The levels of IgE and antigen specific T cells (usually directed to egg, soy, peanut, milk and wheat) is increased. Food allergies generally subside by 3 years of age [9]. Induction of eczema lesions and localized isolation of allergen specific T cells in AD patients upon exposure to HDM (30-50%), *Dermatophagoides pteronyssinus* and other aeroallergens confirm the pathogenicity of environmental allergens in AD [9].

1.1.8.3 Infections

Eczema lesions in most patients with AD are colonised with *Staphylococcus aureus.* Overgrowth of this microorganism and secreting superantigens (staphylococcal enterotoxins (SE) SEA, SEB, and toxic shock syndrome toxin (TSST)-1) can induce an exacerbation of clinical symptoms [66, 67]. Superantigens activate allergen specific T cells, macrophages and induce specific IgE antibody production [68]. The level of anti-superantigen antibodies directly correlates with disease severity. One mechanism for colonization of *S.aureus* in AD lesions is up regulation of IL-4 which in turn increases fibronectin expression. IL-4 directly amplifies cytokine production by T cells. SEB can also induce upregulation of IFN-γ and ICAM-I on keratinocytes and promotes allergen-specific T cell activation at the site of inflammation [69]. As discussed earlier decreased levels of anti-microbial peptides also facilitate the colonisation [70].

Atopic patients are also more susceptible to viral infections such as human papillomavirus (HPV), herpes simplex virus (HSV)-1 (eczema herpeticum), varicella zoster virus (VZV), vaccinia virus (VV) (eczema vaccinatum), coxsackie A virus and molluscum contagiosum virus (MCV) (eczema molluscu latum).

Malassezia (formerly known as *Pityrosporum*) is part of the normal flora of the skin, found especially in hair follicles. 30-80% of patients with AD develop specific IgE antibodies [71]. Malassezia yeasts can induce cytokine production by keratinocytes [72] or directly activate mast cells [73].

Exacerbation of atopic reactions associates with temporal changes in skin microbiota composition. During flares not only pathogenic species of *Staphylococcus* (*S.aureus*) increase but also commensal species *S.epidermidis* become abundant. The numbers of *Streptococcus, Propionibacterium*, and *Corynebacterium* reduces during flare ups and increases following treatment [61]. Improvement following treatment correlated with diversity of skin microbiota. Even intermittent treatment associated with greater skin microbiota composition [74].

1.1.9 Genetics of Atopic Dermatitis

AD is genetically a complex disease. Monozygotic and dizygotic twins show 0.72-0.86 and 0.21-0.23 concordance suggesting an important role for genetic factors [75, 76]. The role of mutations in the FLG gene has been discussed above, but there are other loci which have also been implicated. Amongst the main characteristics of AD are increased levels of type 2 cytokines in eczema lesions and high serum IgE antibodies to common environmental allergens. In fact, case control studies show an association between -590C/T polymorphism in the IL-4 gene promoter region that increases activity of IL-4 promoter. Furthermore, IL-4 receptor subunit polymorphisms (16q12), IL-13 coding region variants, and functional mutations in RANTES promoter region (17q11) have all been reported in AD.

Co-stimulatory molecules CD80 and CD86 are encoded by chromosome 3q21 and found to have a linkage for AD in Scandinavian and German children [77]. Loci on chromosomes 1q21, 17q25, and 20p were found to contain susceptibility genes for both AD and psoriasis.

Genome wide association studies (GWAS) have identified a significant association between AD and an allele on chromosome 11q13.5 (C11orf30) encoding the β chain of FcεRI which has also been reported in Crohn's disease [78]. Epidermal differentiation complex (EDC) located on chromosome 1q21 has high linkage association with AD as described above [79].

SPINK 5 which encodes serine proteinase inhibitor, Kazal type 5, and expressed in outer layers of the epidermis has strong association with Netherton disease. It is known to protect against allergens with serine proteinase activity [80].

Polymorphisms of CD14 and R753Q polymorphism of the TLR2 are also associated with development of allergy probably due to alteration in recognition of microbial products [81]. There is a 2 fold increase in risk of AD in children with C2722 polymorphism of caspase recruitment domain containing protein 15 that alters LPS recognition pathway [82].

1.1.10 Immune responses in AD skin

Not long ago the skin has been viewed only as a passive physical epithelial barrier, but a diverse range of pathological disorders from different types of autoimmune and allergic reactions to cutaneous malignancies and infections clearly indicates the existence of a vital complex immune surveillance system that maintains homeostasis in the skin.

1.1.11 Innate Immune system

The innate immune system is the first line of defence against pathogens. It acts rapidly and non-specifically and includes cells that lack specific antigen receptors. The skin functioning as a physical barrier, the expression of antimicrobial peptides (AMPs, as above) and pattern recognition receptors (PRR) are important components of innate immune system in the pathogenesis of AD.

1.1.12 Cells of the Innate Immune System

1.1.12.1 NK cells

NK cells were extensively examined in atopic dermatitis [83]. NK cells are the innate producers of type 1 cytokines such as IFN-γ and TNF-α. They are phenotypically and functionally defective in AD lesions. The frequency of CD56+ CD16+ NK cells inversely correlated with serum levels of IgE [83]. NK cells were defective in production of IFN-γ and TNF-α whereas IL-4 production was not affected [84]. The role of other members of the innate lymphoid cell family will be further discussed below.

1.1.12.2 Dendritic cells

Various subsets of DC reside in the skin and are believed to contribute to the pathogenesis of AD. Two different subsets of mDC (DC1) are identified in AD lesions; Langerhans cells and inflammatory dendritic epidermal cells (IDEC). Langerhans cells express Birbeck granules which contain langerin protein and CD1a. Both subsets express the high-affinity receptor for immunoglobulin E (IgE) (FcεRI) which is up-regulated in eczema lesions [85]. Plasmacytoid dendritic cells (pDC or DC2) are a major subset of dendritic cells which are important in innate immune responses due to the production of anti-viral type I IFNs. Two subset of pDCs have been identified; IL-3R^{dim} CD11c^{++} with monocytic features and IL-3R^{++} CD11c^{-} with plasmacytoid

properties [86]. Although the frequency of pDCs was increased in the circulation, the lesions show decreased numbers in the skin [87].

1.1.12.3 Keratinocytes

Epidermal keratinocytes are an important source of anti-microbial peptides [70], chemokines and cytokines following inflammation or mechanical stress (e. g. scratching). They produce high amounts of chemotactic cytokine CCL5 (RANTES) in response to IL-2, IFN-γ and TNF-α. They produce a unique profile of epithelial cytokines, IL-33, IL-25 and TSLP (thymic stromal lymphopoietin) that can induce dendritic cells and innate lymphoid cells to produce type 2 cytokines and prime TH2 cells [88].

In atopic dermatitis, the ability of keratinocytes to produce anti-microbial peptides is thought to be diminished which makes patients potentially more prone to infection [9]. T cell induced apoptosis of epidermal keratinocytes also contributes to the pathogenesis of AD inflammation. The production of IFN-γ by activated T cells in AD lesions induces up regulation of Fas-receptor (FasR) on keratinocytes. When the levels exceed a threshold of around 40,000 per cell the binding of Fas ligand induces apoptosis of keratinocytes [89]. Moreover, in approximately 28% of patients with AD (n=192) IgE antibodies reactive to epithelial cell line (A431) and primary human keratinocytes were identified suggesting a role for autoantibodies in AD [90].

1.1.12.4 Eosinophils

Increased infiltration of eosinophils is observed in AD lesions with average of 2.8 cells/mm2 (range 0–90.3) [91, 92]. The increase in eosinophil numbers and granularity correlates with disease severity, epidermal hyperplasia and level of spongiosis. Eosinophil products including eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN, EPX), and eosinophil major basic protein (MBP) are also deposited in higher levels in lesions [93]. Eosinophils are activated by various cytokines, including IL-3, IL-2 and granulocyte-macrophage colony stimulating factor (GM-CSF).

Activated T cells produce IL-4 that activates keratinocytes, fibroblasts and endothelial cells to produce eotaxin which attract CCR3 expressing eosinophils [94]. CCR3 also binds to eotaxin-2, eotaxin-3, RANTES, MCP-2, MCP-3, and MCP-4 [95] released by epithelial cells. Furthermore, IL-5 by activated T cells is a major eosinophil attractant. It induces chemotaxis, activation and delayed apoptosis. Expression of adhesion molecules VCAM-1 and VLA-4 on endothelial cells are essential for preferential recruitment of eosinophils [92, 96].

1.1.13 Innate Immune Receptors

The innate immune system employs germ-line encoded pattern recognition receptors (PRR) to identify pattern associated molecular pattern (PAMPs) and danger associated molecular patterns (DAMPs). PRRs include Toll like receptors (TLRs), C-type lectin receptors (CLRs) that express on the cell surface and Retinoic acid-inducible gene I (RIG-1) and NOD-like receptors (NLRs) that are expressed in the cytoplasm [97]. Polymorphisms in the expression of PRRs can alter the function of innate immune cells. Pro-inflammatory monocytes in AD patients have reduced capacity to produce IL-1β and TNF-α when stimulated with TLR-2 agonists [98]. Gain of function mutations in the TLR-9 promoter region, C-1237T are associated with the 'intrinsic or non-allergic' form of AD. Clinical manifestations of intrinsic AD is similar to the extrinsic form but sensitisation to common environmental allergens is not evident in these patients and serum levels of IgE are normal [99]. TLR9 is expressed on B cells and promotes TH1 biased IgG responses which correspond to reduced type 2 cytokines and IgE in intrinsic AD [100].

NOD-1 (CARD-4) and NOD-2 (CARD-15) receptors recognise cell wall peptidoglycans in *S.aureus* and other organisms. Specific genetic variants of NOD-1 and NOD-2 have been seen in patients with atopic disorders [101]. T allele at rs1077861 or A allele at rs3135500 genetic variants are associated with decreased and increased risk of asthma respectively. Individuals with A allele at position rs5743266 or a T allele at rs2066842 had reduced risk of allergic rhinitis [101].

1.1.14 Adaptive Immune Mechanisms of Atopic Dermatitis

Adaptive immunity is the second line of defence against microbial pathogens. It requires rearranged receptors to identify specific antigens [102]. The role of adaptive immunity in the pathogenesis of AD is well established. Allergen specific TH2 cells are the predominant infiltrating cell population in AD lesions. Up regulation of endothelial adhesion molecule E-selectin and cutaneous T-cell-attracting chemokine (CCL27) facilitate recruitment of T cells to eczema lesions. Thymus and activation-regulated cytokine (CCL17) and macrophage-derived chemokine (CCL22) have also been shown to recruit CCR-4 expressing TH2 cells. The levels of these chemokines appears to be correlated with disease severity [103]. TH2 cells are major producers of type 2 cytokine and are believed to be responsible for class switching to IgE. $RAG2^{-/-}$ mice (lack T and B cells) and $TCR\alpha^{-/-}$ (lack αβ T cells) showed decreased amount of dermal IL-4 mRNA and lower cell infiltration in response OVA sensitisation[104]. Moreover, treatments that alter T cell function are highly effective in AD which supports adaptive immune contribution to the pathogenesis of this disease. Ciclosporin A is a potent calcineurin inhibitor that is highly effective in treatment of AD. It inhibits the proliferation and function of T cells [105]. Tacrolimus and pimecrolimus are other calcineurin inhibitors that have successfully been used in treatment of AD [106].

1.1.14.1 T lymphocytes in atopic dermatitis

Increase in serine proteinase activity induces conversion of proinflammatory cytokines IL-1α and IL-1β into active forms which along with epithelial barrier dysfunction and increased TEWL initiate a signalling cascade that activates TH2 cells [107]. Increased permeability of SC, higher antigen exposure and decreased anti-microbial peptides lead to bacterial colonisation and increased allergen reactivity [69]. On the other hand, CD14, TLRs and NOD polymorphisms prevent effective recognition of microbes and may cause sustained TH2 responses. IL-4 release by TH2 cells stimulates CCL18 production by dendritic cells. CCL18 attracts $CD4^+$, $CD8^+$ T cells, B lymphocytes, dendritic cells and amplify type 2 responses [108]. The clinical severity of AD correlates with CCL18 levels in the serum. Increased telomerase activity and shortened telomere length of T cells in AD patients suggest that T cells are chronically activated in this disease [109]. Furthermore, an experimental immunization system in humans demonstrated an intrinsic tendency of skin in AD patients towards programming for TH2 responses. Epidermal delivery of 2,4-dinitrochlorobenzene (DNCB) in AD patients (with and without filaggrin mutation) and healthy individuals showed similar penetration but the clinical response was lower in AD patients. Although, all groups mount DNCB specific TH1 responses, they were reduced in AD patients. AD patients also developed DNCB specific TH2 responses suggesting specific inclination of atopic skin for TH2 programming [110].

There is a compelling body of evidence that type 1 cytokines play a role in the chronic phase of AD [111, 112]. Type 1 cytokines in chronic AD have stimulatory effects on keratinocytes. IFN-γ up-regulates MHC class I and II expression on keratinocytes which increases antigen presentation and subsequent activation of T cells. IFN-γ increases Fas mediated apoptosis of keratinocytes and reduction of epithelial spongiosis contributing to remodelling changes in chronic AD [113]. The IL-5, GM-CSF, IL-12, and IFN-γ mRNA expressing cells are increased in chronic phase whereas IL-4 and IL-13 levels reduce [114]. The increase in IL-5 levels contributes to eosinophil survival and function in lichenified AD.

The factors responsible for this biphasic response are still being investigated. IDECs may play an important role as they persist in chronic AD lesions and are capable of producing the type 1 polarising cytokine, IL-12 [115]. Interestingly, in the chronic phase of AD type 1 and type 2 cytokines have synergistic effects. IL-13 and IFN-γ both up regulate CCL22 and attract $CD4^+$ $CCR4^+$ T cells [116].

Other subsets of T cells also play a role in pathogenesis of AD. IL-17 mRNA is higher in acute lesions compared to chronic eczema. There is an increased infiltration of TH17 T cells in the papillary dermis of AD skin samples which is more prominent in acute lesions [117]. $CD4^+$ $CD25^+$ $FOXP3^+$ T cells were found in higher numbers in the blood of AD patients compared to healthy controls and psoriatic patients [118]. In lesions, FOXP3+ T cells were similar or higher than healthy controls [119].

1.2 Type 2 promoting cytokines

Both innate and adaptive immune mechanisms play a role in pathogenesis of atopic dermatitis. Type 2 cytokines in the acute phase of AD control proinflammatory responses by regulating activation of TH1 cells, eosinophils, mast cells and dendritic cells [22]. Keratinocytes also contribute to inflammatory reactions by producing cytokines, chemokines, AMP and proteases. Keratinocytes express key regulators such as cytokines IL-25 and IL-33 and chemokine TSLP (thymic stromal lymphopoietin) upon mechanical stress or stimulation with allergens and microbes. IL-25, IL-33 and TSLP initiate a cross talk between innate and adaptive immune systems by promoting type 2 cytokine production either directly or by polarizing dendritic cells [120, 121].

1.2.1 IL-33

IL-33 is a member of the IL-1 family of cytokines and has a role in pathogenesis of several allergic conditions, inflammation, autoimmunity and fibrosis [122]. Precursor IL-33 is synthesised and stored in specialised secretory lysosomes. Pro-IL-33 is cleaved by caspase-1 which transports to the cell membrane by lysosomes and releases active IL-33 [121]. IL-33 increases production of type 2 cytokines in vitro and induces splenomegaly, eosinophilia and production of immunoglobins in vivo. It can also help in maturation and activation of $CD34^{+}$ mast cells in concert with TSLP [123]. It has recently been recognised as an epithelial system defence mechanism and

designated ‘epithelial alarmin’. IL-33 mediates its effect by interacting with ST-2 (suppression of tumorigenicity 2) also known as IL-1RL1 coupled with IL-1 receptor accessory protein (IL-1RAP). ST2 is expressed on T cells, mast cells, NK cells, basophils, eosinophils and type 2 innate lymphoid cells [124]. IL-33 - ST2 interaction leads to the activation of STAT-5, MAP Kinase, Akt and NFκB signalling pathways [125].

IL-33 associated with both pro-inflammatory and anti-inflammatory processes. IL-33 activated type 2 cytokine producing cells during helminth infection in the gut and inhibited type 1 responses. Interestingly, exogenous IL-33 induced TSLP production in infected caecum [126]. IL-33 also prompted production of IL-6 and IL-1β, TNF-α, MCP-1, and PGD2 in primary bone marrow derived mast cells and P815 mastocytes through T1/ST2 receptor [127]. ST-2 can also be shed from the cell surface in a soluble form (which lack the transmembrane and signalling motifs) and act as a ‘decoy’ receptor which can bind to IL-33 and inhibit its reaction before it binds to ST2 receptor on the cell surface [128]. Soluble ST2 acts as a biomarker in myocardial infarction and asthma [128].

There is an increasing body of evidence that IL-33 plays a role in pathogenesis of AD. IL-33 levels are higher in the keratinocytes and endothelial cells of AD patients [122]. 2 and 1.8 fold increases in levels of ST-2 and IL-1RAcP were observed in lesional compared to non-lesional skin samples of AD patients. IFN-γ and TNF-α induced IL-33 expression in skin fibroblasts, HaCaT keratinocytes, primary macrophages, and HUVEC endothelial cells [129]. Sub-cutaneous administration of IL-33 in mice

induced infiltration of eosinophils, monocytes, lymphocytes and increased expression of IL-13 mRNA and led to fibrosis. Expression of extracellular matrix associated genes including collagen VI, collagen III, and tissue inhibitor of metalloproteases-1 were altered [130]. Ovalbumin (OVA) allergen sensitisation in mice up-regulated ST2 and IL-33 expression and induced eosinophilia and mast cell infiltration which were inhibited by topical application of calcineurin inhibitor, Tacrolimus. In AD patients, HDM (house dust mite) and SEB (staphylococcal enterotoxin B) challenge increased IL-33 and ST2 expression [129]. There is a significant genetic association between −26999G/A single nucleotide polymorphism (SNP) in distant promoter region of ST-2 gene and increased risk of development of atopic dermatitis. Having one −26999A ST2 allele correlated with increased levels of soluble ST2 and total IgE serum in AD patients [131].

1.2.2 IL-25

IL-25, also known as IL-17E, is a member of IL-17 family receptors and has a role in regulating type 2 responses [132]. It is produced by epithelial cells and immune cells such as alveolar macrophages, eosinophils, basophils, NKT cells, T cell lymphocytes, mast cells and intraepithelial lymphocytes. IL-25 mediates its effector function by interacting with a dimer of IL-17RB and IL-17RA receptors. IL-17RB$^{-/-}$ and IL-17RA$^{-/-}$ mice did not respond to IL-25 and both strains were resistant to pulmonary inflammation in response to intranasal delivery of IL-25 [133]. Unlike broad expression of IL-25, the expression of its receptor is restricted to naïve T cells,

memory TH2 lymphocytes and ILCs [134]. Over-expression of IL-25 induces eosinophilia, goblet cell and epithelial cells hyperplasia and production of type 2 cytokines. Concurrently, it can inhibit TH17 differentiation by inducing the expression of IL-13 by dendritic cells and inhibiting IL-23 release by macrophages [134]. IL-25 can also enhance TH2 differentiation by up-regulating IL-4 m-RNA expression after activation of naïve T lymphocytes [135]. Furthermore, upon T cell receptor activation, IL-25 inhibits down regulation of GATA-3, c-maf and JunB in memory TH2 cells [136]. AD lesional skin showed increased expression of IL-25 and its receptor IL-17RB compared to non-lesional skin biopsies [137, 138]. In primary keratinocytes, allergen exposure can stimulate IL-25 production and IL-25 treatment down-regulates filaggrin expression [132]. These data indicate that IL-25 not only induces type 2 reactions but also augments barrier defects by directly acting on keratinocytes [132].

1.2.3 TSLP

Thymic stromal lymphopoietin (TSLP) is expressed by epithelial cells especially keratinocytes. Its production is augmented by type 2 cytokines and TNF-α, and inhibited by IFN-γ and TGF-β [139]. Increased levels of TSLP were observed in eczema lesions of patients with AD. TSLP can activate Langerhans cells and induce transmigration [140]. Over expression of TSLP under keratin 14 (constitutively) or keratin 15 (inducible) promoters leads to development of dermatitis lesions, dermal infiltration of $CCR4^+$ TH2 cells, increased type 2 cytokines and serum IgE levels [141].

In $TSLP^{-/-}$ mice, ovalbumin challenge does not induce up-regulation of type 2 cytokines. TSLP induces differentiation of IL-4 producing TH2 lymphocytes from naïve T cells [142]. Triggering of the T cell receptor in TH2 cells increased expression of TSLP receptor [143]. CD11c+ dendritic cells constitutively express TSLP receptor and when primed by TSLP increase thymus and activation-regulated chemokine (TARC) and macrophage derived chemokine (MDC) production, promote TH2 differentiation, leading to increased production of IL-4, IL-5, IL-13 and TNF-α [136].

1.3 Type 2 cytokines

1.3.1 IL-4 and IL-13

IL-4 and IL-13 share 20-25% sequence homology and some functions. They induce IgE isotype class switching in B cells and contribute to up-regulation of IgE receptors on eosinophils, basophils and mast cells [9]. Cross-linking of IgE receptors activates these cells and supports the inflammatory outcomes of AD. IL-4 and IL-13 also induce up-regulation of adhesion molecules on endothelial cells such as vascular cell adhesion molecule 1 (VCAM-1), which stimulates adhesion of eosinophils and basophils by binding to very late activation 4 (VLA-4) on monocytes [144]. IL-4 and IL-13 up-regulate CCL22 and MMP-9 on keratinocytes which induce migration of $CCR4^{+}$ TH2 cells [116]. These cytokines down-regulate expression of filaggrin, AMPs and may contribute to epithelial barrier defect and increased susceptibility of AD patients to infections [145].

There are some differences in sequential expression of IL-13 and IL-4. IL-13 is expressed in both acute and chronic phases of AD but significant up-regulation is observed in sub-acute and chronic lesions; whereas IL-4 is less prominent at this stage. The frequency of IL-13 expressing cells in the peripheral blood of children with AD correlated with disease severity [146]. IL-13 mediates its effect through a heterodimeric receptor composed of IL-4Rα and IL-13Rα1 [147]. Primary human keratinocytes and HaCaTs express functional IL-13 receptor [148]. Over expression of IL-13 under keratin 5 promoter in the skin developed clinical and histopathological manifestations of AD with infiltration of T cells, mast cells, eosinophils, macrophages, and Langerhans cells, increased expression of cytokines and skin remodelling with fibrosis [149]. Transgenic mice over-expressing IL-4 develop AD like inflammation that mimics all features of AD lesions [150]. In IL-$4^{-/-}$ mice epidermal hyperplasia was similar to the wild type mice but there were fewer infiltrating T cells and eosinophils. IL-4 knock out mice showed reduced antigen uptake suggesting a role for IL-4 in compromising epidermal barrier integrity [151].

Dupilumab is a fully humanized fully human monoclonal antibody that blocks interleukin-4 and interleukin-13. Its efficacy has been shown in asthma patients with elevated eosinophil levels. A 12-week monotherapy with Dupilumab reduced EASI score (eczema area and severity score) by 50% in AD patients. Skin lesions were nearly cleared and pruritus greatly improved [152].

1.3.2 IL-5

IL-5 is essential for development, recruitment, proliferation and survival of eosinophils [153]. There is an increased level of IL-5 in eczema lesions of AD patients which correlates with serum IgE levels. Allergen exposure in IL-5 knock out mice showed decreased eosinophilia and epidermal hyperplasia [154] but clinical features of AD can be restored by IL-4 and IL-13. Lack of CCR3 which is required for recruitment of eosinophils had no effect on development of AD.

1.4 Innate lymphoid cells

Recent advances in the field of immunology have identified a novel family of CD45 expressing hematopoietic effector cells. These cells have phenotypical features of lymphoid cells but lack rearranged antigen specific surface receptors specific to adaptive immune cells and are termed innate lymphoid cells (ILCs)[155]. ILCs are essential for lymphoid organogenesis, tissue homeostasis and repair, protection against viral and helminth infections [156-158]. They reside in the blood, spleen, intestine, liver, fat associated lymphoid clusters (FALC), and mesenteric lymph nodes of human and mice. Their development depends on the expression of the transcriptional repressor Id2 that regulates the activity of helix-loop-helix protein E47 and RORC. Cytokines that signal through the common γ chain of IL-2 receptor and Jak3 are essential for their maintenance.

ILCs are thought to be able to influence adaptive immune responses as they reside in the interface of T and B cell zones in the splenic follicles of mice and can express co-stimulatory molecules essential for T cell priming and survival; CD40 ligand and CD30 ligand [159]. Each distinct functional subset produces cytokines that were previously thought to be specific to adaptive immune system lineages. Based on their cytokine profile and functional characteristics, they can be divided into three main groups (Figure 1.1) [155], although recent studies on lineage relationships and common precursors of ILCs make this classification debatable [160].

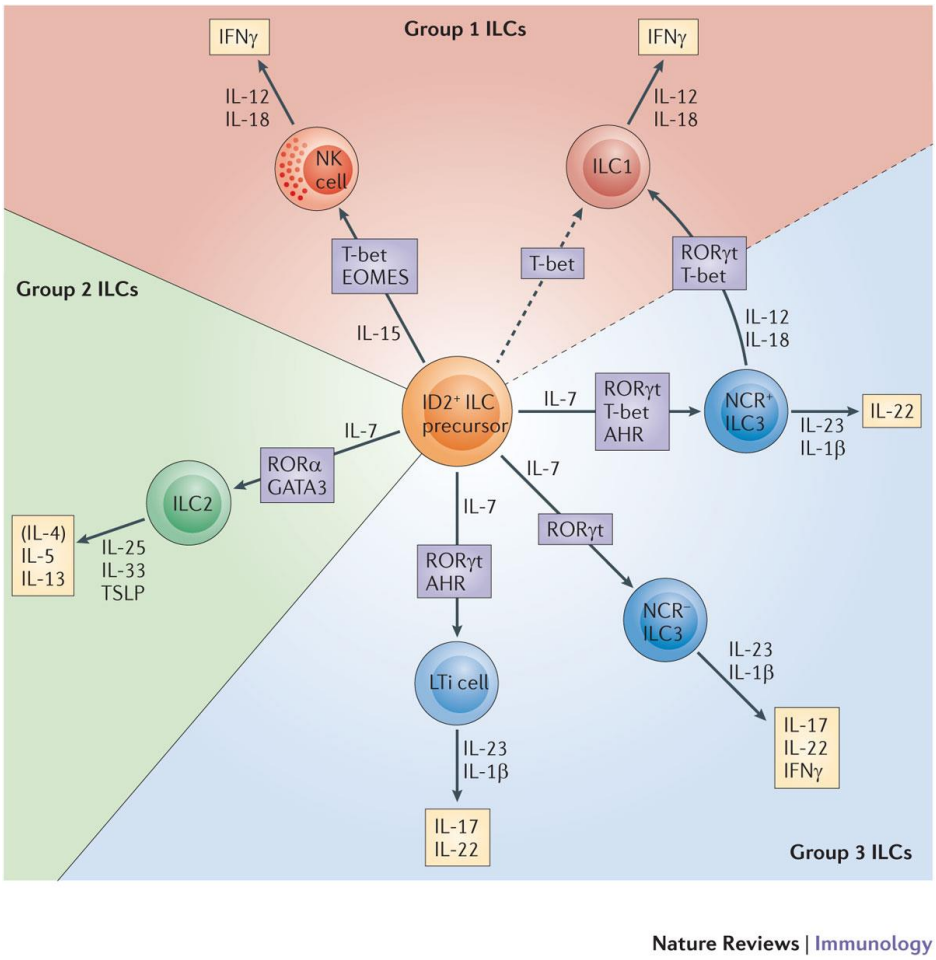


Figure 1.1: Classification of ILCs into three groups on the basis of their functional characteristics.

Innate lymphoid cells are divided into 3 main groups. Group 1 ILCs express T-bet and EOMES and produce interferon-γ (IFNγ). Group 2 ILCs are able to produce type 2 cytokines, IL-4, IL-5 and IL-13. ILC2 express transcription factors RORα and GATA-3. Group 3 depend on RORγt transcription factor and produce the TH17 cell-associate cytokines IL-17 and IL-22. Adapted from reference [155]

1.4.1 Group 1 ILCs

The most studied prototype of this family is group 1 ILCs including NK cells and type 1 innate lymphoid cells (ILC1) which were identified in 1975 and 2012, respectively [161-163]. They express transcription factors T-bet, Nfil3 (E4BP4) and Eomes [164] but c-Kit. IL-15 and IL-12 are required for their development and function. NK cell subsets include cytolytic effectors of the innate immune system and can produce IFN-γ, TNF-α, MIP1-α, MIP1-β and RANTES. They are believed to be responsible for defence against intracellular pathogens, tumours and viruses, but may contribute to aberrant inflammation in certain settings. Unlike NK cells, ILC1 lack granzyme B and perforin but express CD103 [165] and CXCR3 [163]. IL-7Rα+ IL-12Rβ2+ IL-1R+ type 1 ILCs produce IFN-γ in response to IL-12 and are enriched in inflamed mucosal tissue such as tonsils, intestine and in diseased tissue including the lamina propria of patients with Crohn's disease [165] and inflamed lungs of patients with chronic obstructive pulmonary disease (COPD) [166]. Interestingly type 1 ILCs are absent from foetal gut and develop after colonisation of the intestine with commensal bacteria [163]. These findings indicate their potential role in protection against certain bacteria in homeostatic conditions and their involvement in the pathogenesis of inflammatory bowel disease and COPD. The origin of group 1 ILC is not clear; however several studies have showed that RORɣt– IFN-ɣ producing ILC1 can originate from NKp44+ group 3 ILC under the influence of IL-12 and IL-15 [163, 167].

1.4.2 Group 2 ILCs

The production of key type 2 cytokines, IL-13, IL-4 and IL-5 in response to epithelial cytokines IL-25 and IL-33 in $Rag^{-/-}$ mice led to the discovery of group 2 ILCs. When I started my DPhil, three separate groups had recently reported lineage negative cells (CD3, CD4, CD8α, TCRαβ, TCRɣδ, CD5, CD19, B220, NK1.1, Ter119 (Ly76), Gr-1 (Ly6g), Mac-1 (Itgam), CD11c (Itgax) and FcεRIα) that expressed c-Kit (CD117) and T1/ST2, CD90 (Thy-1), CD45 and IL-7Rα (CD127) in mice. They were designated nuocytes [158], innate helper type 2 (IHC)[168] and natural helper cells (NHC)[169] but demonstrated similar functional characteristics. Concurrently, Saenz described a similar population of multi-potent progenitor type 2 (MPP type2) cells but unlike other populations, MPP type 2 cells exhibited progenitor capacity and could differentiate to myeloid and lymphoid lineage descendants [170]. Therefore, it has been speculated that they might be precursors of type 2 ILCs [171]. Recently the term ILC2 was proposed to group type 2 cytokine producing ILC in to a single family [155]. Lack of RORγt expression and IFN-ɣ production differentiate this group of innate cells from LTi and ILC1, respectively. ILC2s were described in fat associated lymphoid clusters (FALC), mesenteric lymph nodes (mLN), intestine and gut associated lymphoid tissues (GALT), liver and spleen. Bearing IL-17RB (IL-17BR, IL-25R), ST-2 (IL-33R) and TSLP receptors ILC2 cells respond to epithelial cytokines including IL-25 (IL-17E), IL-33 and TSLP, by producing type 2 cytokines such as IL-13, IL-4, IL-5, IL-9.

Human ILC2s were subsequently discovered in healthy human lung parenchyma and broncho-alveolar lavage (BAL) fluid of patients receiving a lung transplant as lineage negative cells (CD3, TCRαβ, CD11c, CD11b, CD56, CD19) that express IL-7Rα and ST2 subunit of the IL-33 receptor [172]. Spits *et al.* reported lineage negative (CD3, CD4, CD11c, CD14, CD19, CD34, CD123, TCRαβ, TCRγδ, BDCA2, and FcεRI), CD45hi, CD127+ and CD117+ cells in peripheral blood, foetal gut and inflamed nasal polyps of patients with rhinosinusitis. They also express prostaglandin D2 receptor (CRTH2), CD161 (KLRB1), CD7 and CD25. In response to epithelial cytokines and IL-2 these cells produce large amounts of IL-13 and IL-5 but not IL-17A or IL-22 [173, 174].

The protective role of ILC2 was seen during helminth infections as they provide predominant early source of type 2 cytokines [158, 169]. ILC2 represent a vital source of IL-13 for expulsion of the gut helminth, *Nippostrongylus brasiliensis* [158, 169, 170] by inducing goblet cell hyperplasia, eosinophilia and intestinal smooth muscle cell contraction. ILC2 also contribute to homeostasis and allergic responses in the airways. Using IL-$4^{+/eGFP}$ IL-$13^{+/Tom}$ dual reporter mice, it was shown that ILC2s were the major source of type 2 cytokines in Ovalbumin induced allergy and after intranasal administration of IL-25 and IL-33 [175]. Moreover, depletion of lung resident ILC2 in mice after infection with H1N1 influenza virus A resulted in impaired airway epithelial integrity and lung function, consequently exaggerated thermodysregulation and higher total protein concentration in the broncho alveolar lavage (BAL) fluid. Such function is predominantly mediated by amphiregulin production by ILC2, a wound-healing modulator of the epidermal growth factor family [172].

1.4.3 Group 3 ILCs

Group 3 ILCs include lymphoid tissue inducers (LTi cells), NCR+ ILC3 (NK22, NCR22, ILC22) and NCR- ILC3 (ILC17) [176]. Type 3 ILCs are important in inflammation, anti-microbial protection, mucosal immunity and homeostasis. LTi cells express c-Kit, IL-7Rα, IL-1R, IL-23R and lymphotoxin-β (LTβ), CCR6, and aryl hydrocarbon receptor (AHR) [177]. They initiate lymphoid structure formation and induce expression of VCAM-1 and ICAM-1 on mesenchymal cells through LTβR and TNFR signalling during embryogenesis and produce IL-17A [178]. After birth, LTis contribute to the formation of solitary lymphoid follicles and Peyer's patches. After birth splenic LTis produce IL-22 and IL-17A [177] in response to yeast cell wall product zymosan which suggests that they contribute to host defence. Furthermore, they also support class switching to IgA and are thus important for adaptive immune responses [179].

Two phenotypically distinct RORɣt dependent subsets of ILC3 were characterised recently, and based on the expression of natural cytotoxicity receptors, NKp46 in mice and NKp44 in human, they were divided into NCR^+ [176] and NCR^- ILC3 [155]. Postnatal NCR^+ ILC3 derived from tonsils largely produce IL-22 and small amounts of IL-17. They are thymus independent and reside in the intestine, dermis, tonsils and mLNs. $NKp44^+$ $ROR\gamma t^+$ $IL\text{-}22^+$ are diminished in germ free mice which suggests that their maintenance and functional properties are largely dependent on commensal bacteria [180]. IL-22 produced by $ROR\gamma t^+$ILCs is essential in protection against *Citrobacter rodentium* induced acute colitis in mice [180]. The expression of IL-22 in NCR^+ ILC3 is negatively regulated by epithelial cytokine IL-25 as intestinal

inflammation and epithelial damage by administration of dextran sodium sulfate (DSS) that concomitantly increased IL-23 and reduced IL-25, induced population expansion and IL-22 production by RORγt^{+}ILCs [181]. The NCR^{-} ILC3 population in mice expresses SCA-1, IL-23 receptor, transcription factor RORγt and high levels of Thy-1. The cells accumulate in the gut during *Helicobacter hepaticus* induced colitis. Stimulation of NCR^{-} ILC3s with IL-23 induces production of IL-17 and IFN-γ [156].

Recent studies shows that type 3 innate lymphoid cells can regulate adaptive immune responses. NCR^{-} ILC3 expresses high levels of major histocompatibility complex class II (MHCII) whereas NKp46^{+} ILC3 express minimal levels of MHCII. Although ILC3 can process and present antigen, they cannot induce T cell proliferation of naïve T cells as they lack co-stimulatory molecules, CD80, CD86, and CD40. Instead they appear to reduce T cell responses to commensal flora. Lack of RORγt^{+} ILCs induces spontaneous mild colitis, splenomegaly, shortened intestine and crypt elongation [182].

1.4.4 ILC2 origin and Transcription factors

Type 2 innate lymphoid cells arise in bone marrow from common lymphoid progenitors (CLP) at the double-negative stage 1 (DN1) and stage 2 (DN2). Their presence in thymus deficient Foxn1$^{nu/nu}$ (nude) mice confirms that they do not require thymus for their development [174, 183]. Unlike most previous studies that categorized NK and LTi cells in group 1 and group 3 ILCs respectively, recent work by Constantinides *et al.* showed a common ILC progenitor (ILCP) in foetal liver and adult

bone marrow that can differentiate into ILC1, ILC2 and ILC3 but not NK and LTi cells. ILCP were Lin^- IL-7Rα^+ c-Kit^+ $\alpha4\beta7^+$ and phenotypically similar to precursors of LTi. High levels of PLZF, a transcription factor associated with NKT cells, as well as high levels of Id2, GATA3 and TOX were found in ILCP. PLZF+ ILCP arise from an α4β7 IL-7Rα+ population from which NK and LTi progenitors are also known to develop. However, interestingly depletion of PLZF altered ILC development but did not affect NK and LTi cells which shows that NK and LTi have distinct precursors [160]. Furthermore, Nfil3 which is essential for development of adaptive immunity and NK cells is also required for maturation of Peyer's patches, ILC2 and ILC3s. $Nfil3^{-/-}$ mice failed to develop effective immune responses to *Citrobacter rodentium* [184].

ILC2s require IL-7, IL-33 and signalling through tyrosine kinase receptor Flt-3 [183]. $Jak3^{-/-}$ mice are deficient of ILC2 which confirms their requirement for signalling through common ɣ chain of IL-2 [174]. T cell factor-1 (TCF-1) essential for normal T cell lineage specification and is also required for development of ILC2. In $Tcf7^{-/-}$ mice the frequency of ILC2 is 5% of wild type mice, and the remaining ILC2 are functionally compromised. Transient Notch signalling is another vital requirement for ILC2 development that acts upstream of TCF-1 but does not require the HES-1 pathway [185]. Forced expression of TCF-1 can bypass Notch requirement by up-regulating GATA-3 expression. GATA-3 maintains the expression of IL-17RB, IL-2R, IL-1RL1 receptors and production of IL-13 and IL-5. Its effect is intrinsic and dose dependent [185, 186]. Upon activation with IL-33 or a combination of IL-2 and IL-25, it binds to the IL-5 and IL-13 promoter in a p38 dependent manner [187]. GATA-3 induces the expression STAT-5 and increases responsiveness to IL-33 and TSLP [188]. Unlike TH2

cells, the expression of GATA3 and production of type 2 cytokines in the lung resident ILC2 in mice are STAT-6 and STAT-3 independent [189]. STAT-6 regulates the proliferation of these cells and induction of eosinophilia in response to Alternaria challenge.

Consistent with the lymphoid origin of ILC2, the transcription factor Ikaros is necessary for their development [174]. Although in one report a trace of RAG-1 expression was detected in these cells, mature ILC2s do not express any rearranged antigen receptors [190] and unlike other members of ILC family, their development is largely dependent on RORA but not RORC expression [183]. The precise interaction between GATA-3 and RORA is not clear; Mjösberg *et al.* showed that RORA is not regulated by GATA-3 and these two transcription factors possibly work in parallel during development of ILC2, whereas Wolterink *et al.* observed the lack of RORα expression in the absence of GATA-3 [186, 188]. Although RORA and GATA-3 have a pivotal role in development of ILC2, RORA is not essential for cytokine production and maintenance of mature ILC2 [187]. Lung ILC2 were observed in germ free mice and therefore thought to be independent of commensal bacteria for their development [172]. Although a degree of plasticity has been shown in ILC3 and ILC1 populations as a proportion of RORɣt^{+} NKR^{+} LTis can down regulate RORɣt and produce IFN-ɣ [167], it has yet to be determined whether ILC2 have any plastic characteristics (summarised in Figure 1.2).

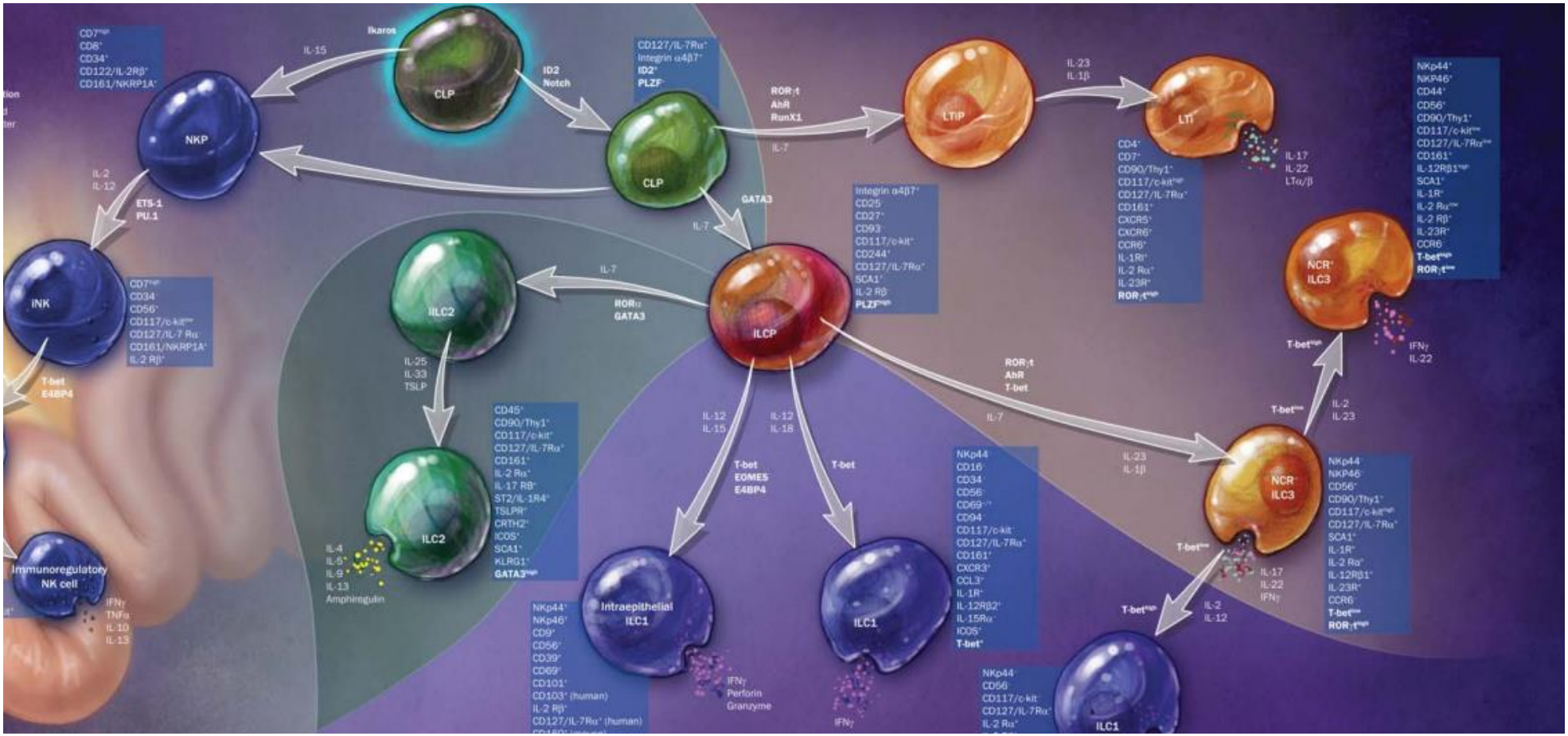

CD7high
CD8−
CD34+
CD122/IL-2Rβ+
CD161/NKRP1A+
Ikaros
IL-15
CLP
NKP
ID2
Notch
CD127/IL-7Rα+
Integrin α4β7+
ID2+
PLZF−
CLP
RORγt
AhR
RunX1
IL-7
LTiP
IL-23
IL-1β
LTi
CD4+
CD7+
CD90/Thy1+
CD117/c-kitlow
CD127/IL-7Rα+
CD161+
CXCR5+
CXCR6+
CCR6+
IL-1Rl+
IL-2 Rα+
IL-23R+
RORγtlow
IL-17
IL-22
LTα/β
NKp44+
NKP46+
CD44+
CD56+
CD90/Thy1+
CD117/c-kitlow
CD127/IL-7Rαlow
CD161+
IL-12Rβ1low
SCA1+
IL-1R+
IL-2 Rαlow
IL-2 Rβ+
IL-23R+
CCR6+
T-betlow
RORγtlow
IL-2
IL-12
ETS-1
PU.1
iNK
CD7high
CD34−
CD56+
CD117/c-kitlow
CD127/IL-7 Rα+
CD161/NKRP1A+
IL-2 Rβ+
T-bet
E4BP4
GATA3
IL-7
iILC2
RORα
GATA3
iLCP
Integrin α4β7+
CD25−
CD27+
CD93−
CD117/c-kit+
CD244+
CD127/IL-7Rα+
SCA1+
IL-2 Rβ−
PLZFhigh
NCR+
ILC3
T-bethigh
IFNγ
IL-22
IL-25
IL-33
TSLP
ILC2
CD45+
CD90/Thy1+
CD117/c-kit+
CD127/IL-7Rα+
CD161+
IL-2 Rα+
IL-17 RB+
ST2/IL-1R4+
TSLPR+
CRTH2+
ICOS+
SCA1+
KLRG1+
GATA3high
IL-4
IL-5+
IL-9+
IL-13
Amphiregulin
RORγt
AhR
T-bet
IL-7
IL-12
IL-15
IL-12
IL-18
T-bet
EOMES
E4BP4
T-bet
IL-23
IL-1β
T-betlow
IL-2
IL-23
NCR−
ILC3
NKp44−
CD16−
CD34−
CD56−
CD69−/+
CD94−
CD117/c-kit−
CD127/IL-7Rα+
CD161+
CXCR3+
CCL3+
IL-1R+
IL-12Rβ2+
IL-15Rα+
ICOS+
T-bet+
NKp44+
NKP46+
CD56+
CD90/Thy1+
CD117/c-kitlow
CD127/IL-7Rα+
SCA1+
IL-1R+
IL-2 Rα+
IL-12Rβ1+
IL-23R+
CCR6−
T-betlow
RORγtlow
T-betlow
IL-17
IL-22
IFNγ
T-betlow
IL-2
IL-12
Immunoregulatory
NK cell
IFNγ
TNFα
IL-10
IL-13
Intraepithelial
ILC1
NKp44+
NKp46+
CD9+
CD56−
CD39+
CD69+
CD101+
CD103+ (human)
IL-2 Rβ+
CD127/IL-7Rα+ (human)
IFNγ
Perforin
Granzyme
ILC1
IFNγ
NKp44−
CD56−
CD117/c-kit−
CD127/IL-7Rα+
IL-2 Rα+
ILC1

Figure 1.2. Innate Lymphoid Cell Lineage Pathway. Innate lymphoid cells originate from common lymphoid progenitors (CLP) in foetal liver and adult bone marrow. CLP express various transcription factors and differentiate into LTi progenitor (LTiP), NK cell progenitor (NKP) and ILC progenitor (ILCP). ILCP up-regulate T-bet and differentiate into ILC1. It can increase expression of GATA-3 and ROR-α and become ILC2. Up-regulation of ROR-γt gives rise to ILC3. Adapted from R&D systems ILC poster.

1.4.5 ILC2, allergic airway inflammation and lung infection

Airway hyper-reactivity (AHR) in response to common environmental allergens is the hallmark of asthma. Several studies have shown that intranasal administration of IL-25 and IL-33 as well as ovalbumin, alternaria and house dust mite induce allergic asthma, airway inflammation and accumulation of lineage negative cells that express IL-33R in lungs, mediastinal lymph nodes and BAL fluid [175, 189, 191]. Most allergens e.g. HDM, plant pollens, and air pollutants (diesel, smoke and ozone), viral infections and some bacterial species contain glycolipids that can activate NKT cells and induce the production of IL-33 by alveolar macrophages and type II pneumocytes. IL-33 in turn activates ILC2 and NKT cells and induces AHR independent of adaptive immunity [192]. Similarly airway infection with influenza A virus subtype H3N1 induced airway hyper-reactivity (AHR) by stimulating alveolar macrophages to produce IL-33 independent of NKT cells and therefore activating ILC2s through IL-33 - IL-13 axis [192, 193].

Moreover, depletion of lung resident ILC2 in mice after infection with H1N1 influenza virus A resulted in impaired airway epithelial integrity and lung function, consequently exaggerated thermodysregulation and higher total protein concentration in the broncho alveolar lavage (BAL) fluid. Such function is predominantly mediated by amphiregulin production by ILC2, a wound-healing modulator of the epidermal growth factor family [172].

Asthma attacks can be triggered by rapid production of mediators by innate immune components including ILC2 and eosinophils. Alternaria is a fungal allergen and

associated with near-fatal asthma exacerbations. Taylor and colleagues have shown that rapid production of IL-33 by airway epithelium following intranasal alternaria challenge induced the proliferation of ILC2s and increased expression of ST2 and IL-4R, production of type 2 cytokines and amphiregulin [189]. Cysteinyl leukotrienes (CysLTs) are potent smooth muscle contractors and can be produced by dendritic cells and macrophages in response to zymosan, HDM and peptidoglycans. Lung resident ILC2s can express CysLT1R and produce IL-13 and IL-5 in response to stimulation with LTD4, LTC4 and LTE4 [194, 195].

Eosinophilic pleural effusion (EPE) following primary spontaneous pneumothorax (PSP) is another inflammatory lung condition associated with increased levels of IL-5, IL-4, IL-33, TSLP, eotaxin-3. Kwon and colleagues reported the infiltration of type 2 ILCs in pleural effusion of PSP patients in response to IL-33 released following mechanical stress. ILC2 in this condition can potentially trigger and amplify eosinophilic inflammation independent of adaptive immunity [196].

1.4.5 ILC2 and skin

Last year, Kim *et al.* provided the first evidence on the presence of an ILC2 population in mouse and human skin. In mice, they reported a population of Lin^- $CD25^+$ $ST2^+$ c-Kit^+ $CD127^+$ $ICOS^+$ that did not express ILC3 associated markers CD4, NKp46 and RORɣt. Although they found a similar population in healthy human skin (Lin^- $CD25^+$ IL-33^+) these cells were negative for CRTH2 and CD161, previously described markers of ILC2 in humans [173], and they only acquired the expression of these markers in atopic dermatitis (AD) lesions. Therefore it raised the possibility that this population is either a distinct population of ILCs in human skin or is in a different stage of activation.

Roediger *et al.* demonstrated potential immunosurveillance activity of ILC2 in mouse skin. They reported a unique and abundant population of $CD45^+$ $CD11b^-$ $CD90^{hi}$ $CD3^-$ $CD2^-$ c-Kit^- IL-$17RB^+$ ILC2s in the dermis of naïve mice and called them 'dermal ILC2' (dILC2). DILC2s expressed integrin αEβ7 (CD103) and comprised 5-10% of $CD45^+$ cells. Using 4C13R dual reporter mice in which single alleles of IL-13 and IL-4 were substituted with dsRed and AmCyan respectively, they showed that homeostasis of the skin in the steady state was mainly controlled by $CD3^-$ $NK1.1^-$ dILC2 rather than TH2 cells as they were the main producers of IL-13. Although dILC2s were unable to produce IL-4 under homeostatic conditions, they acquired this capacity upon stimulation with TSLP. Furthermore, intravital multiphoton microscopy showed that $CXCR6^+$ ILC2 are mainly aggregated in the close vicinity of blood vessels. They constantly patrol the skin local microenvironment with rapid migration (5μm/min)

but with intermittent long interactions with dermal mast cells that lasted 20 to 30 minutes. Interestingly mast cells were not essential for development and maintenance of dILC2 as mast cell deficient mice (B6-Kit $^{W-sh/W-sh}$ and WBB6F1-Kit $^{W/W-v}$) had intact dILC2, but IL-13 produced by dILC2 was able to regulate mast cells.

Anti-CD90.2 antibody is routinely used to deplete ILC2 in mice. This method has been successfully used in the skin by Kim *et al.* [197] although Roediger *et al.* [174] could not deplete $CD103^+$ dILC2 from the skin using this method and only the ILC2 population in the spleen was depleted, which suggests that $CD103^+$ dILC2 population might represent a distinct sub-population of ILC2 in the skin. Therefore instead of using a depletion strategy to study ILC2 function in the skin, they activated dILC2 *in vivo* using complexes of IL-2 and JES6-1. Consistent with their immunomodulatory function, dILC2 underwent proliferation and produced large amounts of IL-13 and IL-5. IL-13 suppressed the IgE dependent release of inflammatory cytokines by mast cells in a dose dependent manner [174].

1.4.6 Innate lymphoid cells, atopic dermatitis and psoriatic skin inflammation

Given increased levels of type 2 cytokines, IL-13, IL-4 and IL-5, in acute atopic eczema lesions and enhanced production of epithelial cytokines IL-33, IL-25 and TSLP, it is plausible that group 2 innate lymphoid cells contribute to the pathogenesis of atopic dermatitis. Indeed, ILC2 were found in higher proportions in the lesions of patients with atopic dermatitis while the frequency of the cells was similar in the blood of both healthy and atopic individuals (explained in detail in results chapters) [197, 198].

Type 2 innate lymphoid cells contribute to inflammation in mouse models of AD-like inflammation. hK14mIL33tg mice in which IL-33 is expressed under the keratin 14 promoter developed spontaneous atopic dermatitis-like inflammation of the skin at 6-8 weeks of age in specific pathogen-free (SPF) conditions. Similar to AD lesions, the pattern of IL-33 expression in this model was confined to the epidermal cell nuclei. Dermatitis lesions in hK14mIL33tg mice were associated with a substantial increase in the concentration of IL-13, IL-5, RANTES/CCL5 and Eotoxin 1/CCL11, whereas the levels of TSLP, IFN-γ and TNF-α were not altered. Increased degranulating IgE^{+} $c\text{-}Kit^{+}$ mast cells as well as a significant infiltration of eosinophils were observed in the lesions and blood of transgenic mice (7.4 and 4.5 fold higher expression compared to wild type mice respectively). Concurrently skin lesions and regional lymph nodes were enriched for Lin^{-} $ST2^{+}$ $Sca\text{-}1^{+}$ ILC2 producing IL-5 and IL-13 [199].

Interestingly treatment of $Rag1^{-/-}$ mice with IL-2 and anti-IL-2 complex (JES6-1) for 2-3 weeks induced spontaneous skin inflammation around ears, eyes, mouth and tail. Extensive accumulation of neutrophils, eosinophils and increased degranulation of mast cells were observed in these lesions which were caused in part by dILC2. DILC2 showed activated phenotype with higher expression of CD25, ICOS, CD69, ST2 and enhanced production of IL-13 and IL-5 [174].

Topical application of a form of vitamin D3, calcipotriol (MC903), induces ear thickening, xerosis, elevated IgE and histopathological changes comparable to AD lesions and is recognised as an experimental murine model of atopic dermatitis. Coincident with ear thickening, increased infiltration of ILC2 was observed in the ear

pinna and draining lymph nodes of treated mice [197, 198] which was independent of adaptive immunity as $Rag1^{-/-}$ mice still developed AD-like inflammation. Treatment of $Rorc^{-/-}$ mice with calcipotriol induced the same level of inflammation which ruled out the involvement of RORγt dependent ILC3s [197]. Depleting ILC2 using anti-CD90.2 [197, 198] or anti-CD25 antibodies [197] in $Rag1^{-/-}$ mice significantly ameliorated inflammation and histopathological changes observed in this model.

It is noteworthy to mention that as well as ILC2s, populations of NCR^- ILC3 (26.5±8.6%) and $CD161^+$ ILC1 (24.8 ±11.0% of $CD45^+$ Lin^- $CD127^+$ ILC) were observed in normal human skin [200] (Figure1). There is a noticeable population of $NKp44^+$ ILC3s in cultured dermal explants, although rare in freshly isolated cells. NCR^+ ILC3s differentiated from NCR^- ILC3 upon culture with IL-23, IL-1β and express IL-22.

Blood and lesional skin biopsies of patients with psoriatic skin inflammation showed enrichment of NCR^+ ILC3 although similar frequencies of $CD161^+$ ILC1 and $CRTH2^+$ ILC2 were observed [200, 201]. These cells were an innate source of IL-22. One report found that the frequency of $NKp44^+$ ILC3 correlated with disease severity using PASI score [200] whereas another report could not show a similar finding [201]. Treatment of one psoriatic patient using anti-TNF monoclonal antibody (adalimumab) showed substantial drop (75%) in frequency of $NKp44^+$ ILC3 and equivalent increase in population of NCR^- ILC3s. This reduction inversely correlated with disease severity (PASI score 21.2 to 13.6) which shows the potential importance of NCR^+ ILC3 in the pathogenesis of psoriasis.

1.4.7 Other roles of ILC2

1.4.7.1 Visceral adipose tissue ILC2

ILC2s have also been implicated in metabolic homeostasis. They reside in perigonadal visceral adipose tissue (VAT) and spontaneously produce IL-5 and IL-13. Depletion of ILC2s, as shown in IL-5 or IL-13 deleter mice, resulted in profound loss of eosinophils and alternatively activated macrophages (AAM) which lead to adiposity and insulin resistance in mice challenged with high fat diet (HFD) [202].

1.4.7.2 Bee venom PLA2 and ILC2

Phospholipase A2 (PLA2) is a major allergen in venoms. Using IL-4-IRES-eGFP (4get) reporter mice, Noah et al demonstrated that subcutaneous delivery of bee venom induced infiltration of $CD4^+$ T cells in the draining lymph node and increased antigen specific IgE and IgG1 responses. These responses were believed to be dependent on PLA2 as heat inactivation of PLA2 eliminated responses. While conditional $MyD88^{-/-}$ mice that lack MyD88 in DC had normal responses, type 2 responses were reliant on MyD88 expression in T cells. PLA2 mediated reactions through cleavage of membrane phospholipids and production of lysophospholipids (LPC) which in turn induced production of IL-33 and activated ILC2s. TH2 responses and ILC2 induction by bv-PLA2 were ST2 dependent [203].

1.4.7.3 Interaction between ILC2 and T cells

Recent studies demonstrated mutual interaction between ILC2 and T cells. ILC2s in humans and mice express MHC-II and co-stimulatory molecules CD80 and CD86. Co-

culture of ILC2s and T cells in the presence of antigen, induced type 2 cytokine production in T cells. Furthermore, *N. brasiliensis* infection of two mouse models deficient in ILC2 showed delayed worm expulsion and a dramatic decrease in IL-13 and IL-5 producing $CD4^+$ T cells. Moreover, IL-2 released from T cells promotes ILC2 proliferation and cytokine production [204].

1.4.7.4 ILC2 sensing of malnutrition

Malnutrition is the leading cause of immune suppression. A recent study by Spenser et al revealed that nutrient deficiency does not cause generalised immune suppression as previously thought; instead it activates a distinct pathway of the innate immune system. Reduction in vitamin A and its metabolite retinoic acid (RA) are dietary alarm signals that are sensed by type 2 innate lymphoid cells and leads to proliferation, IL-13 production and goblet cell hyperplasia. Although TH1, TH17 and ILC3 cells diminish under vitamin A deficiency, increased type 2 responses by ILC2 help in barrier integrity and defence against helminth infections. Vitamin A deficient mice and those treated with RA inhibitors were resistant to infection with *Trichuris muris* which was dependent on IL-13 by ILC2 and independent of adaptive immune system [205].

1.5 Aims and Objectives.

The overall aims and objectives of this thesis address the phenotype and functions of type 2 innate lymphoid cells in the skin and in patients with atopic dermatitis. At the time of starting my DPhil, there were no data on ILC2 and the skin, but given the known presence of epithelial cytokines IL-33, IL-25, TSLP and increased expression of type 2 cytokines IL-4, IL-5 in atopic dermatitis lesions this raised the possibility that ILC2 may be contributing to the cutaneous inflammation. Specifically, I aimed to test the hypothesis that ILC2 are present in human skin and contribute to the pathogenesis of dermatitis. Furthermore I aimed to investigate the associated underlying mechanisms. This hypothesis would be tested by addressing the following objectives:

- To characterise type 2 ILC population in human skin
- To evaluate cytokine expression and cell content of allergen challenged and unchallenged atopic dermatitis skin compared to healthy controls
- To define the role of ILC2 in a mouse model of dermatitis
- To identify a barrier sensing mechanism of ILC2 (KLRG1 Ecadherin interaction)
- To evaluate the effect of prostanoid lipid mediator prostaglandin D2 (PGD2) on CRTH2 expressed by ILC2
- To characterise the role of natural cytotoxicity receptor 3 (NKP30) on ILC2

Chapter 2. Material and Methods

2.1 Patients and Samples

Normal adult human skin surplus tissue was obtained from surgical procedures according to GCP guidance with ethical approval of the Oxford Research Ethics Committee. Blood samples were collected from patients exhibiting symptoms of varying severity recruited through the department of Dermatology, Churchill Hospital, Radcliffe Hospitals NHS Trust, Oxford, UK, under the ethical approval of Oxford Clinical Research Committee. All patients were characterised using the validated UK working party criteria for diagnosis of atopic dermatitis [206]. Control samples were obtained from healthy volunteers. All data and samples collated were stored anonymous and confidential, with informed consent gained from all donors.

2.2 Blood Separation

Venous blood was collected using BD Vacutainer® Safety-Lok™ Blood Collection Set with Pre-Attached Holder (368654) in heparin coated tubes (BD vaccutainer 368480) to prevent coagulation, diluted 1:1 with RPMI-1640 media (R8758, Sigma Aldrich, UK) supplemented with penicillin/streptomycin (DE17-603E, BioWhittaker, Lonza Group Ltd, Switzerland) and L-glutamine (BE17-605E, BioWhittaker, Lonza Group Ltd, Switzerland), then layered on top of Ficoll-Hypaque medium (1114547 Lymphoprep, Axis-Shield PoC AS, Norway). The blood was centrifuged for 20 minutes at 2000 rounds per minute (rpm), ensuring that the brake application was switched off. The PBMC layer was aspirated into a new tube and diluted with RPMI media and centrifuged for 10 minutes at 1600 rpm. This washing process was repeated, then an

aliquot of cells were counted in a 1:1 dilution with 0.4% Trypan blue solution (T8154, Sigma-Aldrich, UK) using Hycor Kova Glasstic counting chamber slides (87144, Hycor Biomedical Inc., USA). The cells were resuspended in an appropriate volume of R0 supplemented with 10% human AB serum (H4522 Sigma-Aldrich, UK) (R10H – see Table 2.1) to achieve the desired concentration.

2.3 Isolating immune cells using established methods

After removing the subcutaneous fat, skin biopsies were cut into small pieces (0.5x0.5mm) and incubated in 5mM EDTA/HBSS with vigorous shaking at 4°C. After 2 hours the supernatants were spun down and the skin fragments were crushed through 40µm strainer and pooled to obtain the maximum number of cells [207]. For Collagenase D digestion, skin biopsies were prepared as described above and incubated in RPMI 1640 medium containing 1mg/ml collagenase D on a shaker at 37°C. After 30 minutes the skin fragments were washed with cold 10mM EDTA to stop digestion. The remaining tissue were homogenized through 40µm nylon mesh and spun down [207, 208].

2.4 Serum separation and specific IgE testing

Fresh venous blood was collected in BD serum tubes (Increased Silica Act Clot Activator, Silicone-Coated Interior, BD 367812). Samples were centrifuged at 3000 rpm for 10 minutes and the serum layer aliquoted into 1.5ml Eppendorf Tubes® 3810X and stored at -80°C. Specific IgE to common allergens were measured at the Department of immunology, Churchill Hospital, Oxford using the Pharmacia Unicap system.

2.5 Cell Culture

2.5.1 ILC2 cell culture

Purified ILC2 were sorted into 96-well plates at the density of 100 cells per well and re-suspended in mixed lymphocyte reaction (MLR) of gamma-irradiated peripheral blood mononuclear cells (PBMCs) from 3 healthy volunteers ($2x10^6$ cells/ml) coupled with 100 IU/ml of IL-2 in RPMI 1640 (Sigma) supplemented with 10% heat inactivated human serum and 2 mM l-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin (R10H). Every 2-3 days thereafter, half of the medium was replaced with equal volume of R10H and 100IU IL-2. Every 4 weeks the cultures were fed with MLR mixed with IL-2.

2.5.2 Keratinocytes

Keratinocyte line (HaCaT) was cultured in tissue culture flasks (Corning Incorporated, USA) D10 at 37°C with 5% CO2 and split on reaching confluence (approx. every 3-4 days). To split cells, media was removed and adherent cells washed with warm trypsin-EDTA (BE17-161E, Sigma-Aldrich, UK) to remove any media, as trypsinisation is blocked by the presence of FCS. 2 mls of trypsin were added to the flask for 45 minutes at 37°C with 5% CO2 until all adherent cells were suspended in the trypsin, applying gentle tapping if necessary. The trypsin was diluted with 5 times its volume of warm D10. Cells were then centrifuged at 300g for 5 minutes, the supernatant discarded, and resuspended in warm D10 and split appropriately into flasks.

2.5.3 K562, Jurkat and THP-1 cell lines

K562, Jurkat and THP-1 cell lines were cultured in RPMI-1640 supplemented with 10% FCS, Amino acids (MEM Non-Essential Amino Acids Solution 11140-050 Life Technologies) and HEPES (83264 SIGMA). Cells were maintained at $0.2x10^6$/ml density.

2.6 Enzyme linked immunospot assay (ELISpot)

2.6.1 IFN-γ and IL-4 ELISpots

IFN-γ ELISpot (Mabtech, 3420-2A, Sweden) and IL-4 ELISpot (Mabtech, 3410-2A, Sweden) were performed according to the manufacturer's protocol. To prepare coating buffer, each carbonate-Bicarbonate Buffer capsules (C3041 SIGMA) were dissolved in 100ml of purifies H_2O. The IFN-γ/IL-4 specific capture antibody was diluted in the coating buffer to 15 µg/ml. $Multiscreen_{HTS}$-IP filter plates (Millipore, MSIPS4510) were soaked with 50 µl/well 35% ethanol for 1 minute, and then rinsed with distilled water 5 times. The wells were then coated with 50 µl/well of the diluted capture antibody, sealed with parafilm and stored at 4°C overnight. Plates were washed six times with R0 and subsequently blocked with R10 for 1 hour at 37°C/5% CO2. T cell lymphocytes were added to the plate at the concentration of between $5x10^4$ and $2x10^4$ cells per well. Phorbol-12-myristate-13-acetate (PMA) (79346, Sigma-Aldrich, UK) (50 ng/ml) / ionomycin (I3090, Sigma-Aldrich, UK) (250 ng/ml) was used as a positive control at 2 µg/ml per well and R0 alone was used as a negative control. All conditions were set up in duplicate wells and the mean number of spot forming units was used in analysis. The plate was incubated overnight at 37°C with 5%

CO2. After incubation, the plate was washed 6 times with 200 µl of PBS-0.05% Tween (4370829, BDH chemicals, UK) per well. IFN-γ/IL-4 detection antibody was diluted in PBS to a concentration of 1 µg/ml and 100 µl of the diluted antibody was added to each well and incubated for 2-4 hours at room temperature. The washing step was repeated and then 100 µl/well of diluted streptavidin (1 µg/ml) was added and the plates were incubated at room temperature for a further 1-2 hours. The washing step was repeated for a final time and 100 µl of developing buffer (as per manufacturers' instructions – 170-6432, Biorad, UK) was added per well, and the plate incubated for 10-20 minutes at room temperature until spots appeared. Plates were then washed with tap water and inverted on paper towels to dry.

2.6.2 IL-13 ELISpot

IL-13 ELISpot assays were completed with an identical method to IFN-γ and IL-4 except that the IL-13 capture antibody was added at 10µg/ml dilution.

2.7 Enzyme-linked Immunosorbent Assay (ELISA)

AD patient and healthy control samples were stimulated with recombinant human IL-25 (10, 40, 100 ng/ml; Peprotech), IL-33 (10, 40, 100ng/ml; Peprotech), TSLP (10, 40, 100 ng/ml; Peprotech), IL-2 (100 unit/ml; Peprotech), PMA (10 ng/ml; 79346, Sigma-Aldrich, UK) and Ionomycin (2 µmol/l; I3090, Sigma-Aldrich, UK), PGD2 (1ng-1mg; Enzo life sciences) and TM30089 (CAY10471) for specified time period. Supernatant was collected for human IL-13, IL-22, IL-17 ELISA Ready-SET-Go eBiosciences 88-7439-86, 88-7522 and 88-7176 respectively. Corning Costar 9018 (or Nunc Maxisorp®) ELISA plates were coated with 100µl/well of capture antibody and incubated

overnight at 4°C. The plates were then washed with 300µl/well with PBS-Tween (0.05%) 5 times with 1 minute soak intervals using an ELISA plate washer. The wells were then blocked by 100 µl/well 1X diluted assay buffer for 1 hour at room temperature. After 3 washes, 100 µl/well supernatant was added in duplicates. The plates were gently tapped to remove air bubbles, covered with an adhesive strip and incubated for overnight at 4°C. The plates were washed with PBS-Tween (0.05%) 5 times with 1 minute soaking intervals. The secondary antibody was diluted in PBS and 100 µl were added per well. The plate was covered with an adhesive strip and incubated again for 1 hour at room temperature. After adding the streptavidin-peroxidase conjugate, the plate was covered with an adhesive strip and incubated again for 30 minutes at room temperature. The washing step was repeated again and then 100 µl of tetramethyl benzidine (TMB) substrate solution was added to each well, the plate covered with foil and incubated for 5 minutes at room temperature. The reaction was stopped with 100 µl of stop solution (H2SO4) and tapped gently to remove air bubbles. The plate was then placed in the spectrophotometer and the absorbance measured at 450 nm using a BioRad Microplate reader (model 680) and software (BioRad, UK).

2.8 Depletion of $CD3^+$ T cells and $CD14^+$ monocytes

$CD3^+$ and $CD14^+$ cells were depleted from PBMCs using MACS bead separation system. PBMCs were resuspended in 80µl/10^7 cells of MACS buffer (0.5% BSA/0.2mM EDTA in PBS). 20µl CD3 or CD14 beads was added to every 10^7 cells and incubated for 15 minutes at 4°C. Cells were washed in 2ml/10^7 cells of MACS buffer for 10 minutes

at 300g. The pellets were resuspended in 500ml of MACS buffer. Cell suspension was passed through LS column on a MACS magnet and washed 3 times with 3ml buffer. CD3 and CD14 depleted cell suspension was collected, washed and resuspended in appropriate media.

2.9 Flow Cytometry and cell sorting

For FACS surface staining the cells were labelled by following anti human antibodies (table 2.1) purchased from Biolegend unless stated otherwise: CD3 (SK7;BD biosciences), CD19 (SJ25C1; BD biosciences), CD123 (FAB301C; R&D systems), CD11b (DCIS1/18), CD11c (BU15; Abcam), CD8 (RPA-T8), FceRI (AER-37 (CRA-1)), CD14 (MϕP9; BD biosciences), CD4 (MEM-241), CD45 (H130), ICOS (C398.4A), CD56 (B159), CRTH2 (BM16; Miltenyibiotec), IL-7Rα (A019D5), CD25 (BC96; eBiosciences), live/dead violet (L34955; Invitrogen), NKp30 (clone: AF29-4D12), NKp30 blocking antibodies (Clone 210845 R&D systems), Phospho-IkappaBalpha (Ser32/36 Cell Signalling 9246) and Anti-B7-H6 antibody (ab121794). The right concentrations of antibodies were added and the cells were incubated for 30 minutes at 4°C. After 2 washes, the samples were acquired using FACSDiva or Summit software on LSRFortessa or CyAn flow Cytometer, respectively. FlowJo and Summit software were used for further data analysis.

ILC2 were purity sorted after depletion of T cells and monocytes using CD3 and CD14 coated MicroBeads (Miltenyi Biotec 130-050-101 and 130-050-201) and staining with the above antibodies on a MoFlo™ XDP cell sorter or a BD FACSARIA III or BD FACSAria™ Fusion. Single staining of lineage cocktail is shown in Figure 2.1.

Antigen	Clone	Supplier
CD3	SK7	BD Biosciences
CD19	SJ25C1	BD Biosciences
CD123 (IL-3R)	32703	R&D Systems
CD11b	DCIS1/18	Abcam
CD11c	BU15	Abcam
CD8	RPA-T8	BioLegend
FcεRI	AER-37 (CRA-1)	BioLegend
CD14	MφP9	BD Biosciences
CD4	MEM-241	Abcam
CD45	H130	BioLegend
CD56	NCAM	BioLegend
CRTH2	BM16	Miltenyi Biotec
IL-7Rα (CD127)	A019D5	BioLegend
LD		Invitrogen
CKIT	104D2	eBioscience
CD161	DX12	BD Biosciences
IL17RB (IL-25R)	MM0374-5P14	Novus Biologicals
CXCR6	K041E5	BioLegend
KLRG1	RabPolyclonal ab77524	Abcam
CD3	UCHT1	Beckman coulter
TSLP-R (CRLF-2)	eBio1A6	eBioscience
ST-2 (IL-33R/IL-1RL1)		
ICOS	C398.4A	BioLegend
CD25 (IL-2R)	BC96	eBioscience
NKp30 (NCR3/CD337)	AF29-4D12	Miltenyi Biotec
NKp30 blocking antibodies	210845	R&D systems
Phospho-IKBα	9246	Cell Signalling
B7-H6	ab121794	Abcam
CLA	HECA-452	Biolegend
CCR4	TG6/CCR4	Biolegend
CCR6	FAB195F	R&D systems
CCR10	6588	Biolegend

Table 2.1 antibodies, clone and suppliers used for FACS sorting ILC2

Antigen	Clone	Supplier
CD45RO	UCHL1	BD Biosciences
γδ T cell receptor	B1.1	eBiosciences
IL-13	JES10-5A2	BD biosciences
IL-22	22URTI	BD biosciences
IL-17	eBio64DEC17	eBiosciences

Table 2.1 antibodies, clone and suppliers used for FACS sorting ILC2

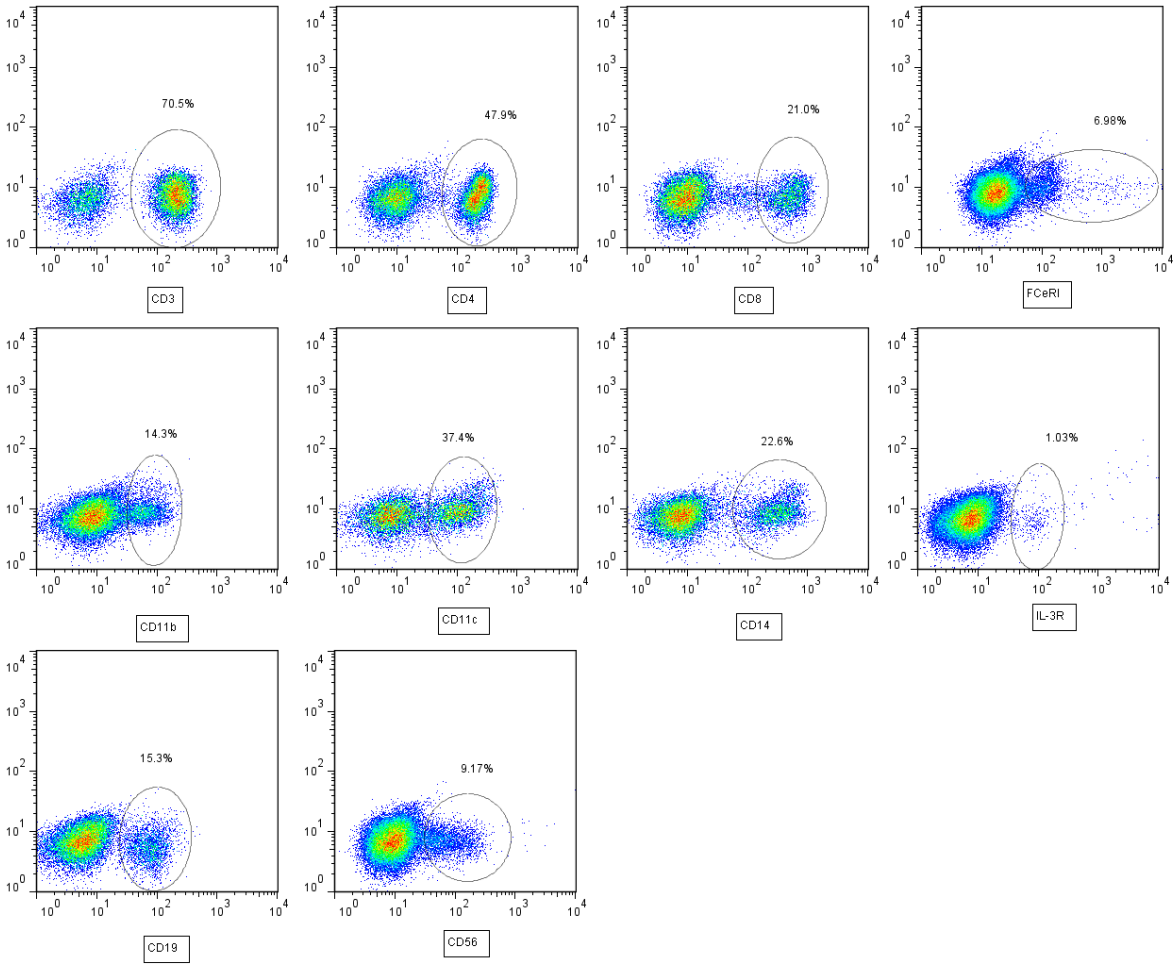


Figure 2.1 Single staining of lineage markers. PBMC were stained with lineage markers to identify different populations.

2.9.1 Flow cytometry studies of isolated skin T cells

Isolated T cells were analysed using anti human CD3 (SK7; BD biosciences), and combination of skin homing receptors; cutaneous lymphocyte associated antigen CLA (HECA-452 Biolegend), CCR4 (TG6/CCR4 Biolegend), CCR6 (FAB195F R&D systems), CCR10 (6588 Biolegend). To compare memory and naïve T cells were stained with CD45RA (HI100 BD Biosciences) and CD45RO (UCHL1 BD Biosciences). Monoclonal antibodies against γδ T cell receptor (B1.1) was obtained from eBiosciences. The samples were acquired using FACSDiva or Summit software on LSRFortessa or CyAn flow Cytometer, respectively. FlowJo and Summit software were used for further data analysis.

2.10 Intracellular Staining

Cells were stimulated with one or combination of following cytokines; recombinant human IL-25 (10, 40, 100 ng/ml; Peprotech), IL-33 (10, 40, 100ng/ml; Peprotech), TSLP (10, 40, 100 ng/ml; Peprotech), IL-2 (100 unit/ml; Peprotech), PMA (10 ng/ml; 79346, Sigma-Aldrich, UK) and Ionomycin (2 µmol/l; I3090, Sigma-Aldrich, UK), PGD2 (1ng-1mg; Enzo life sciences) and TM30089 (CAY10471) for specified time period. For intracellular cytokine staining (ICS), monensin (3 µmol/l) was added for the last 6 hours of the culture to the medium. The cells were washed twice with PBS via centrifugation at 1500 rpm for 5 minutes. Antibodies against surface markers were added to each sample, and then the cells were incubated for 30 minutes at 4°C in the dark. The cells were washed twice. The cells were permeabilized using

Cytofix/*Cytoperm*™ kit (BD *554722*) and stained for production of cytokines using IL-13 (JES10-5A2;BD biosciences), IL-22 (22URTI; BD biosciences), IL-17 (eBio64DEC17) and incubated for 30 minutes at 4°C in the dark. Cells were washed twice again and fixed with 0.5% formaldehyde and acquired using Summit or FACSDiva software on CyAn (Dako, Denmark) or BD LSRFORTESSA flow Cytometer. FlowJo and Summit software were used for further data analysis.

2.11 Multiplex bead array

2.11.1 Luminex and Magpix

Multiplex bead arrays were performed according to manufacturer's instructions, briefly 25µl of culture supernatant was incubated for 1 hour with anti-cytokine antibody coupled beads. After 3 washes, samples were incubated with biotinylated detection antibody for 1 hour before Streptavidin-phycoerythrin incubation for further 30 minutes. Fluorochrome conjugated beads were enumerated using *Luminex® 200TM system* (Bio-Rad).

2.11.2 QuantiGene Plex assay

ILC2 cells were treated with various conditions as described for indicated duration. Total RNA of the cells were extracted using an RNeasy Mini kit, and then the mRNA levels of selected genes (IL-3, IL-4, IL-5, IL-8, IL-9, IL-13, IL-21, CSF1, GM-CSF, and GAPDH) in the RNA samples were measured using a QuantiGene 2.0 Plex Assay kit with magnetic beads following the manufacturer's instruction, briefly 20 µl RNA

sample was shaken with Bead Mix for 20 h at 54°C, and then with 2.0 Pre-Amplifier Reagent for 1 h at 50°C followed by incubation with 2.0 Amplifier Reagent for another hour at 50°C. The sample was further incubated with Label Probe Reagent for 1 h at 50°C before Streptavidin-phycoerythrin incubation for further 30 minutes at room temperature. Fluorochrome conjugated beads were quantified using a Bio-Plex 200 System (Bio-Rad).

2.12 Quantitative Polymerase Chain Reaction (Q-PCR)

RNA extraction, reverse transcription, pre-amplification and qPCR were performed using Ambion® Single Cell-to-CT™ Kit (Life technologies 4458236) for ex-vivo assays according to manufacturer's instructions. For cultured cells RNA extraction was performed using RNeasy plus Mini Kit (Qiagen 74134). cDNA was prepared using Omniscript RT kit.

The following gene expression assays were purchased from Applied Biosystems: GATA3 (Hs00231122_m1), IL-5 (Hs01548712_g1), TSLP-R (Hs00845692_m1), KLRG1 (Hs00929964_m1), CRTH2 (Hs00173717_m1), RORC (Hs01076122_m1), CD161 (Hs00174469_m1), IL-13 (Hs00174379_m1), GAPDH (Hs99999905_m1), ST-2 (HS00545033-m1), IL-4 (Hs00174122_m1), RORα (HS00536545_m1), NKp30a (Hs01553310-g1), NKp30b (Hs01561746-g1) and NKp30c (Hs01553311-g).

Primers and probes used were as follows: IL-4, 5'-CACCGAGTTGACCGTAACAG-3' and 5'-GCCCTGCAGAAGGTTTCC-3' with probe 16 generating a 72-bp amplicon; IL-5, 5'-GGTTTGTTGCAGCCAAAGAT-3' and 5'-TCTTGGCCCTCATTCTCACT-3' with probe 25 generating a 66-bp amplicon; IL-13, 5'-AGCCCTCAGGGAGCTCAT-3' and 5'-

CTCCATACCATGCTGCCATT-3’ with probe 17 generating a 84-bp amplicon; CRTH2, 5’-CCTGTGCTCCCTCTGTGC-3’ and 5’-TCTGGAGACGGCTCATCTG-3’ with probe 43 generating a 95-bp amplicon; IL1RL1, 5’-ttgtcctaccattgacctctacaa-3’ and 5’-gatccttgaagagcctgacaa-3’ with probe 56 generating a 75-bp amplicon; IL17RA, 5’-catcctgctcatcgtctgc-3’ and 5’-gccatcggtgtatttggtgt-3’ with probe 85 generating a 85-bp amplicon; IL17RB, 5’- gcccttccatgtctgtgaat-3’ and 5’- ccggccttgacacacttt-3’ with probe 64 generating a 82-bp amplicon; IFNγ, 5’- ggcattttgaagaattggaaag-3’ and 5’-tttggatgctctggtcatctt-3’ with probe 21 generating a 112-bp amplicon; IL-17A, 5’-tgggaagacctcattggtgt-3’ and 5’- ggatttcgtgggattgtgat-3’ with probe 8 generating a 84-bp amplicon; IL-17F, 5’- ggcatcatcaatgaaaacca-3’ and 5’- tggggtcccaagtgacag-3’ with probe 10 generating a 94-bp amplicon; and GAPDH, 5’-AGCCACATCGCTCAGACAC-3’ and 5’-GCCCAATACGACCAAATCC-3’ with probe 60 generating a 66-bp amplicon. Reactions were carried out in a Applied Biosystems 7500 Fast Thermal Cycler.

2.13 Chemotaxis assay

For measurement of cell migration, ILC2 cells were resuspended with RPMI 1640 media; 25 µl of cell suspension and 29 µl test samples prepared in RPMI 1640 or mast cell supernatants were applied to the upper and lower chambers respectively in a 5 µm-pore sized 96-well ChemoTx plate (Neuroprobe, USA). After incubation at 37°C for 60 min, any cells remaining on top of the filter were wiped off, and the migrated cells in the lower chambers were collected. The cells were quantified by reaction with

a Cell Titer-Glo Luminescent Cell Viability Assay kit and then measured using a FLUOstar OPTIMA luminescence plate reader (BMG LabTech).

2.14 Suction blister technique

HDM extract was delivered by intra-epidermal skin prick test to the upper arm of the volunteers, briefly a drop of HDM extract was applied to the skin and a 2mm lancet was used to inject the extract into the epidermis and superficial dermis. Approximately 0.07µg HDM extract (ALK) were injected into the skin during this process which is 0.001% of the amount administered during HDM immunotherapy [209]. Allergic reactivity was defined on the basis of an immediate urticarial response. After approximately 2 hour, suction blister cups were applied to the site of injection with vacuum pressure of 250-450 mmHg. Blisters were formed within 30-90 minutes. At defined time points, fluid was aspirated using a 30-gauge needle. Fluids were then centrifuged at 1500 rpm for 5 minutes at 4°C and cell pellets were re-suspended in R10H and counted using 0.4% trypan blue.

2.15 E-cadherin plate bound experiments

Streptavidin-coated microwell plates from Millipore (20-183) were first coated with biotinylated Fc receptor (ebio) and then used to immobilize recombinant human E-Cadherin Fc Chimera (R&D systems 648-EC-100) or isotype control. Each step was performed at the concentration of 1 µg/ml overnight at 4°C. $5x10^4$ PMA/Ionomycin or

IL-25, IL-33 activated ILC2 were cultured on E-cadherin or isotype control coated plates. After 24 hours, cells were washed and RNA was extracted.

2.16 B7H6 plate bound assay

Coat Corning Costar 9018 (Nunc Maxisorp®) were coated with indicated concentration of recombinant human B7H6 Fc chimera protein (R&D systems 7144-B7-050) or isotype control overnight at 4°C. $5x10^4$ ILC2 were cultured on B7H6 or isotype control coated plates. After 24 hours the supernatants were collected for cytokine analysis using ELISA or Luminex.

2.17Statistical Analysis

The unpaired t-test, Mann Whitney U test, Spearman's rank correlation, the Kruskal Wallis Test with Dunn's multiple comparison, were performed as appropriate using GraphPad Prism version 6.0 for Windows (GraphPad software, San Diego, California, USA).

Chapter 3: Phenotype and function of ILC2 in skin and in AD patients

3.1 Enhanced isolation of lymphoid cells from human skin

3.1 Introduction

A diverse repertoire of T cells and B cells reside in the skin and indeed it has been estimated that the number of resident T cells in unchallenged skin are almost 2×10^{10} which is nearly double the number of T cells in the circulating blood [210]. These predictions have been based on estimates from normal skin sections, but intact *ex vivo* isolation of T cells at such frequencies has not previously been obtained.

Established methods using EDTA or collagenase D produce a low yield of cells [207, 210] so other approaches have introduced a culture step, for example on the surface of Cellfoam three-dimensional growth matrices for 21 days [211]. Although such culture approaches represent a significant step forward for certain applications, they can introduce potential *in vitro* changes to the frequency of the isolated cells. Furthermore, skin-derived T cells are imprinted with the tissue specific homing receptors including cutaneous lymphocyte antigen (CLA) and chemokine receptors, CCR4, CCR6[212], CCR10 [213, 214], to provide effective and quick trafficking of T cells to the site of inflammation, and it is not clear to what extent the culture step can

modulate phenotype and function. Effective isolation of lymphoid cells from the skin is vital for maximising information obtained from skin samples in order to define their role in health and disease. It is clear that T cells play a role in diverse skin surveillance and pathology [215, 216] and understanding their role may contribute to the development of novel therapeutic approaches [217]. Herein I wished to develop a new method that gives rise to a far larger number of intact T cells *ex vivo* from human skin than has previously been possible.

3.1 Aim

I aimed to identify enhancements of existing methodologies to increase effective isolation of skin immune cells.

3.1 Results

3.1.1 Collagenase P enzymatic treatment dramatically increases skin cell isolation yield

T cells were isolated from normal healthy adult skin using EDTA (n=4) and collagenase D digestion (n=4) as previously described [207, 208]. In addition, an alternative approach was used where skin biopsies were first washed with cold PBS and subcutaneous fat was removed. The biopsies were cut into < 0.5mm pieces, and incubated in RPMI 1640 (Sigma) supplemented with 10% heat inactivated foetal calf

serum (FCS) and 2 mM l-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin (R10H) and 1mg/ml collagenase P (Roche 11213865001) overnight at 37°C. The mixture was then pipetted up and down several times to homogenise the tissue further. To reduce the free DNA fragments, endonuclease deoxyribonuclease I (DNase I) was added at 200 Kunitz unit/ml (Roche 10104159001) for 15 minutes at room temperature. The tissue was passed through 100µm followed by 70 µm nylon mesh strainers (VWR 734-0004 and 734-0003) and washed with ice-cold 10mM EDTA solution (10x the volume of collagenase solution). After spinning for 20 minutes at 300g at 4°C, the supernatant was discarded and the cell pellet was re-suspended in cold RPMI medium containing 10% FCS and passed through a 40µm tissue strainer (VWR 734-0002). Larger samples were further purified using Ficoll density gradient purification.

The cells were then counted using 0.4% trypan blue exclusion method. On average, EDTA treatment and collagenase D treatment resulted in the isolation of 2,130±889 and 6,417±927 cells/cm^2 respectively from the skin biopsies, while the new approach based on collagenase P treatment dramatically increased the number of isolated cells to 303,234±68321 cells/cm^2 (Figure 3.1.1).

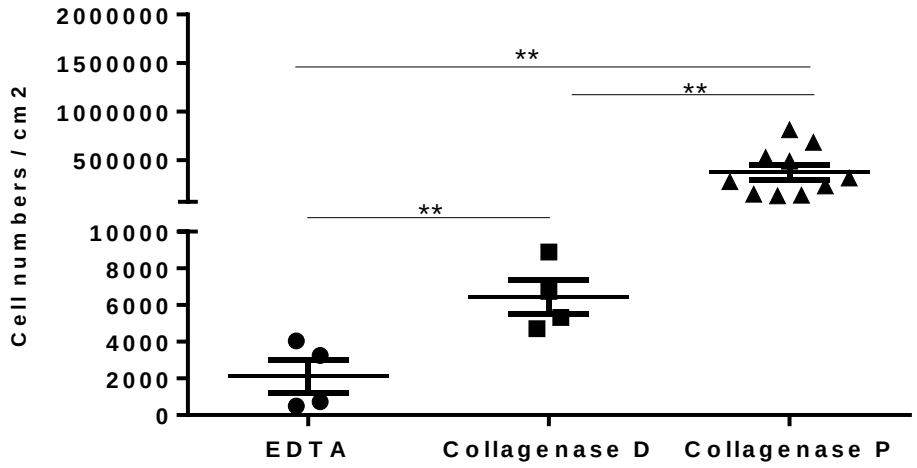


Figure 3.1.1 Collagenase P enzymatic treatment dramatically increases skin lymphoid cell isolation yield. (a) Skin samples were cut into small pieces and treated with EDTA, collagenase D or collagenase P. Frequencies of isolated cells were measured using 0.4% trypan blue exclusion. EDTA treatment and collagenase D treatment resulted in the isolation of 2,130±889 and 6,417±927 cells/cm^2 respectively, while collagenase P treatment increased the number of isolated cells to 303,234±68321 cells/cm^2.

3.1.2 Cell surface phenotype of skin-derived T cells

Depending on naïve T cells first encounter with antigen, activated effector memory cells are imprinted with preferential homing markers. Selective imprinting of T cells is essential for effective recruitment and robust immune responses in tissues and is influenced by the microenvironment and professional antigen presenting cells (APC) [208]. To evaluate the phenotype of T cells isolated using collagenase P treatment the expression of skin homing marker cutaneous lymphocyte antigen (CLA) [218] and chemokine receptors including CCR4 [219], CCR6 [220], and CCR10 [221] was compared. More than 90% of T cells isolated from the skin express CLA and CCR10. CCR4 and CCR6 were expressed on over 70% of T cells. Collagenase P did not alter the expression of homing markers on T cells isolated from the skin, whereas the levels were lower on isolated cells using collagenase D digestion (Figure 3.1.2, 3.1.3). To ensure that our method did not alter the cell surface expression of CD3 and CD8 markers when compared to EDTA or collagenase D, we investigated expression on the skin resident T cells [222, 223]. CD3 and CD8 were expressed at similar levels and proportions in the cells isolated by different methods (Figure 3.1.4). γδ T cells are another important subset of T cells that are believed to contribute to psoriasis skin inflammation, in part by producing IL-17 [216]. They are known to be expressed in small numbers in human skin and so we wished to examine whether the new method could detect such a minor population. The frequency of γδ T cells (3.63±0.77% of live cells) detected following collagenase P treatment of healthy skin donors was also similar to earlier reports which used other methods of skin T cell isolation (Figure 3.1.5) [224, 225].

Figure 3.1.2

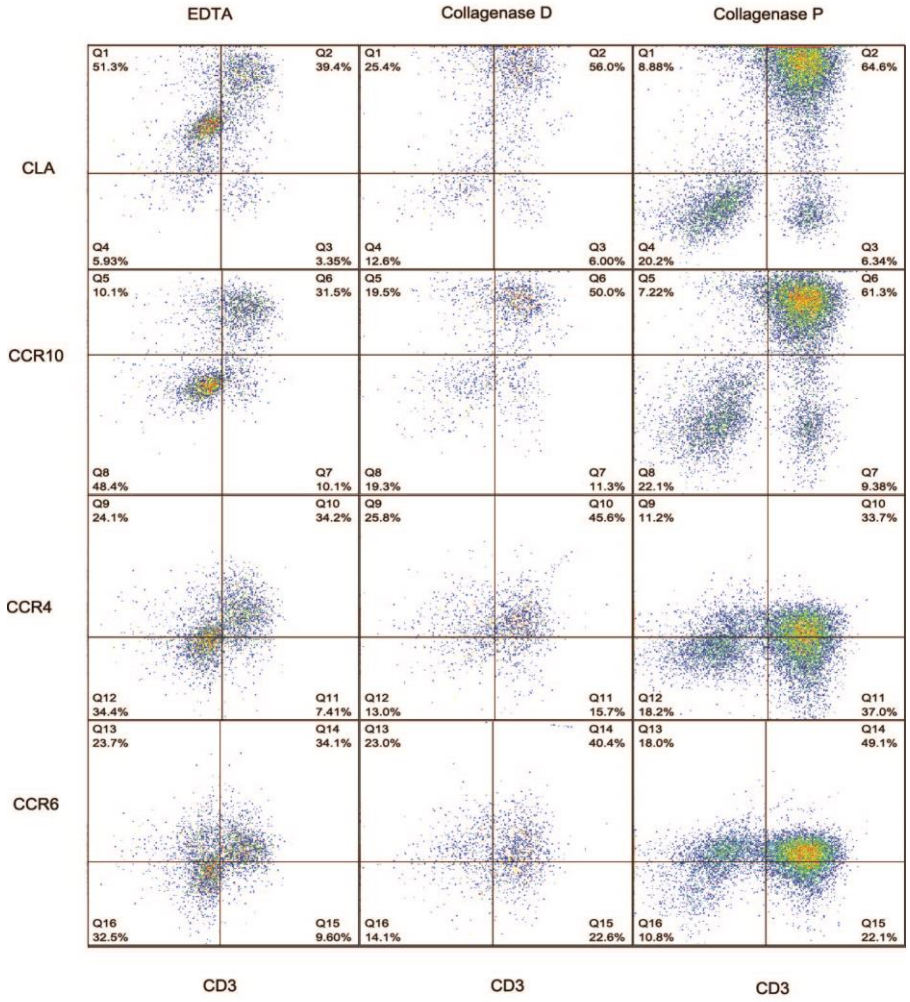


Figure 3.1.3

	EDTA	COLLAGENASE D	COLLAGENASE P
	n=4	n=4	n=10
CLA	93.4±3.2	90.7±4.7	90±5.8
CCR10	91.8±4.5	91.2±3.5	94.9±3.2
CCR4	82.4±3.2	83.2±7.4	84.5±8.7
CCR6	85.7±4.8	81.6±4.2	85.1±3.6

Figure 3.1.2 Expression of skin homing markers, CLA, CCR10, CCR4 and CCR6 were compared on lymphoid cells using the different methods of cell isolation

Figure 3.1.3 Mean frequencies of skin homing markers using different methods on $CD45^+$ $CD3^+$ cells are summarized

Figure 3.1.4

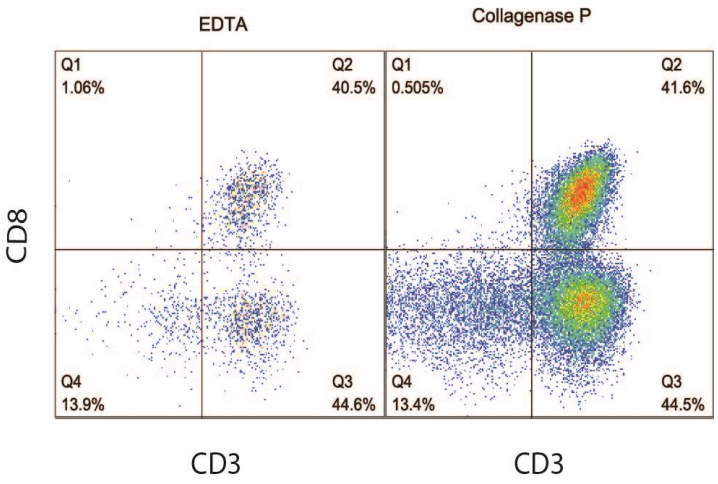


Figure 3.1.4 Live $CD45^+$ Cells isolated by EDTA and collagenase P treatment were stained for CD3 and CD8 expression. Similar frequency of $CD8^+$ and $CD8^-$ cells were observed using the different methods.

Figure 3.1.5

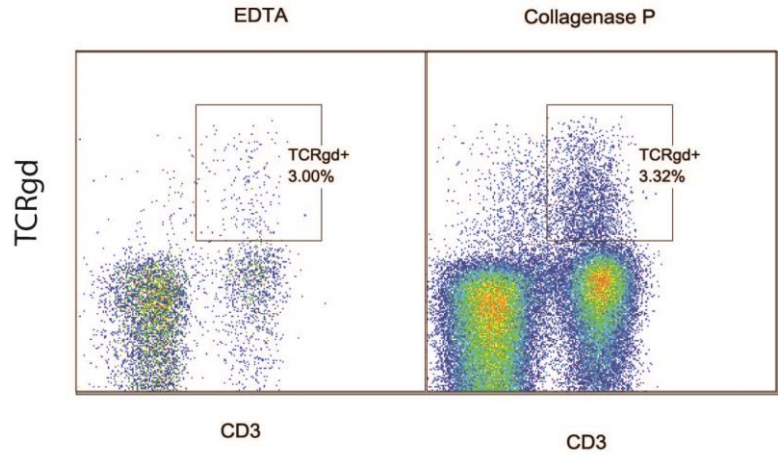


Figure 3.1.5 Frequencies of $CD3^+$ γδ T cells isolated by collagenase P treatment were similar to the EDTA method

3.1.3 Skin resident T cells have memory phenotype and show rapid effector function

Further examination of cell surface phenotype showed that most skin resident T cells have a memory phenotype (Figure 3.1.6). Next different sub population of T cells in the skin were investigated for their functional integrity after collagenase P digestion [226]. Cells isolated using the new method were stimulated with PMA/ionomycin for 16 hours and cytokine-producing cells were quantified using cytokine based ELISA. IFNɣ was produced by activated T cells in response to PMA/ionomycin stimulation for 16 hours (Figure 3.1.7) and isolated cells were also able to produce IL-17, IL-22 and IL-13 (Figure 3.1.7). These investigations demonstrate that the cells isolated *ex vivo* from human skin show rapid effector function and that cells producing different cytokines can be identified.

Figure 3.1.6

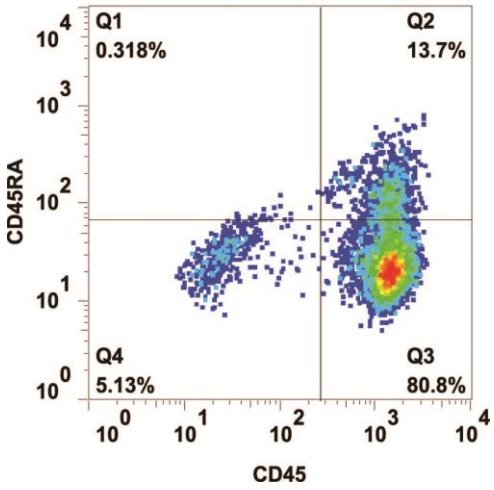


Figure 3.1.6 Skin resident T cells have memory phenotype. Less than 10% of T cells isolated by the new method of collagenase P treatment show naïve phenotype and express CD45RA.

Figure 3.1.7

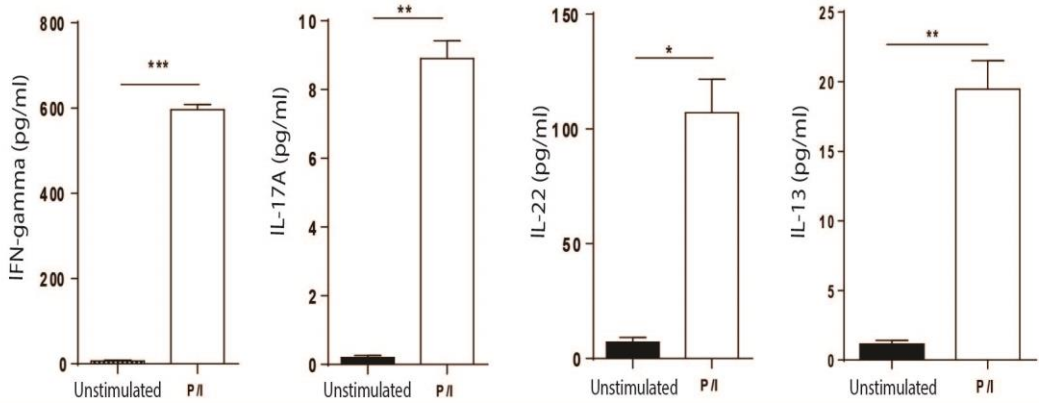


Figure 3.1.7 *Ex vivo* PMA/ionomycin activated T cells isolated by collagenase P treatment express IFN-γ, IL-17A, IL-22 and IL-13 as measured by ELISA. * P< 0.02 ** P< 0.002 ***<0.0002

3.2. An ILC2 population resides in human skin

3.2 Introduction

Atopic dermatitis (AD) is a common pruritic inflammatory skin disease that is associated with barrier dysfunction and Th2 cell adaptive immune responses to common environmental allergens. It is a disease with complex genetic and environmental susceptibility factors. Whilst it is likely that many genetic loci are involved, the association of filaggrin null mutations with atopic dermatitis has provided a major step forward in our understanding of disease pathogenesis [37]. Filaggrin is expressed in keratinocytes and is thought to have a role in skin barrier function, cutaneous pH and hydration [26, 227]. However, little is known how an inherited epidermal abnormality leads to a compromised skin barrier, skin inflammation and related atopic disorders, although high levels of IL-13 and IL-4 are known to be expressed in lesions of atopic dermatitis [9, 197].

Whilst Th2 cells have been characterised as producers of the cardinal cytokines IL-4, IL-5 and IL-13 in atopic dermatitis [9], the recent discovery of innate lymphoid cells (ILC) raises the question of their potential involvement as innate sources of type-2 cytokines in this disease. As described above, several recent studies have identified a family of CD45-expressing hematopoietic effector ILC that link the innate and adaptive arms of the immune system [159, 173, 228]. Such ILC are found in the blood, spleen, intestine, liver, lung, fat-associated lymphoid clusters (FALC), and lymph nodes of mice [158, 168-170, 173]. An ILC subset that produces type-2

cytokines (IL-5, IL-9, IL-13), and which is independent of RORγt has been designated as the type-2 ILC or ILC2 [155, 228]. ILC2 are negative for lineage markers of T cells and B cells, but in mice they express c-Kit (CD117), ST2, CD90, and the hematopoietic and lymphoid markers CD45 and IL-7Rα (CD127). Consistent with their expression of IL-17RB (IL-17BR, IL-25R) and ST2 (IL-33R) receptors, these cells respond to IL-25 and IL-33 by producing type-2 cytokines, and in mice ILC2 have been shown to induce goblet cell hyperplasia and eosinophilia, and contribute to protection against helminth infections [158, 159, 168, 169].

In mice, lung-resident ILC2 have also been demonstrated to contribute to airway hyperreactivity, induced by viral or allergen challenge [169, 172, 173, 175, 191]. However, ILC2 also serve to restore epithelial integrity and lung function after infection with the H1N1 influenza virus, predominantly by producing amphiregulin, a regulator of wound healing [172]. The human counterparts of ILC2 were recently reported in human lung parenchyma and bronchoalveolar lavage (BAL) fluid, and defined as lineage negative cells that express IL-7Rα and the ST2 subunit of the IL-33 receptor [172]. More comprehensively, Spits *et al.* reported $CD45^{hi}$, $CD127^{+}$ and $CD117^{+}$ cells in peripheral blood, foetal gut and the inflamed nasal polyps of patients with rhino-sinusitis. The cells also expressed CRTH2 and CD161 and, in response to epithelial cytokines, produced large amounts of IL-13 and IL-5, but not IL-17A or IL-22 [173].

Recently, ILC2-like cells have been reported within mouse and human atopic lesional skin and, at least in mice, recruitment of ILC2-like cells to sites of inflammation was

demonstrated to be dependent on TSLP and independent of IL-33 [197]. However, transgenic mice with IL-33 expressed under the keratin 14 promoter developed a spontaneous atopic dermatitis-like inflammation of the skin which associated with ILC2 infiltration [199]. A further murine study identified ILC2 in the skin which were present at a 30% frequency of T cells and were IL-7 and IL-2 dependent. Using intravital microscopy, it was shown that these ILC2 interacted with mast cells and produced IL-13 [174]. It is therefore unclear to what extent human and murine skin-derived ILC2 are dependent on IL-33. This is an important question for understanding disease and for directing future therapeutic activity.

3.2 Aim

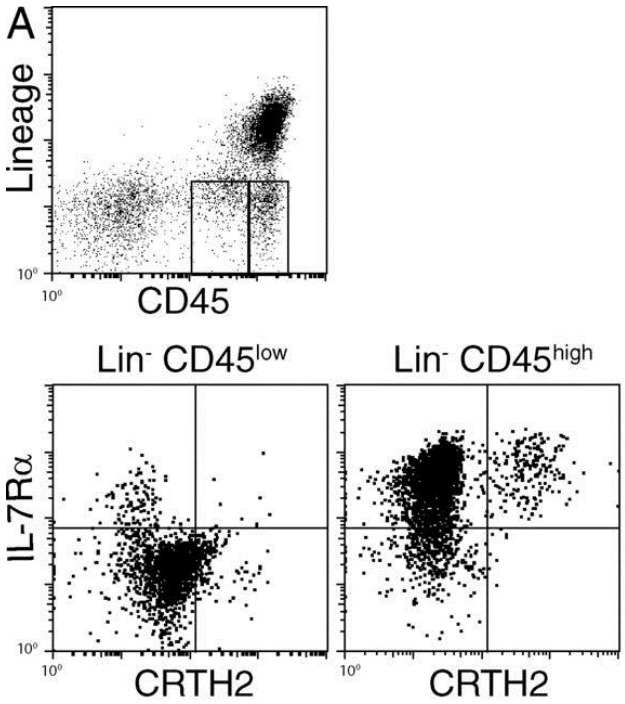
Our aims were to identify type 2 ILCs in human skin, study their phenotypical and functional properties and further Investigate signals that regulate ILC2 function and gene expression.

3.2 Results

3.2.1 ILC2 identification and phenotype analysis

To investigate the presence of ILCs in human skin, lymphoid cells were isolated from skin biopsies of healthy adult donors (n=15). After excluding cells that expressed common lineage markers (CD3, CD4, CD8, CD14, CD19, CD56, CD11c, CD11b, FcεRI, TCRγδ, TCRαβ and CD123), we observed that many $CD45^+$ cells (52.78 ± 33.26%) express the lymphoid marker IL-7Rα, and therefore are likely to belong to the ILC

family (Figure 3). To specifically identify the ILC2 sub-population, ILCs were stained for the nan ILC2 marker CRTH2. Almost all CRTH2-expressing cells exhibited high levels of surface CD45, which is similar to the population of ILC2 reported in fetal and adult human intestines [173] (Figure 3.2.1). Skin-resident ILC2 expressed high levels of c-kit, ICOS, CD161 and CD25 whereas they were negative for IL-23R, CD1a, and γδ (Figure 3.2.2).



3.2.1 ILC2 are resident in human skin. Freshly isolated leukocytes from the human skin were stained with the combination of ILC surface markers including lineage markers (CD3, CD4, CD8, CD14, CD19, CD56, CD11c, CD11b, FceRI, TCRgδ, TCRαβ and CD123), CD45 and IL-7Rα. Flow cytometric analysis of IL-7Rα and CRTH2 expression on lineage negative $CD45^{hi}$ and lineage negative $CD45^{low}$ cells (n=15).

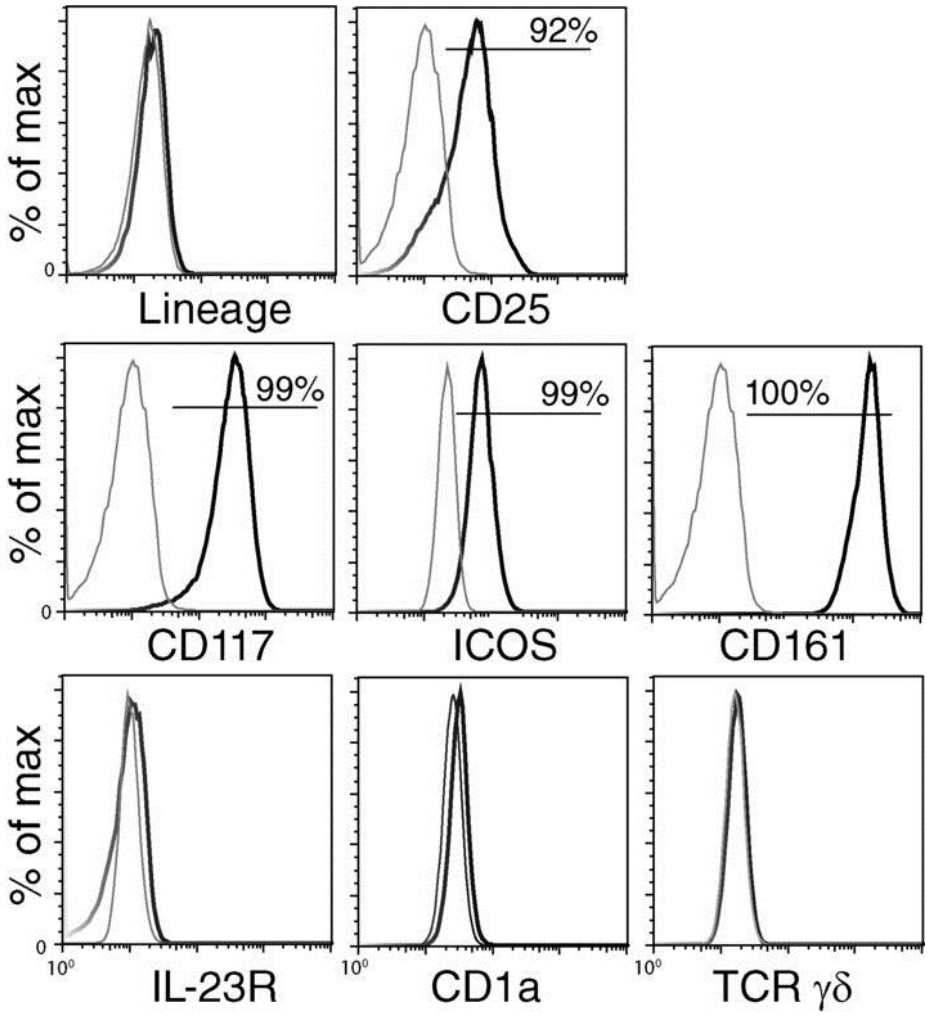


Figure 3.2.2 Expression of the indicated cell surface markers on $Lin^{-}CD45^{hi}IL\text{-}7R\alpha^{+}CRTH2^{+}$ cultured ILC2 from the skin (n=3). Black plots show relevant marker; grey show isotype control. Bars show percentage of cells expressing relevant marker.

To further characterise ILC2s in humans, the expression of MHC class I specific receptors that are widely expressed by cytotoxic effectors of NK cells were analysed. These receptors belong to two main classes of structurally distinct proteins: killer cell lectin-like receptors (KLRs) and killer cell immunoglobulin-like receptors (KIRs) [229].

ILC2s do not express any of the KIR receptor family (KIR2DL1/S1, KIR3DL1/S1, and KIR2DL2/S2/L3/S4) and KLR family receptors, NKG2A and NKG2C. They also appeared to be negative for the NK associated surface markers, NKp46 and CD56 (Figure 3.2.3). It remains possible that ILC2 can express these markers under different conditions, but currently we believe that expression levels are low or absent on skin-derived ILC2.

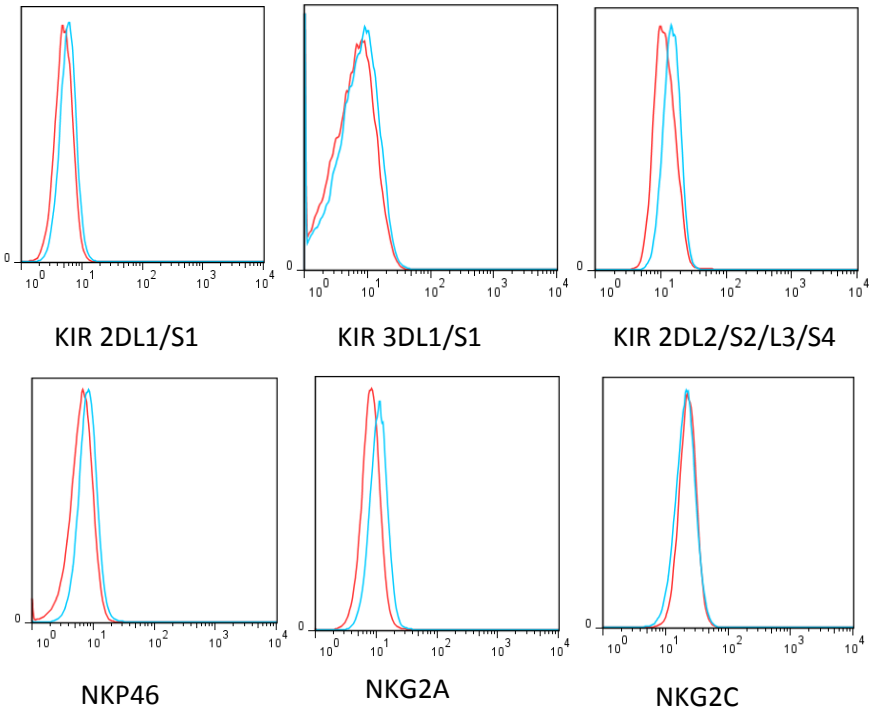


Figure 3.2.3 ILC2s do not express any of the KIR receptor family (KIR2DL1/S1, KIR3DL1/S1, and KIR2DL2/S2/L3/S4) and KLR family receptors, NKG2A and NKG2C. They also appeared to be negative for the NK associated surface markers, NKp46

3.2.2 Analysis of transcription factors in skin derived and blood derived ILC2

ILC2 differentiation and expansion is largely dependent on the transcription factors RORα [183] and GATA3 [230]. Using quantitative PCR, the transcripts of these genes were analysed in purified ILC2 isolated from human skin. Skin resident ILC2 expressed low levels of the *RORC* transcripts, while expressing relatively high amounts of *RORA* and *GATA3* transcripts. Interestingly, when compared to similarly purified ILC2 from the blood, *RORA* and *GATA3* expression in skin resident ILC2 were significantly higher (P value = 0.04 and 0.009, respectively) (Figure 3.2.4).

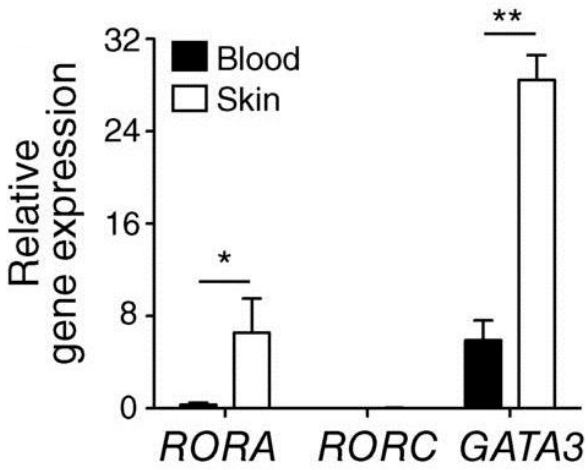


Figure 3.2.4 CD45hi IL-7Rα+CRTH2+ were sorted and cultured from blood and skin, and RORA (n = 8, where n is number of donors), RORC (n = 5), GATA3 (n = 3), and (D) AREG (n = 3) gene expression relative to GAPDH was measured by RT-PCR.

3.2.3 Skin derived ILC2 express skin homing markers

It has been proposed that ILC2 derive from common lymphoid progenitors in the bone marrow and mature ILC2 circulate in the blood and home to the tissues [173, 183]. As the skin is a major site of antigen encounter and long-term immune surveillance, recruited leukocytes in the skin are imprinted with tissue-specific homing receptors such as cutaneous lymphocyte antigen (CLA) and chemokine receptors including CCR4, CCR6, CCR10 which provide effective and quick trafficking of memory cells to sites of inflammation [231]. We found that a significant proportion of ILC2 derived from healthy donors express the skin-homing receptors CLA, CCR10 and CCR4 (Figure 3.2.5). Taken together these data indicate that skin-infiltrating/resident ILC2 may have a phenotype that is distinct from circulating ILC2.

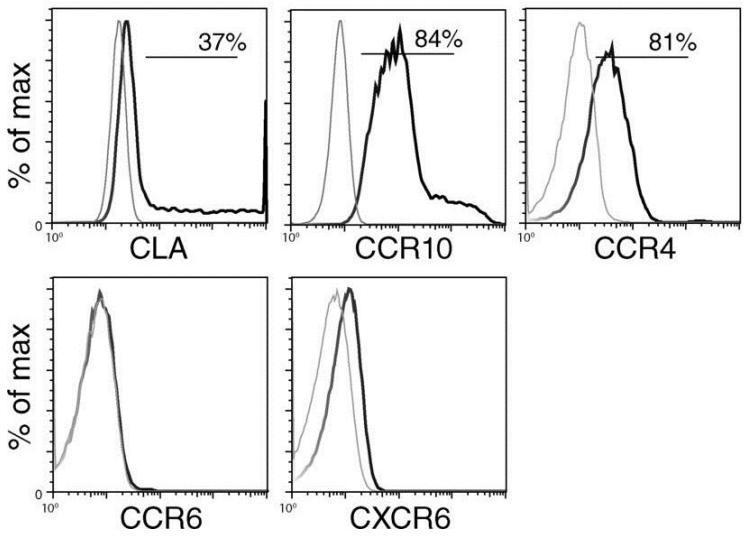


Figure 3.2.5 Lin^- $CD45^{hi}$IL-7R$\alpha^+$$CRTH2^+$ ILC2 were isolated from human skin and expression of the indicated homing receptors was analysed by flow cytometry. Black plots show relevant marker; grey show isotype control. Bars show percentage of cells expressing relevant marker

3.2.4 IL-33 is a potent stimulus for skin-derived ILC2

We next assessed the responses of ILC2, isolated from healthy skin, to treatment with the cytokines IL-25, IL-33 and TSLP since a defining feature of ILC2 is their ability to produce IL-13 and IL-5, but not IL-17A/IL-22 in response to stimulation with IL-25 and IL-33. Using multiplex analysis, the cytokine production following stimulation with PMA/Ionomycin or cytokine combinations at different concentrations was compared (Figure 3.2.6). IL-33 stimulation elicited significant levels of IL-13, IL-6 and IL-5 production (Figure 3.2.6), but induced little IL-4 (Figure 3.2.6). TSLP and IL-25 did not induce significant IL-4, IL-13, IL-6 or IL-5 production when present in isolation, but interestingly were able to enhance cytokine production when present in combination. Increased type-2 cytokine gene expression indicated that this was not simply due to the release of pre-formed cytokines (Figure 3.2.7). The cytokine production of IL-25- and IL-33-activated cells was also evaluated using intracellular cytokine staining. Whilst skin-derived ILC2 produced high levels of IL-13 after stimulation with PMA/Ionomycin or IL-33, they did not express detectable IL-17A and IL-22, (Figure 3.2.8). IL-25 failed to induce IL-13 in these assays. The responsiveness to IL-33 also coincided with an increase of IL-33 receptor expression (Figure 3.2.9).

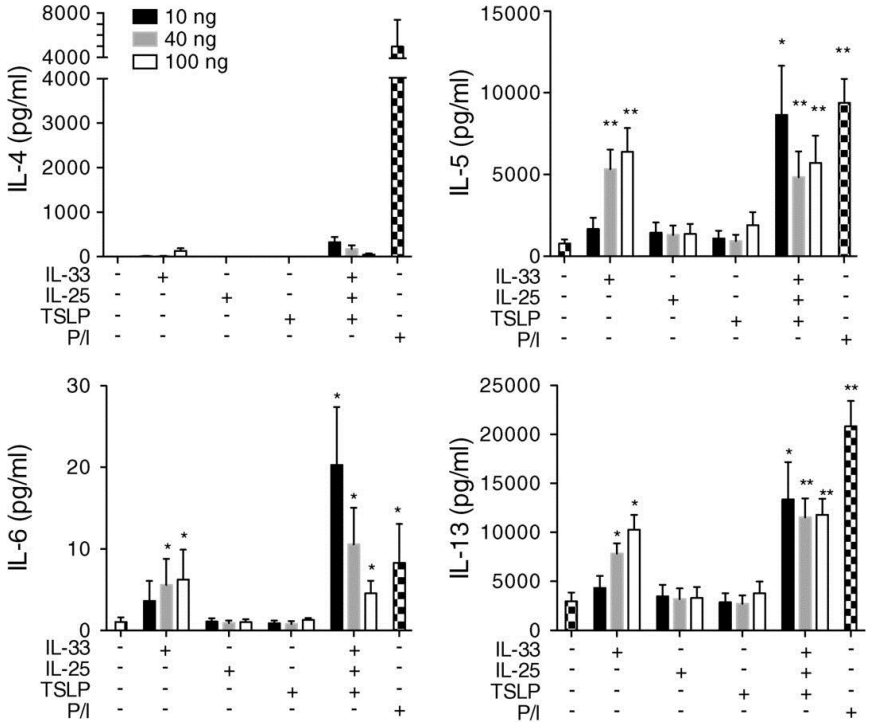


Figure 3.2.6 Cultured skin ILC2 were stimulated with PMA/Ionomycin or IL-25, IL-33 and TSLP alone or in the indicated combinations and at the indicated concentrations. Supernatant was collected after 24 hrs. Levels of IL-4, IL-5, IL-6, and IL-13 were measured by multiplex cytokine analysis. Statistical comparisons are compared to the negative (no cytokine) control.

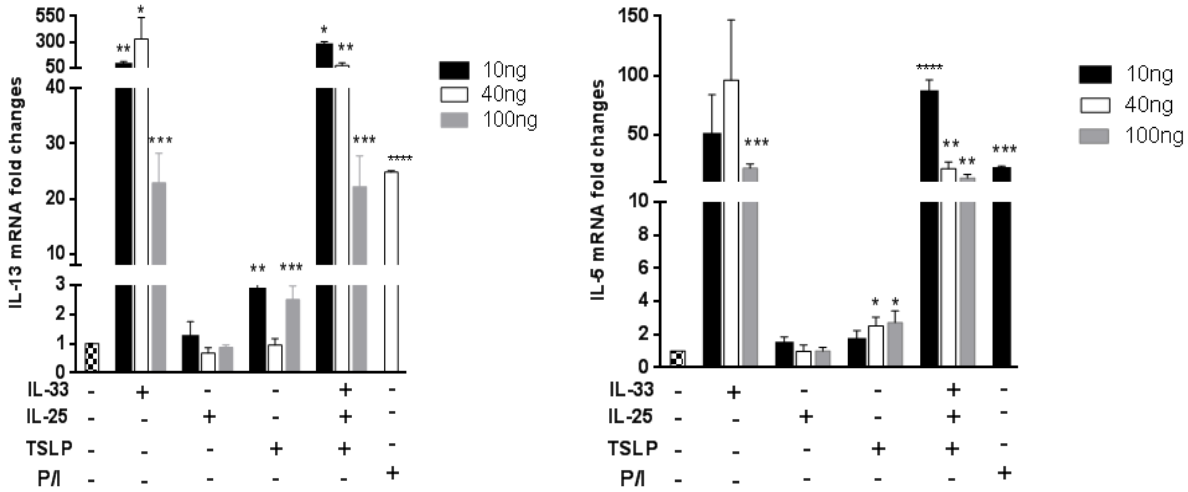


Figure 3.2.7 RT-PCR analysis of the expression of transcripts of IL-13 and IL-5 in activated ILC2. Data representative of three independent experiments.

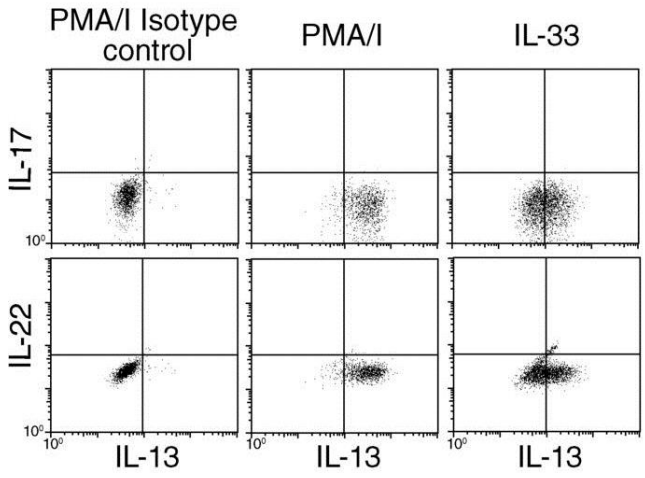


Figure 3.2.8 Skin-derived ILC2 were stimulated with PMA/Ionomycin or IL-33 and levels of IL-13, IL-17A and IL-22 expression were measured using intracellular cytokine staining. Data representative of four independent experiments on cultured cells

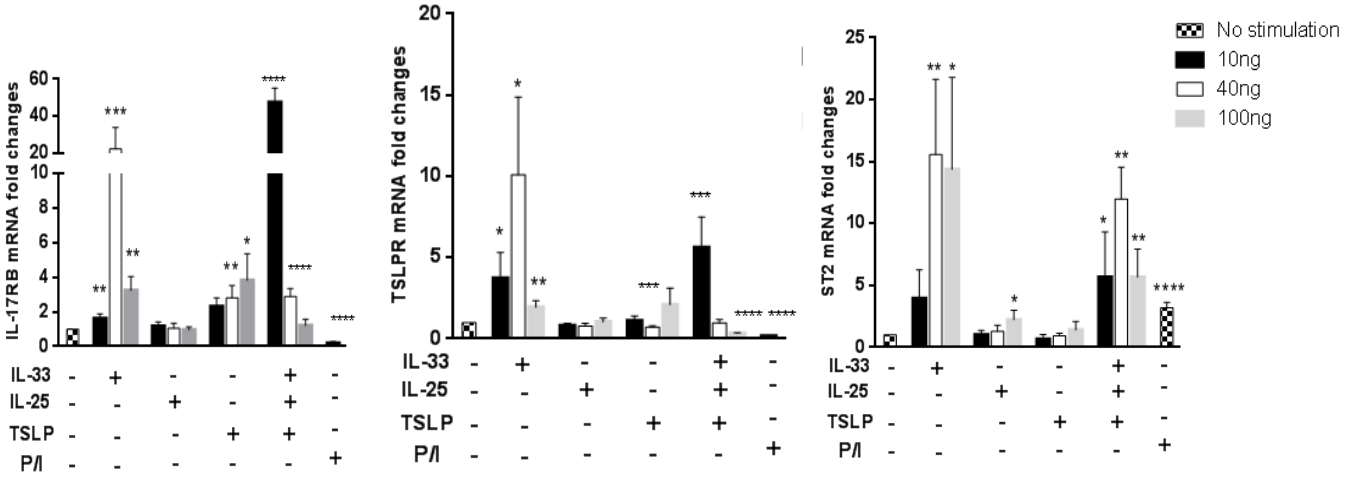


Figure 3.2.9 ILC2 were stimulated for 16 hours with IL-25, IL-33 or TSLP in isolation or combination at the indicated combinations. Expression of *IL1RL1* (IL-33R), IL-17RB (IL-25R) and TSLPR were measured by RT-PCR. Data representative of three independent experiments. Statistical comparisons are compared to the negative (no cytokine) control.

3.2.5 IL-33 attracts ILC2 to the site of inflammation

Disruption of epithelial barrier integrity in the lung and skin induces the production of IL-25, and IL-33 [232]. Consistent with activation in response to IL-25 and IL-33, human ILC2 express IL-17RB (IL-25R) and ST2 (IL-33R) receptors which are up-regulated after stimulation (Figure 3.2.9). Using trans-migration assays we also determined that IL-33, in contrast to IL-25, elicited significant migration of skin-derived *in vitro*-cultured ILC2 (Figure 3.2.10). While migration towards TSLP was also observed, this occurred only at high concentrations (Figure 3.2.10). Thus, IL-33 is a potent stimulus for the activation of human skin-derived ILC2, inducing the up-regulation of its own receptor, enhancing the expression of type-2 cytokine expression and increasing the migratory capacity of these cells.

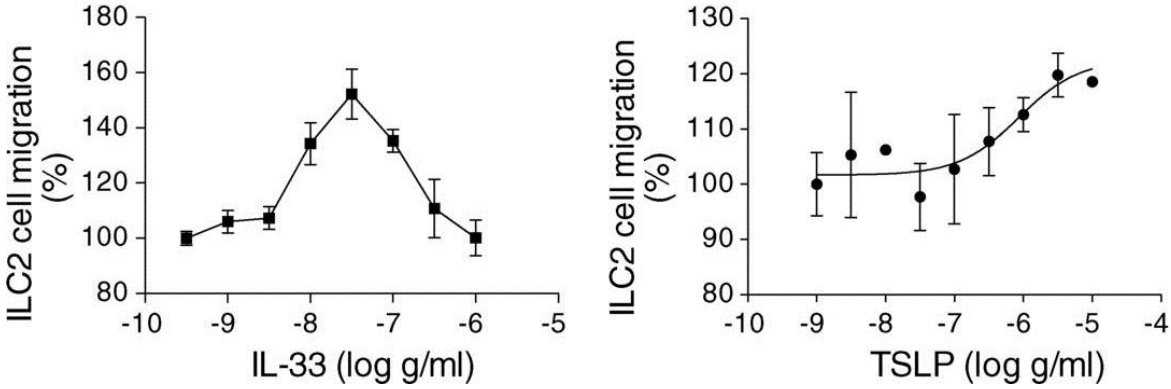


Figure 3.2.10 Chemotaxis of skin-derived ILC2s towards indicated concentrations of IL-33 and TSLP, relative to base line, was measured after 1 hour using transmigration across a 5μM pore membrane. Data are representative of three independent experiments (n = 3).

3.2.6 Amphiregulin

Amphiregulin (AR) is a member of epidermal growth factor family (EGF) that can alter proliferation, apoptosis and migration of epithelial cells, fibroblasts, DC, neutrophils, mast cells and lymphocytes [233]. Amphiregulin mediates its effect through binding to epidermal growth factor receptor (EGFR also known as ErbB1) and triggers activation of ErbB2, ErbB3 and ErbB4 [234]. EGFR is a tyrosine kinase and ubiquitously expressed on many cell types; amphiregulin is the most highly expressed ligand of it on keratinocytes [235]. AR is constitutively expressed in normal tissues and is involved in many physiological processes. Although expressed at low levels in liver, $AREG^{-/-}$ mice developed signs of hepatocellular injury [236]. It is required for development of the female reproductive system and mammary gland and ductal morphogenesis. Loss of TGF-α in gastric mucosa had little effect but $AREG^{-/-}$ mice developed gastrointestinal ulcers [237].

Amphiregulin is also considered a type 2 cytokine and its production is thought to be a defence mechanism during inflammatory processes. Overproduction of AR promotes airway epithelium and smooth muscle over growth and mucous production. AR is required for profibrogenic activity of TGF-β in animal models of pulmonary fibrosis and in patients with idiopathic pulmonary fibrosis [238]. Increased expression of AR has been reported in chronic airway inflammation, liver fibrosis and autoimmune disorders [239].

3.2.6 ILC2 can produce amphiregulin

Activation of murine ILC2 by epithelial cytokines can induce type 2 cytokine production and initiate allergic responses [175, 191] while they can concurrently induce amphiregulin, a wound-healing regulator that aids tissue repair [172]. Therefore, the transcript levels of the amphiregulin gene (*AREG*) in skin resident ILC2 were compared to those purified from the blood. Interestingly, the expression of *AREG* was also significantly higher in the skin resident ILC2 (P value =0.02) and was further up-regulated in response to stimulation (Figure 3.2.11). These data on differential expression of *AREG* in skin and blood ILC2 infer the activation state and/or function of ILC2 is distinct at the different anatomical sites.

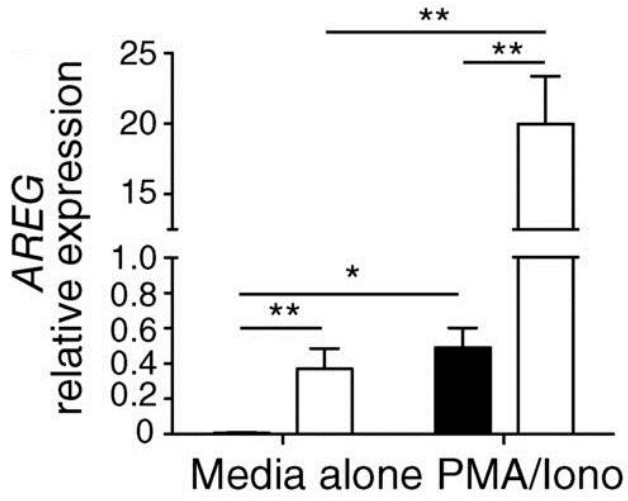


Figure 3.2.11 CD45hi IL-7Rα+CRTH2+ were sorted and cultured from blood and skin, and AREG (n = 3) gene expression relative to GAPDH was measured by RT-PCR.

3.2.7 Enrichment of ILC2 cells in the skin of patients with atopic eczema

It is noteworthy that compared to the paucity of circulating ILC2 in the blood, ILC2 are highly enriched in human skin tissue (0 - 0.18% vs 0.04 - 2.94% of lymphoid cells in blood and skin respectively) (Figure 3.2.12). To examine whether ILC2 cells might contribute to the pathogenesis of AD, we examined peripheral blood and acute lesional skin of adults with AD for the presence of ILC2. There were significantly ($P<0.005$) more ILC2 detected in lesional skin biopsies from atopic patients relative to healthy individuals (Figure 3.2.12). In contrast, there was a similar frequency of circulating ILC2 in the peripheral blood of both groups (Figure 3.2.12). Notably, IL-17RB subunit of IL-25, ST-2 subunit of IL-33 and TSLP receptor were further up-regulated in freshly isolated ILC2 from the skin of patients with AD as compared to healthy controls (Figure 3.2.13). Gene expression analysis on skin samples isolated from either non-involved skin or AD-involved skin clearly demonstrated the up-regulation of gene expression for the IL-17RB (IL-25R), ST2 (IL-33R) and TSLP receptors in the AD skin samples, and this was accompanied with elevated expression of *CRTH2, RORA* and *AREG* mRNA (Figure 3.2.14). Analysis for cytokine gene expression also detected increased levels of *IL33*, *IL25* in the AD samples (Figure 3.2.14). Thus, skin ILC2 were more prevalent in the lesional skin of AD patients and showed a co-incident up-regulation of the receptors for the key ILC2–inducing cytokines IL-25, IL-33 and TSLP, in an environment in which *IL25* and *IL33* gene expression was also elevated.

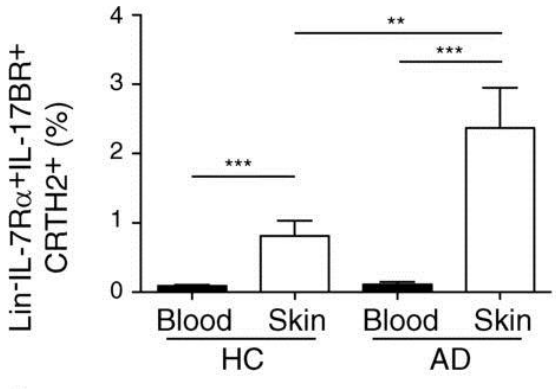


Figure 3.2.12 Frequency of ILC2 (as a percentage of IL-7Rα+ cells) in the blood and skin of healthy controls (HC, n = 15) or in lesional biopsies of the acutely affected skin of patients with atopic dermatitis (AD, n = 8) were measured by flow cytometry

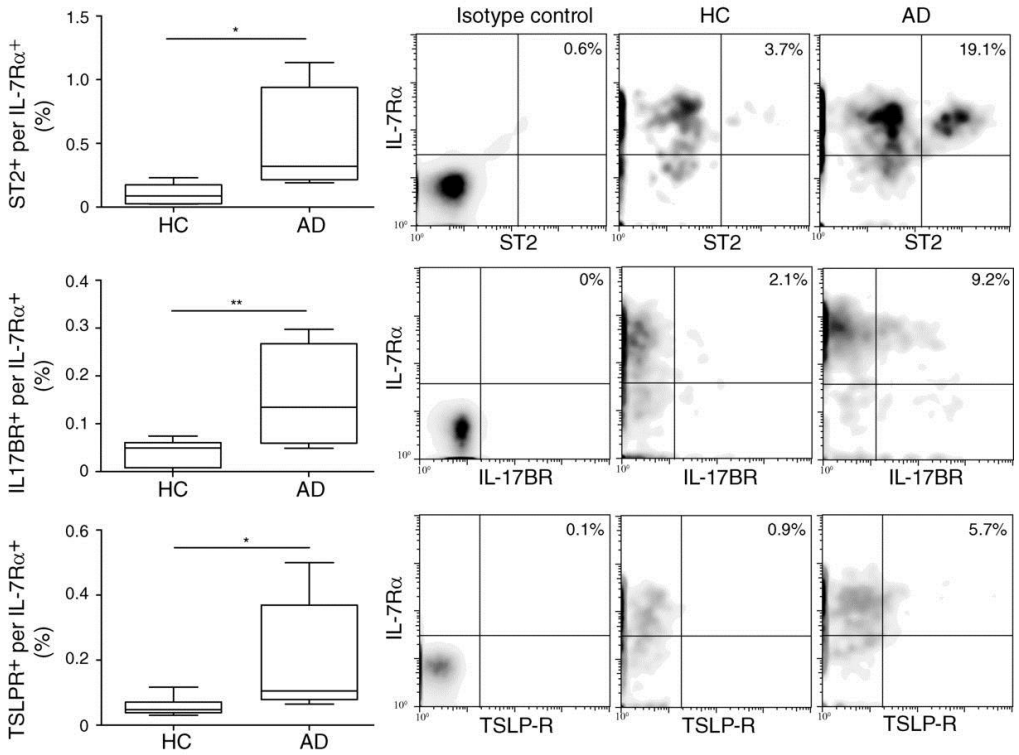


Figure 3.2.13 ILC2 (Lin-CD45highIL-7Rα+) were isolated from the skin of patients with atopic dermatitis and healthy controls and expression of ST2, IL-17BR and TSLPR were analysed by flow cytometry. Representative flow plots are shown on the right. Numbers indicate percentages of cells within top right quadrant of gated populations. B: patients and controls (n=7); C: patients (n=5), controls (n=8); D: patients (n=6), controls (n=7).

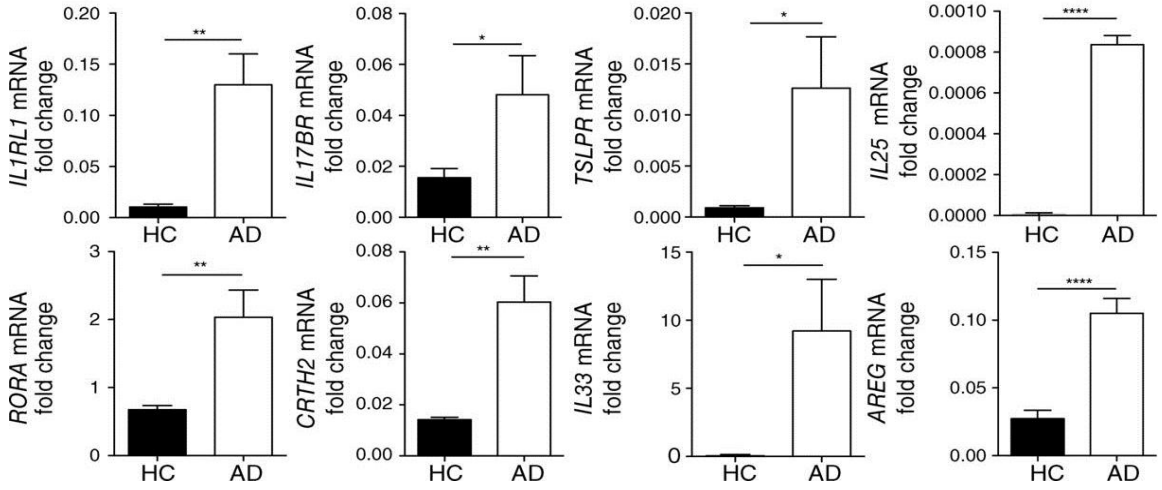


Figure 3.2.14 Acute lesional skin biopsies were taken from patients with AD (*n* = 8) and healthy controls (*n* = 6), and expression of *IL1RL1* (IL-33R), *IL-33*, *IL17BR*, *IL-25*, *CRTH2*, *TSLPR*, *AREG*, and *RORA* was measured by RT-PCR relative to *GAPDH*. *, P < 0.05; **, P < 0.01; ***, P < 0.001, unpaired Student's *t* test

3.3 Cytokine expression and cell content of allergen challenged and unchallenged Atopic dermatitis skin compared to healthy controls

3.3 Introduction

3.3 House dust mite allergy

An IgE mediated reaction to allergens is a fundamental mechanism in the pathogenesis of atopic dermatitis. Among environmental allergens, house dust mite (HDM) is the most common aeroallergen especially in temperate climates. At least 56% of patients with atopic dermatitis, asthma and rhinitis have specific IgE to HDM [240]. House dust mites are arthropods belonging to the subphylum *Chelicerata*, class *Arachnida*, order *Acari*, and suborder *Astigmata*. Worldwide the most common pyroglyphid mites found in homes are *Dermatophagoides farinae* and *D. pteronyssinus* [241]. Mite faeces, enzymes that originate from gastrointestinal tract, mite bodies, saliva and molting enzymes are sources of allergens. House dust mite allergens are divided into 13 groups. Designation is based on the first 3 characters of genus (*Der*), the first letter of species name (*P* or *F*) and a number indicating the order of allergen isolation (Der p1, Der p2) [241]. Group 1 allergens (Der p1) have cysteine proteinase activity; it can cleave CD23/FcεRII on B cells and IL-2R (CD25) on T cells and

increase its allergenicity. Group 2 allergens (Der p2) are nonglycosylated proteins of the male HDM reproductive tract. The enzymatic properties of HDM allergens facilitate their penetration to the compromised epithelial barrier. Most HDM sensitive individuals have IgE specific antibodies to group 1 and/or group 2 allergens. These allergens can survive in the environment after the removal of mites.

3.3 Aim

House dust mite (HDM) extract is one of the most common aeroallergens that associate with exacerbation of AD symptoms [242]. The aim was to study the dynamics of ILC2 recruitment and cytokine profile of allergen-provoked skin using suction blisters to sample skin cells and interstitial fluid before and after HDM allergen delivery in the epidermis of humans and capture early signals of an inflammatory response.

3.3 Patients and Control Characteristics

In total 9 atopic dermatitis (AD), 4 atopic asymptomatic (AA) and 5 healthy control (HC) individuals were recruited. All atopic patients had active eczema lesions, allergen specific serum IgE and satisfied the UK modifications of the Rajka Hanifin criteria. The criteria are an itchy skin condition plus three or more of the following: onset below age 2, flexural involvement, generally dry skin and history of other atopic disorders (summarised in table 3.3.1). The extent and severity of eczema lesions were clinically

defined by detailed history and using SCORAD (**SCOR**ing **A**topic **D**ermatitis) system (Table 3.3.2). Atopic asymptomatic individuals had history of atopic condition and serum IgE responses without active eczema lesions. All individuals were skin prick tested for HDM, cat, dog, grass, tree, *Alternaria alternata* and *Aspergillus*. The specific serum IgE antibodies were also tested. Table 3.3.3 describes the features of groups; including age, SCORAD, and serum specific IgE levels to HDM, cat, dog, grass, tree, *Alternaria alternata* and *Aspergillus* allergens.

In order to qualify as a case of atopic eczema with the UK diagnostic criteria, patient:
Must have: An itchy skin condition in the last 12 months Plus three or more of: i. Onset below age 2* ii. History of flexural involvement iii. History of a generally dry skin iv. Personal history of other atopic disease** v. visible flexural dermatitis as per photographic protocol

Table 3.3.1 The UK refinement of the Hanifin and Rajka diagnostic criteria for atopic eczema http://www.nottingham.ac.uk/dermatology/eczema/Section1-3.html

SCORAD

Area

To determine extent, the sites affected by eczema are shaded on a drawing of a body. The rule of 9 is used to calculate the affected area (A) as a percentage of the whole body.

Head and neck 9%

Upper limbs 9% each

Lower limbs 18% each

Anterior trunk 18%

Back 18%

1% for genitals.

The score for each area is added up. The total area is 'A', which has a possible maximum of 100%.

Intensity

A representative area of eczema is selected. In this area, the intensity of each of the following signs is assessed as none (0), mild (1), moderate (2) or severe (3).

Redness

Swelling

Oozing / crusting

Scratch marks

Skin thickening (lichenification)

Dryness (this is assessed in an area where there is no inflammation)

The intensity scores are added together to give 'B' (maximum 18).

Table 3.3.2 http://www.dermnetnz.org/dermatitis/scorad.html

	Atopic dermatitis	Atopic asymptomatic	healthy control
Number	9	4	5
Age	58	39.25	42
SCORAD	45.8	0	0
Specific IgE			
HDM	64.463	15.075	0.058
cat	26.453	14.07	0.09
dog	20.374	9.92	0.04
grass	+	+	-
tree	+	+	-
alternaria	+	+	-
aspergillus	7.8	0.03	0.048

Table 3.3.3 Description of patient and control subjects.

3.3 Results

We extracted the interstitial fluid and cells infiltrating the blister 26 hours after intra-epidermal administration of HDM extract to the skin. Blister fluid samples were extensively profiled using Magpix array of 32 inflammatory cytokines, chemokines, and growth factors. Infiltrating blister cells were phenotypically characterized using flow cytometry. We observed similar levels of type 1 cytokine (IFN-γ) and TH17 cytokines (IL-17 and IL-23) and granulocyte attracting chemokines (MIP-1α, MIP-1β and IL-8) (Figure 3.3.4).

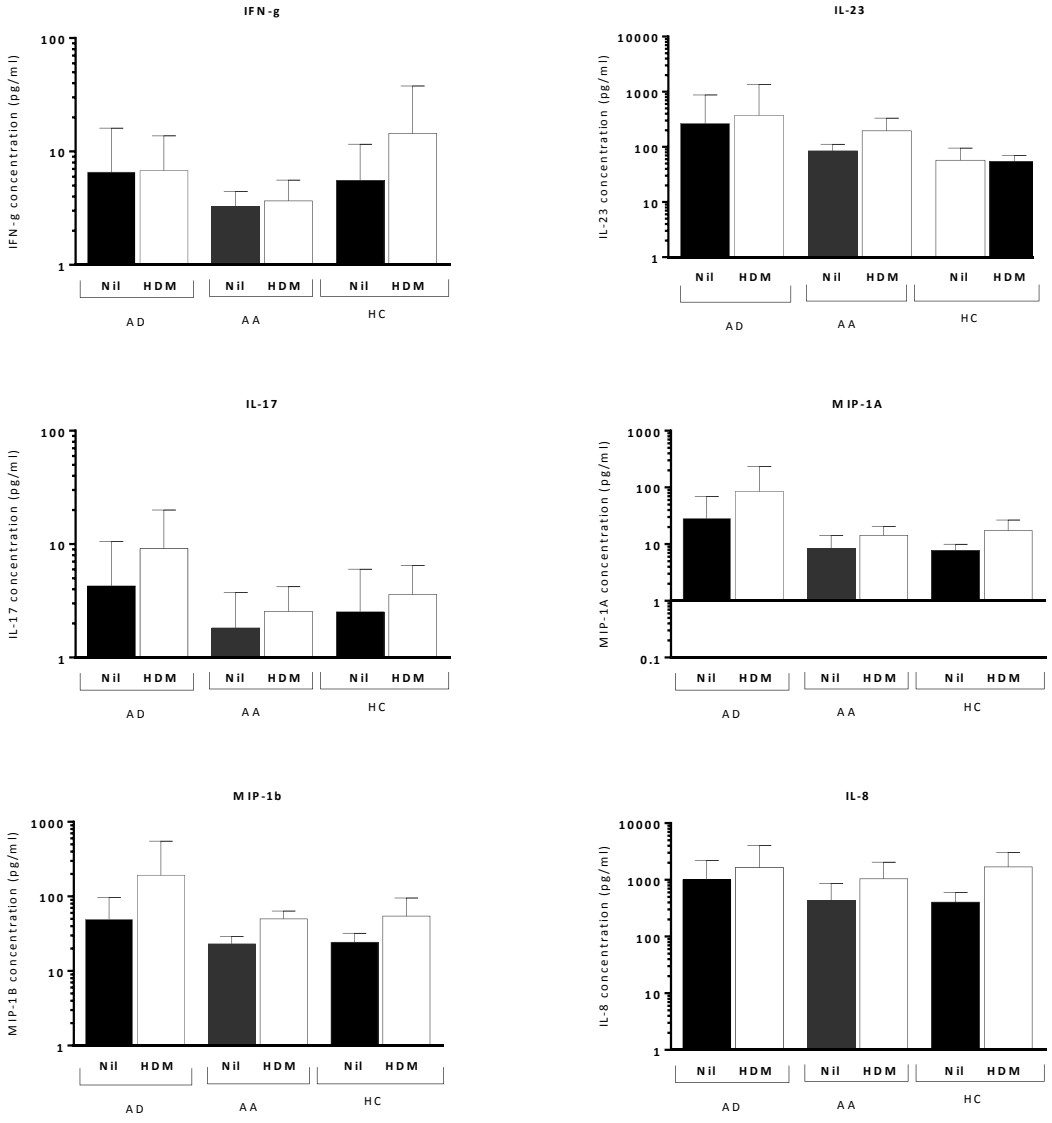


Figure 3.3.4 Levels of IFN-γ, IL-23, IL-17, MIP-1A, MIP-1B and IL-8 within the blister fluid isolated from atopic dermatitis (AD; n=9), atopic asymptomatic (AA; n=4) and healthy donors (HC; n=5) were quantified 24 hours after house dust mite administration into the skin.

3.3.1 Type 2 cytokines and epithelial cytokines

There is a great body of evidence supporting a crucial role for type 2 cytokines in pathogenesis of atopic dermatitis (discussed in detail in introduction). IL-13 and IL-4 has been shown to reduce expression of filaggrin and anti-microbial peptides (HBD-2 and HBD-3) hence contributes to barrier dysfunction and greater susceptibility of AD patients to infections [88, 145, 151, 243]. IL-5 attracts eosinophils to the site of inflammation and positively correlates with allergen specific IgE concentration [153, 154]. Measuring type 2 expression profile of interstitial blister fluid showed significantly higher expression of IL-5 (8.6±0.6 pg/ml), IL-9 (6.9±0.9) and IL-13 (7.7±1.5) in unchallenged skin of atopic patients compared to healthy controls; 2±0.2, 0.02±0.01, 2.5±0.7 pg/ml respectively (Figure 3.3.5). HDM challenge further up-regulated the expression levels of type 2 cytokines; IL-5 (131.5±24.3), IL-9 (81.5±13), IL-13 (310±69 pg/ml) (Figure 3.3.6). The expression pattern of IL-4 varied between atopic individuals after allergen challenge (0-452pg/ml). Atopic asymptomatic donors expressed intermediate expression of type 2 cytokines with slight increase in response to HDM challenge which was not statistically significant (Figures 3.3.5 and 3.3.6). Interestingly, concentration levels of IL-5, IL-9 and IL-13 positively correlated with total serum IgE levels (Figure 3.3.7). The concentration of type 2 cytokines (IL-4, IL-5, IL-9 and IL-13) in the interstitial blister fluid was associated with the severity of AD according to the SCORAD index (Figure 3.3.8).

As previously discussed, epithelial cytokines IL-25, IL-33 and TSLP are produced by epithelial cells and keratinocytes in response to mechanical stress, infection and allergen exposure. Epithelial cytokines induce production of type 2 cytokines IL13, IL-4, IL-5. Given the established role for IL-25 and IL-33 in type 2 inflammation, the involvement of these cytokines in AD is highly probable. Although we could not detect IL-25 in blister fluids, figure 3.3.9 shows significantly higher concentrations of IL-33 (37±4.9) and TSLP (13±3.2) in AD patients.

Following confirmation of high levels of epithelial and type 2 cytokines, the correlation between these cytokines was investigated. Both TSLP and IL-33 demonstrated significant positive correlation with IL-5, IL-13 and IL-9 (Figure 3.3.10).

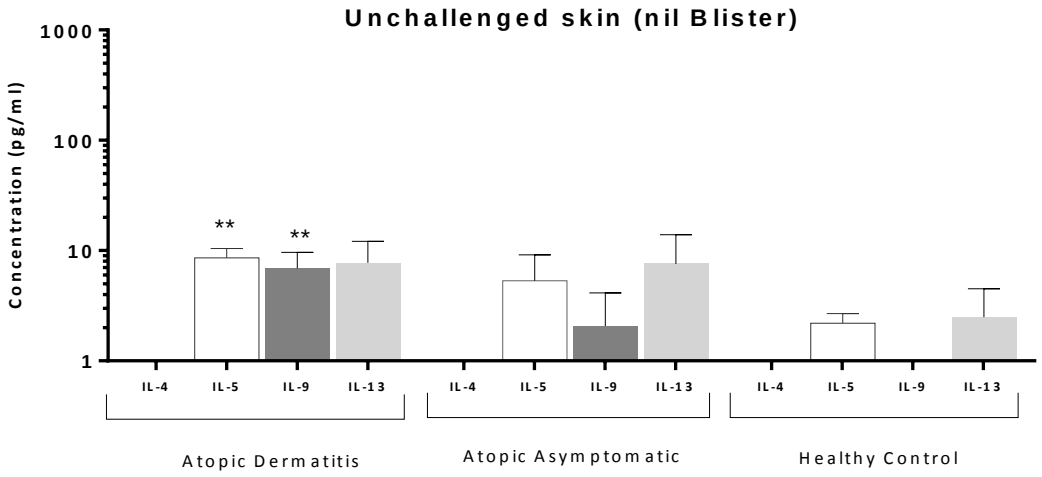


Figure 3.3.5 Levels of IL-4, IL-5, IL-9 and IL-13 within the blister fluid isolated from atopic dermatitis (AD; n=9), atopic asymptomatic (AA; n=4) and healthy donors (HC; n=5).

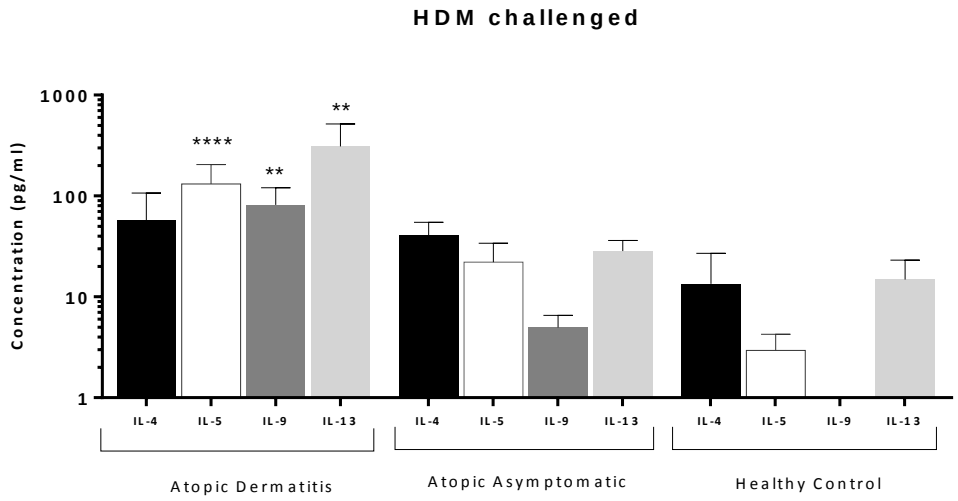


Figure 3.3.6 Levels of IL-4, IL-5, IL-9 and IL-13 within the blister fluid isolated from atopic dermatitis (AD; n=9), atopic asymptomatic (AA; n=4) and healthy donors (HC; n=5) 24 hours after intra-epidermal application of HDM.

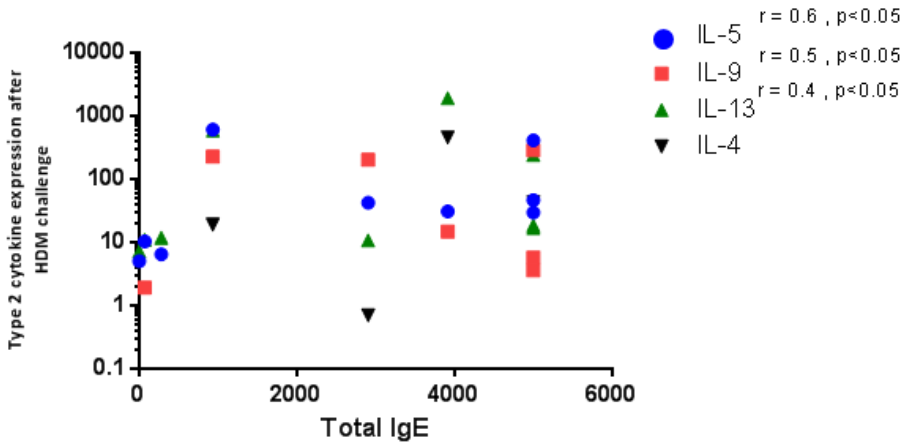


Figure 3.3.7 Levels of IL-4, IL-5, IL-9 and IL-13 within the blister fluid of HDM challenged AD patients were measured and the correlation of each cytokine with total IgE was analysed. IL-5, IL-9 and IL-13 showed significant positive correlation.

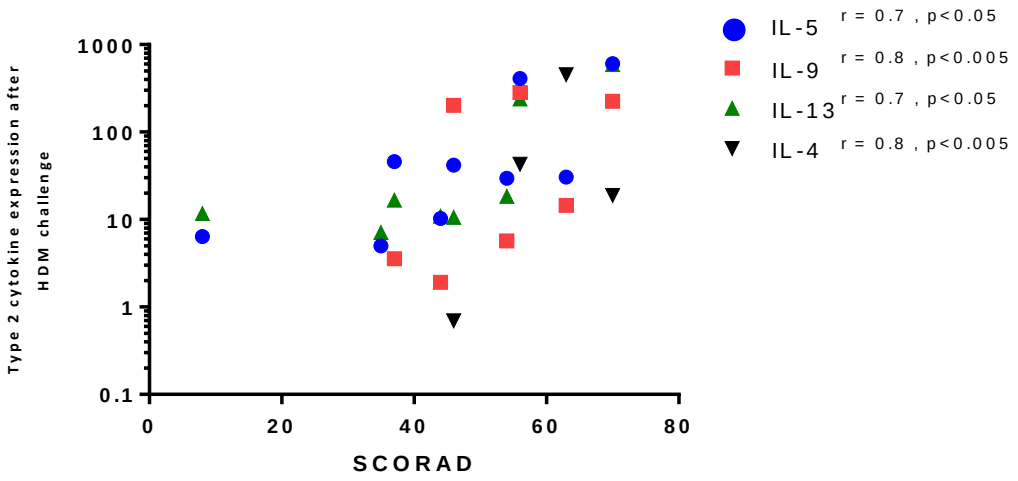


Figure 3.3.8 Levels of IL-4, IL-5, IL-9 and IL-13 within the blister fluid of HDM challenged AD patients were measured and the correlation of each cytokine with SCORAD index was analysed. IL-4, IL-5, IL-9 and IL-13 showed significant positive correlation.

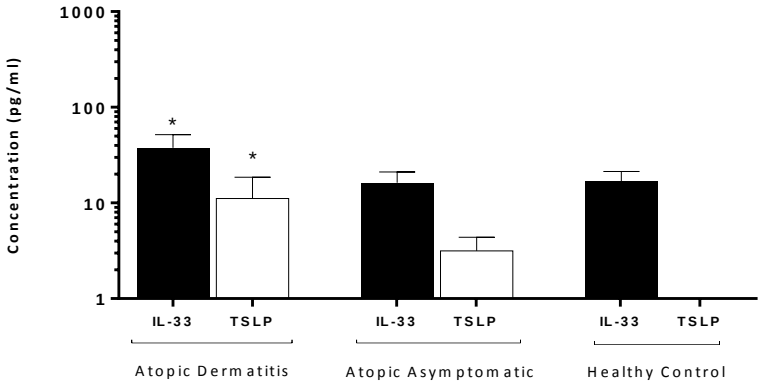


Figure 3.3.9 Higher concentration of epithelial cytokines IL-33 and TSLP was observed in the blister fluid of AD patients compared to healthy controls.

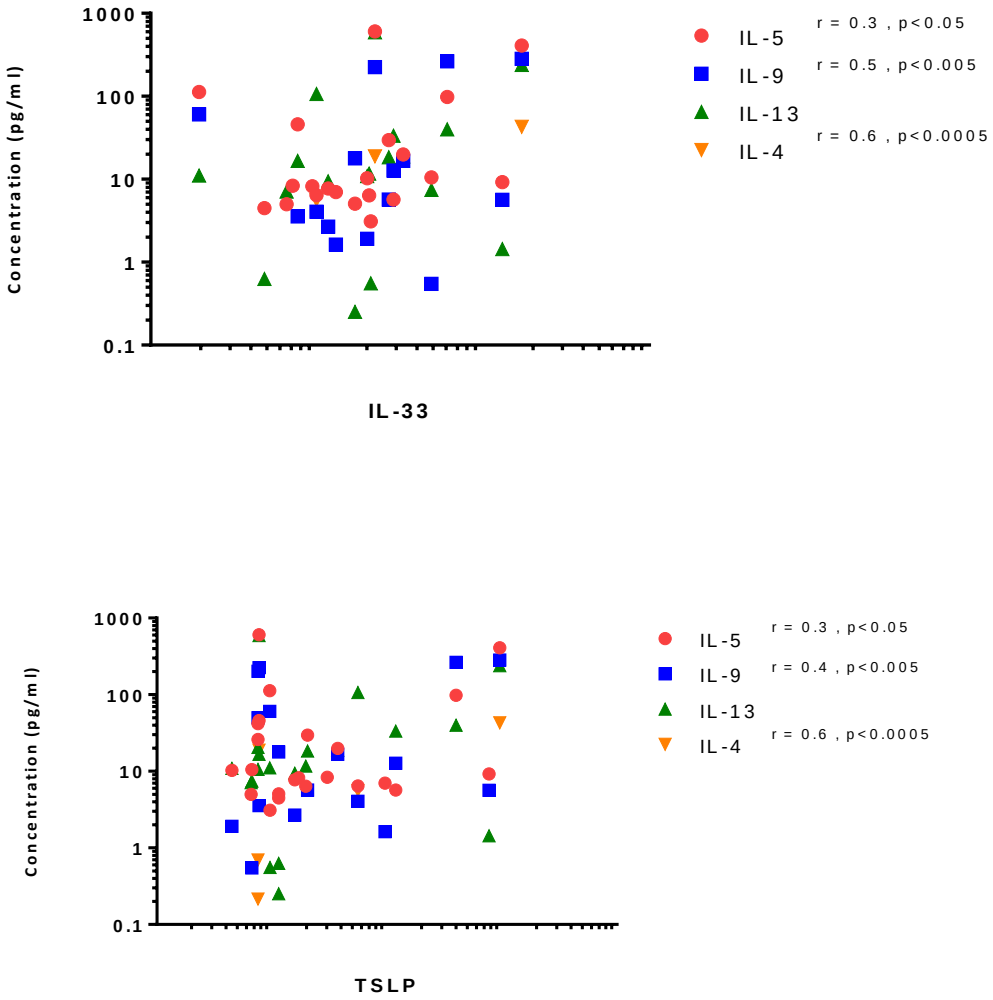


Figure 3.3.10 The concentration of IL-33 and TSLP in the blister fluid of patients with AD correlated with type 2 cytokines, IL-5, IL9 and IL-4.

3.3.2 IL-10

IL-10 is an anti-inflammatory cytokine produced by dendritic cells, monocytes, regulatory and TH2 lymphocytes. It modulates immune responses to infections hence reducing tissue damage. The studies on the expression levels of IL-10 in AD patients are conflicting. In severe AD patients fewer IL-10 producing, allergen-specific $CD4^+$ T cells were found in the blood when compared to patients with mild AD and non-atopic controls [244]. The plasma level of IL-10 was decreased in patients with severe AD [245]. In contrast lipopolysaccharide (LPS) stimulated PBMCs isolated from AD patients had higher capacity of IL-10 production [246]. Interestingly higher expression of IL-10 mRNA was found in the skin of patients with AD compared to psoriatic patients which contributed to the decreased levels of human β-defensin-2 (HBD-2) [52]. Overexpression of IL-10 mRNA was also observed in AD lesions compared to contact dermatitis and tuberculin reaction [247].

Our investigations showed significantly higher expression of IL-10 in unchallenged skin of atopic patients (354±30 pg/ml) (Figure 3.3.11) compared to healthy controls (197±12.4). Interestingly, following HDM challenge, up-regulation of IL-10 was more prominent in asymptomatic and healthy donors compared to unchallenged skin (Figure 3.3.12). IL-10 fold changes following HDM challenge was significantly higher in healthy individuals (2.5±0.1) compared to atopic patients (1.5±0.1) (Figure 3.3.13). Although IL-10 concentration levels were high in AD patients but up-regulation levels were not comparable to the increase in type 2 cytokines.

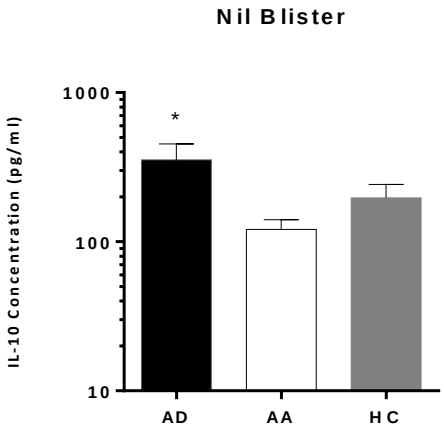


Figure 3.3.11 Higher concentration of IL-10 was observed in the blister fluid of unchallenged AD patients compared to healthy controls

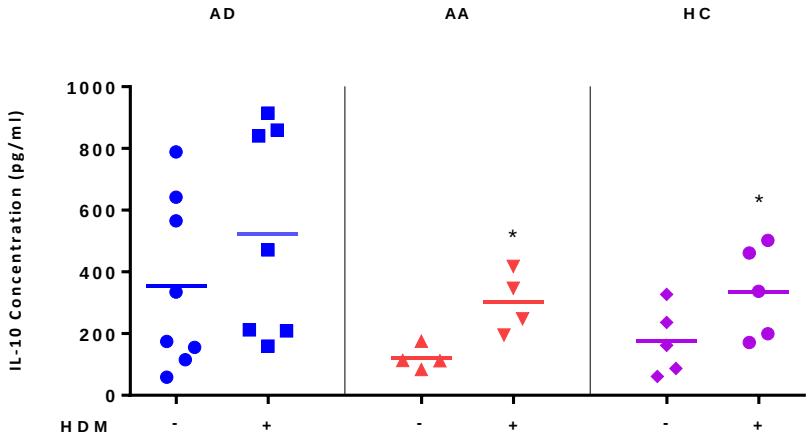


Figure 3.3.12 Up-regulation of IL-10 cytokine following HDM challenge was significantly high in AA and HC donors.

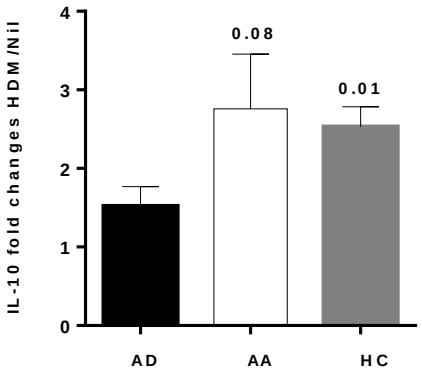


Figure 3.3.13 IL-10 fold up-regulation following HDM challenge was significantly higher in HC donors compared to AD patients.

3.3.3 Eotaxins

Eotaxins are members of the C-C chemokine family and include eotaxin-1 (CCL11), eotaxin-2 (CCL24) and eotaxin-3 (CCL26). Eotaxins are eosinophil selective chemoattractants and bind exclusively to the CCR3 receptor. It has been reported that the serum concentration of eotaxin-3 was significantly higher in patients with AD compared to healthy individuals and psoriatic patients. The serum concentration of eotaxin-3 was positively correlated with severity of AD [248], SCORAD index, eosinophil numbers and serum TARC levels [249]. The transcripts of eotaxin-3 and CCR3 were found in higher concentration in lesional skin biopsies of patients with AD [250]. Eotaxin-3 level was decreased in the serum following treatment with topical corticosteroid in combination with oral antihistamines. Interestingly, no association was observed between AD and eotaxin-2 [249].

Blister protein concentrations of eotaxin-1, eotaxin-2 and eotaxin-3 were investigated. Consistent with previously published data, we found significantly higher concentration of eotaxin-3 (but not eotaxin-1 and eotaxin-2) in unchallenged (99.2±6.68) and HDM challenged skin (162.3±15.5) of AD patients compared to asymptomatic and healthy controls (Figure 3.3.14).

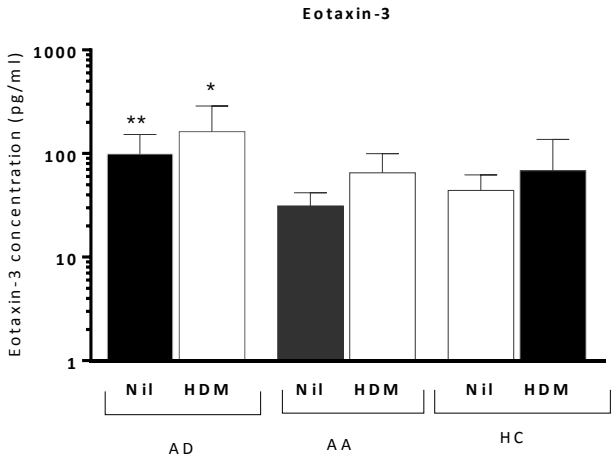


Figure 3.3.14 Higher concentration of eotaxin-3 is observed in unchallenged and HDM challenged blister fluid of AD patients compared to healthy controls

3.3.4 Proinflammatory cytokines

Proinflammatory cytokines are cytokines that induce the cascade of genes encoding mediators that increase during inflammation. These inflammatory mediators are platelet-activating factor (PAF), leukotrienes (LT), and prostaglandins (PG), and nitric oxide synthesized by type 2 phospholipase A2 (PLA2), cyclooxygenase (COX)-2, and inducible nitric oxide synthase (iNO). Endotoxins can induce production of proinflammatory mediators but IL-1, TNF-α, IL-6 and IL-12 (in some cases IFN-γ) cytokines are also exceptionally efficient. The production of proinflammatory cytokines is stimulated by trauma, infections, allergens and activated immune cells [251]. IL-1 uses adaptor protein MyD88 and triggers transcription factors NFkB and AP-1 (*c-jun and c-fos*). IL-1 and TNF-α have synergic effects. Although binding different receptors, the signalling post-receptor cascade is very similar. Except TNF-α induces apoptosis but IL-1 does not [251]. Epidermal keratinocytes constitutively produce IL-1 and stratum corneum is a major reservoir of active IL-1 [252].

Cytokine milieu in atopic dermatitis favours augmented type 2 cytokines and diminished proinflammatory mediators. Human primary keratinocytes cultured from AD patients had reduced intrinsic capacity to produce proinflammatory cytokines compared to healthy controls [253]. The content of IL-1α is remarkably decreased in stratum corneum of AD patients [252]. Significantly lower concentrations of IL-1A were induced after HDM challenge in interstitial blister fluid of patients with AD (213±32) compared to healthy donors (634±35) (Figure 3.3.15). IL-1Ra is IL-1 antagonist that competitively binds to IL-1 receptor and blocks the effect of IL-1 [254,

255]. Considerably more IL-1Ra is required to diminish the effects of IL-1. The ratio of IL-1Ra to IL-1A in stratum corneum in atopic dermatitis and psoriasis patients was higher compared to healthy individuals. Our findings also confirmed the higher IL-1Ra/IL-1A in interstitial blister fluid of unchallenged (41) and HDM challenged skin (52) (Figure 3.3.16).

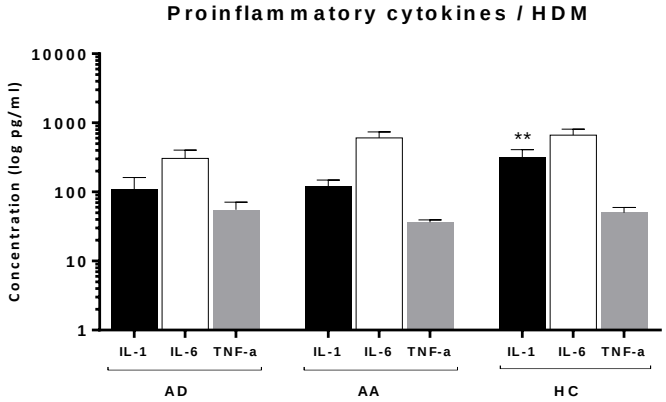


Figure 3.3.15 A significantly higher concentration of IL-1A was induced after HDM challenge in interstitial blister fluid of healthy donors (634±35) compare to patients with AD (213±32)

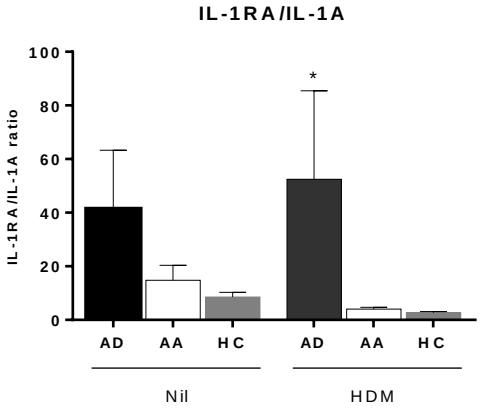


Figure 3.3.16 The IL-1RA/IL-1 ratio is significantly higher in the blister fluid of AD patients following HDM challenge compared to HC individuals

3.3.5 ILC2 infiltrate HDM challenged skin

ILCs reside in the interface of T and B cell zones in the splenic follicles of mice and can express co-stimulatory molecules essential for T cell priming and survival (CD40 ligand and CD30 ligand) [159, 256]. Co-culture of ILC2s and T cells in the presence of antigen, induced type 2 cytokine production in T cells [204]. Although ILCs have been considered to have a role in the crosstalk between the innate and adaptive arms of the immune system, little has been published on the kinetics of their recruitment to the site of inflammation. The suction blister technique was used to sample skin cells before and after allergen delivery in the epidermis of humans. Accompanying the accumulation of inflammatory mediators was the infiltration of cellular immune responses in the blister fluid.

After HDM administration, there was a significant infiltration in to blisters from allergic individuals compared to non-allergic individuals for granulocytes (39.9 vs 4.53, $P<0.01$), monocytes (8.84 vs 1.86, $P<0.01$) and lymphoid cells (13.57 vs 2.55, $P<0.01$). Infiltrating lymphoid cells included CD3, CD56 and ILC2 populations (Figure 3.3.17). ILC2 (Lin- CD45+ CD127+CD25+CRTH2+ ST2+) were clearly observed following allergen challenge (Figure 3.3.18). Furthermore, we observed that ST2 expressing ILC2 infiltrated the skin 26 hours following HDM challenge of humans (Figure 3.3.19).

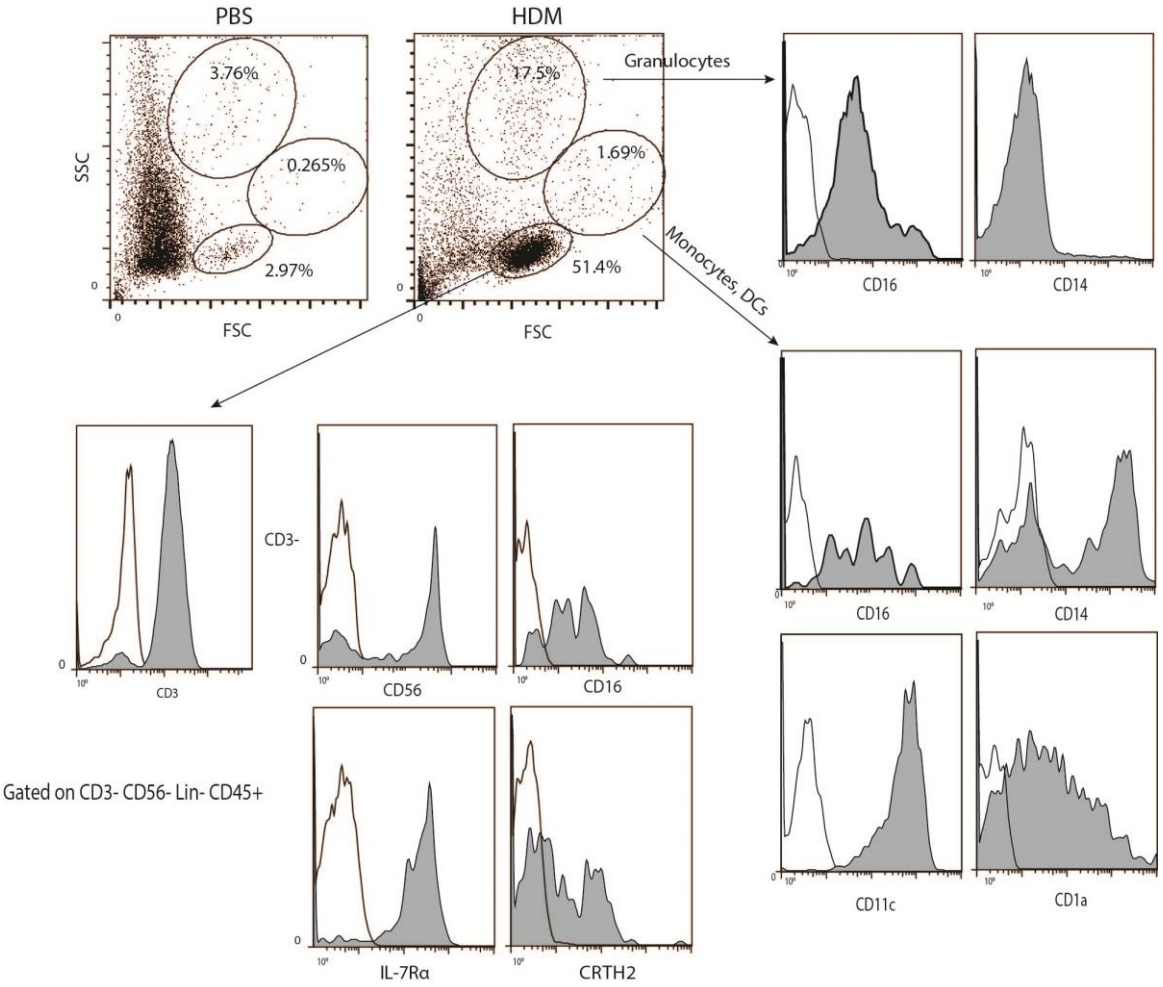


Figure 3.3.17 Cellular infiltrate in to the skin after HDM challenge. Gating strategy to show different populations of cells that infiltrated human skin blisters after house dust mite allergen or PBS challenge.

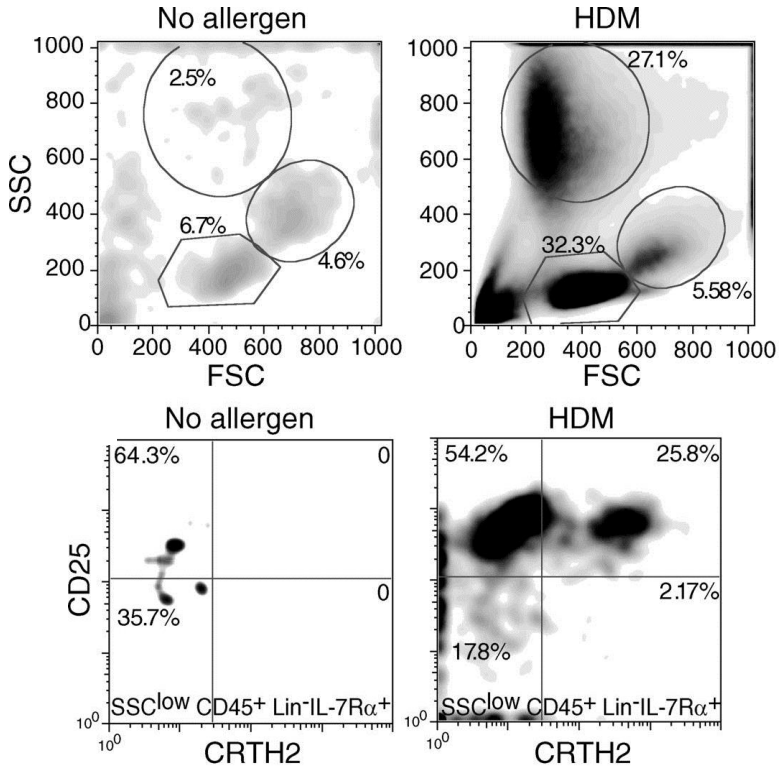


Figure 3.3.18 ILC2 localise to the human skin during an allergic response in vivo. An intraepidermal injection of HDM was given to an HDM-allergic individual and 2 hrs later suction blisters were formed over the site of injection or on an uninjected area of skin. Fluid was extracted 26 hrs later and flow cytometry performed to analyse total cell and ILC2 accumulation expressed as percentage of cells within the plots (shown as numbers within each gated region)

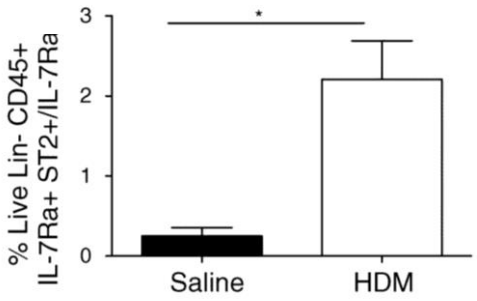


Figure 3.3.19 Infiltration of ST2-expressing ILC2 into human skin 26 hours after house dust mite or saline challenge (n=4).

3.4 The role of KLRG1 on ILC2

3.4 Introduction

3.4 Killer cell lectin-like receptor G-1 (KLRG1)

As mentioned earlier, activating and inhibitory receptors on NK cells are categorized into two distinct groups, immunoglobulin (Ig) - like receptors (belong to Ig superfamily) and C type lectin receptors. Ig-like receptors include KIR family of receptors and C type lectin receptors comprise CD94/NKG2 (KLRD/KLRC), rodent Ly49 (KLRA), NKG2D (KLRK), NKR-P1 (KLRB), and KLRG1. While KIR, CD94/NKG2, NKG2D, and Ly49, mediate their responses through interaction with MHC class I molecules or MHC-I related molecules, KLRG1 is an orphan C-type lectin receptor that binds to three of the members of cadherin family receptors. KLRG1 was originally identified in the rat basophilic leukaemia cell line RBL-2H3. Since antibody mediated binding of KLRG1 to mast cell function associated antigen (MAFA) inhibited the FcεRI crosslinking and related responses, it was termed MAFA [257]. In homeostatic conditions KLRG1 is expressed on 30% of NK population which is further upregulated during infections [258]. In humans KLRG1 is expressed on 50-80% of NK cells, γδ T cells [259], 20% and 40% of $CD4^+$ and $CD8^+$ T lymphocytes respectively [260]. The expression of KLRG1 is largely dependent on the activation stage of effector cells which is mainly studied in $CD8^+$ T cells. Early effector cells are $KLRG1^-$ $CD127^-$, and can differentiate into $CD127^{low}$ $KLRG1^+$ cells. Chronic antigen encounter leads to senescence and upregulation of KLRG1 and CD57 in higher proportion of cells [261].

High expression of KLRG1 on short lived effector CD8+ and low expression on memory precursor CD8+ T lymphocytes has been used for differentiation [262]. In CMV and EBV chronic infections over 92% of $CD8^+$ T cells express KLRG1 [263].

KLRG1 is an inhibitory receptor that mediates signalling through an immune receptor tyrosine-based inhibitory motif (ITIM) in its cytoplasmic domain and recruitment of SHIP-1 and SHIP-2 phosphatases [264]. Phosphatases degrade PIP2 and PIP3 thus regulating PI3K activation and downstream serine-threonine kinase Akt [265].

Crosslinking of KLRG1 on NK cells in mice using antibodies inhibited the cytolytic activity and IFN-γ production [258]. Simultaneous crosslinking of TCR and KLRG1 on murine T cells reduced the calcium influx and IL-2 production [264]. Consistent with mouse studies, in humans KLRG1 ligation to Ecaherin reduced the NK cytolytic activity and IFN-γ release [266]. Using $CD4^+$ T cell hybridoma transduced with retroviral vector encoding KLRG1 demonstrated that ligation of KLRG1 inhibited the NFAT signaling pathway and down regulated CD95 mediated lysis [267]. Furthermore, ligation of CD3/TCR and KLRG1 in spatially linked manner leads to inhibitory activity of KLRG1 [267].

Recently KLRG1 has been identified as a marker of ILC2 in mice [158, 230]. Around 90% of $GATA3^{hi}$ $Sca\text{-}1^{hi}$ intestinal ILC2s in adult mice expressed high levels of KLRG1 and produced IL-13 and IL-5. $KLRG1^-$ population expressed IFN-γ and IL-22. Newborn mice had a few $GATA3^{hi}$ ILC2s that expressed high levels of integrin $\alpha_4\beta_7$, this population increased steadily in the first 2 months after birth. As mice age the

expression of $\alpha_4\beta_7$ decreased and KLRG1 levels increased which suggested that KLRG1 is a marker of mature intestinal ILC2s [230].

3.4 Ecadherin expression and regulation

Cadherins are calcium dependent transmembrane glycoprotein cell adhesion molecules that are categorised into two types. Type I includes epithelial (E), neuronal (N), placental (P), retinal (R) and muscle (M) – cadherins. Vascular endothelial (VE) cadherin is a type II cadherin. Extracellular domains are tandem repeats that form parallel dimers and mediate intrinsic homophilic-binding activity with calcium. Cytoplasmic domain binds to actin cytoskeleton via α-catenin, β-catenin and γ-catenin [268-270]. Cadherin – catenin – actin complex is important in maintaining the mechanical interactions of cells and integrity of cell - cell adherens junctions. Moreover, they play an important role in regulating physiological functions of cells such as proliferation, differentiation and survival. In one study by Zhu and Watt, transfecting normal human keratinocytes with dominant negative cadherin increased cell motility, reduced expression of integrins and cell proliferation and induced terminal differentiation of keratinocytes [271].

Cadherin dependent cell connections are regulated by a wide variety of signals such as growth factors, peptide hormones and cholinergic receptor agonists [270]. Overexpression of Wnt-1 in PC12 pheochromocytoma cells increased steady state level of Ecadherin and plakoglobin, resulted in firmer cell adhesion [272]. Interestingly, epidermal growth factor (EGF) reduced the Ecadherin concentration at cell junctions in adenocarcinoma cell lines [273] whereas thyroid stimulating

hormone (TSH) and Forskolin increased Ecadherin levels in thyrocytes [274]. Activation of M3 muscarinic acetylcholine receptor (mAChR) increased concentration of E-cadherin cell junction in a small cell lung carcinoma (SCLC) cell line [275].

KLRG1 has been shown to recognise three members of the cadherin family; E-, N- and R-cadherin [276]. The first and second extracellular domains of Ecadherin are crucial for KLRG1 ligation [267]. Ecadherin is widely expressed on epithelial cells, keratinocytes, Langerhans cells and epidermal dendritic cells [277].

E-cadherin expression is down-regulated from the surface of keratinocytes in the lesional skin of patients with AD. Several mechanisms are thought to contribute to the low levels of Ecadherin. T cell mediated Fas induced keratinocyte apoptosis cause reduction in concentration of Ecadherin, whereas levels of desmosomal proteins Dsg and Dsc remain intact which leads to spongiosis. Epidermal spongiosis is one the classical histological features of AD lesions. It is characterised by widening of intercellular spaces between keratinocytes and stretching of the intercellular bridges leads to sponge like features and formation of vesicles in the epidermis [89]. In profilaggrin deficient mice ‘flaky tail (ft) mice’, reduced filaggrin levels associated with down regulation of epidermal growth factor receptor (EGFR), E-cadherin, occludin, and SIRT1 in the skin [278].

3.4 Aim

Our aim was to study whether a KLRG1–E-cadherin interaction may alter ILC2 function and act as a suppressive mechanism for dampening the ILC2 response which may act as a possible mechanism underlying barrier sensing by ILC2.

3.4 Results

3.4.1 KLRG1 expression and its binding to E-Cadherin

Although KLRG1 has been studied recently as a marker of ILC2, in the context of mouse ILC2 development within the bone marrow and the periphery [230], the functional role of KLRG1 on ILC2 has not been investigated. We noted that KLRG1 (killer cell lectin-like receptor G1) was also more highly expressed by ILC2 that were freshly isolated from AD samples, as compared to healthy controls (Figure 3.4.1), and that IL-33 and TSLP both up-regulated KLRG1 gene and protein expression on cultured ILC2 (Figure 3.4.2). Moreover, we have determined that E-cadherin is down-regulated from human keratinocytes after shRNA knockdown of the AD-associated filaggrin gene (Figure 3.4.3 previous work done in the lab, Wang and Gutowska-Owsiak).

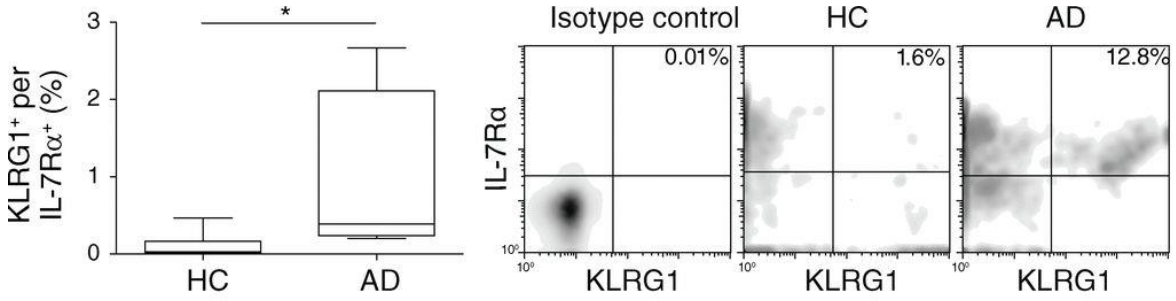


Figure 3.4.1 ILC2 were isolated from the skin of patients with atopic dermatitis (n=4) and healthy controls (n=8) and expression of KLRG1 was analysed by flow cytometry. Representative flow plots are shown on the right. Numbers indicate percentages of cells within top right quadrant of gated populations

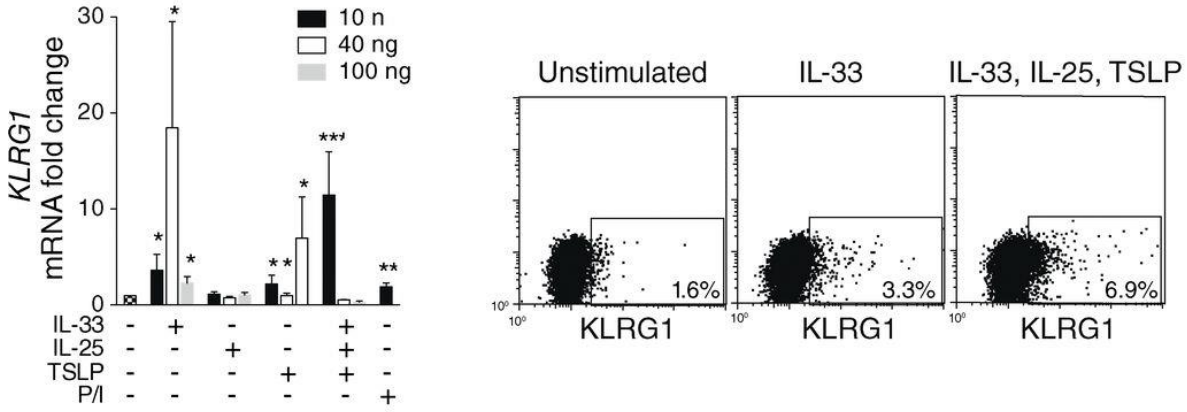


Figure 3.4.2 ILC2 were stimulated with IL-33, IL-25 and TSLP and levels of KLRG1 gene and protein expression were measured by RT-PCR and flow cytometry. Statistical comparisons are compared to the negative (no cytokine) control. Numbers on the flow cytometry plots indicate the proportion of gated cells that express KLRG1 (n=4)

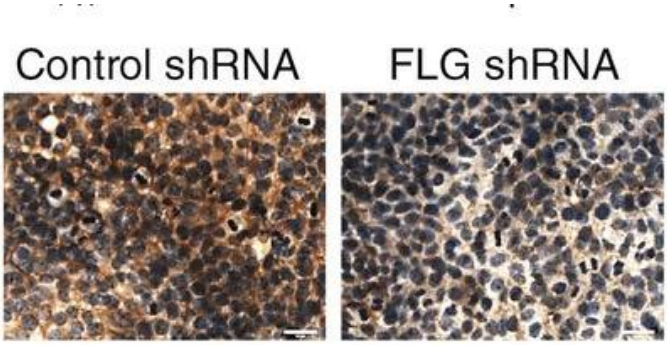


Figure 3.4.3 E-cadherin expression by keratinocytes after control (left panel) or filaggrin (right panel) shRNA knockdown (scale bar equals 50 μm)(n=3)(This work has been done by Danuta Gutowska-Owsiak and Xinwen Wang).

3.4.2 E-Cadherin ligation can inhibit ILC2 cytokine secretion

We hypothesised that null mutations in the filaggrin gene, which are found in 20-40% of patients with severe AD, may contribute to the down-regulation of E-cadherin, leading to a failure to turn-off ILC2 proliferation and cytokine expression.

We therefore investigated whether a KLRG1-E-cadherin interaction may alter ILC2 function and act as a suppressive mechanism for dampening the ILC2 response, PMA/Ionomycin-activated human ILC2 were then cultured with recombinant plate-bound E-cadherin for 24 hours. Supporting the inhibitory role of E-cadherin binding, down-regulation of the expression of *GATA3* was detected, as well as transcripts for the ILC2 signature cytokines *IL13* and *IL5*, and *AREG* (Figure 3.4.4), and reduced ILC2 proliferation (Figure 3.4.5). Furthermore, in the presence of plate-bound E-cadherin,

the production of IL-13 and IL-5 protein was reduced in IL-25, IL-33-activated ILC2 (Figure 3.4.6). Thus, in tissues expressing E-cadherin, the up-regulation of KLRG1 on ILC2 may represent an important mechanism for ameliorating the ILC2-mediated type-2 cytokine-driven inflammatory response, but when E-cadherin is down-regulated, for example in AD, this signal is absent or reduced, resulting in unhindered ILC2-cytokine production. This would represent a novel mechanism of skin barrier sensing.

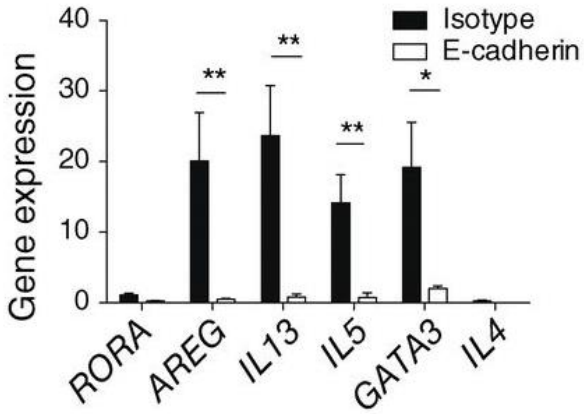


Figure 3.4.4 Expression of RORA, AREG, IL-13, IL-5, GATA3, and IL-4 mRNA relative to GAPDH by ILC2 activated using PMA/I before and after culture with rhE-cadherin (recombinant human E-cadherin) for 24 hours(n=7), as measured by PCR.

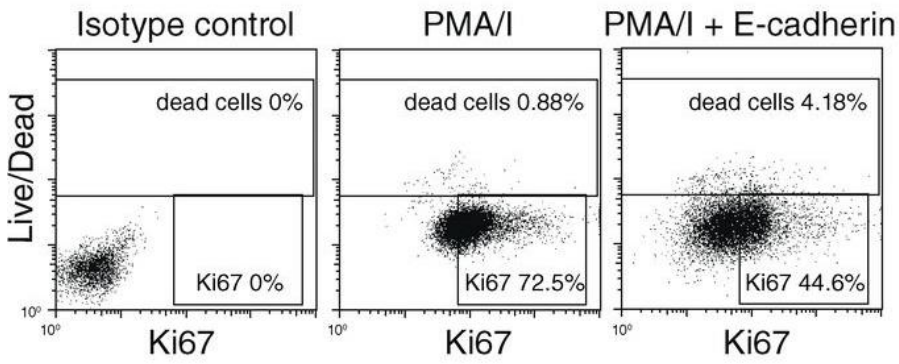


Figure 3.4.5 Ki67 and live/dead staining of ILC2 incubated with PMA/ionomycin in the presence or absence of E-cadherin. Data are representative of three independent experiments. Numbers indicate percentages of cells within the indicated gates.

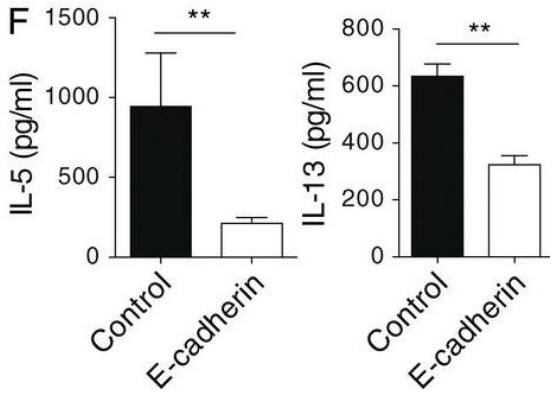


Figure 3.4.6 IL-5 and IL-13 concentration in supernatant from ILC2 activated with IL-25 and IL-33 and incubated in the presence or absence of rhE-cadherin

3.5 Discussion

3.5.1 Enhanced method of isolation of cells from the skin

Effective isolation of lymphoid cells from human skin ex vivo is vital for understanding their role in health and disease, and for informing new therapeutic developments. Longstanding existing methodology can be used to isolate cells from human skin but the frequencies of cells do not approach those predicted by estimates based on skin sections [211]. Alternative approaches have been based on cell culture steps, which have been very useful additions to possible methodologies, but require a degree of experience and laboratory infrastructure, and the culture step may introduce potential artefacts. Therefore there is a need to improve *ex vivo* cell isolation techniques that are able to obtain frequencies of viable cells that reach levels known to be present in human skin [211]. In this section I have defined a new method of isolating large number of cells from skin tissue. Using a collagenase P enzymatic treatment step, yielded far more cells than conventional collagenase D digestion and did not require cell culture [210]. Over 90% of isolated T cells expressed skin homing markers CLA and CCR10 and approximately 70% expressed CCR4 and CCR6 [210]. The expression of surface markers most susceptible to collagenase and DNase were intact, which can be explained by the presence of serum in the digestion media [222, 223]. The functional competence of different subsets of T cells was confirmed by rapid production of IFN-ɣ, IL-13, IL-22 and IL-17 *ex-vivo* after stimulation.

T cells are thought to play a role in the pathogenesis of many common inflammatory skin conditions such as atopic dermatitis, psoriasis and contact dermatitis. Inhibiting T cell function may contribute to the activity of therapeutic intervention. For example, topical steroids are believed to affect T cells via trans repression process by inhibiting production of proinflammatory cytokines (IL-1, IL-6, TNF-α, GM-CSF), chemokines (IL-8, RANTES, MIP-1) and adhesion molecules (ICAM, VCAM, E-selectin) [4]. Novel biological approaches are also believed to act through affecting T cell function. For example, inhibition of IL-17 function through blocking the antibody or its receptor is showing promise in the treatment of psoriasis [217]. Furthermore, it is becoming increasingly clear that modulating inhibitory molecule activity PDL-1 known as check points on T cells can reverse the immunosuppressive microenvironment created by tumour cells and dramatically influence the prognosis of skin malignancies such as melanoma [279].

We have developed a novel approach to isolate lymphoid cells from human skin that greatly increases the number of viable cells obtained *ex vivo*. We anticipate that the method will be of value to those studying lymphoid populations of skin resident cells including T cells or innate lymphoid cells in small patient samples [198, 280].

3.5.2 An ILC2 population resides in human skin

In this section we have shown that type 2 innate lymphoid cells represent a resident population of ILCs in the skin of both humans and mice. Similar to their counterparts in other tissues, they are characterised by a lack of lineage markers, but expression of IL-7Rα, CRTH2 and CD45 [173] and the ILC2 specific transcription factors, RORα and GATA3 [159, 183, 230, 281]. Interestingly CRTH2 was expressed on a greater proportion of ILC2s taken from the foetal gut [173] as compared to the ILC2s here taken from the skin, which could represent differential organ specific CRTH2 expression. Furthermore, the lack of RORγt and NKp46 further confirms that they are distinct from other members of the ILC family: ILC3, LTi or conventional NK cell populations [159, 228]. Although, it remains possible that ILC2 can express the latter receptors under specific conditions, their expression levels are low or absent on skin-derived ILC2. Human skin ILC2 have also recently been described by Kim *et al* (Kim et al., 2013), however our data presented here go on to show that human skin-derived ILC2 express the cytokine receptors ST2 (IL-33R), TSLPR and IL-17BR (IL-25R). Furthermore, ILC2 were also positive for skin homing markers which would support the possibility that in addition to skin residence, these cells can also home to human skin in inflammatory settings. We observed that ILC2 were enriched within atopic dermatitis acute lesional skin and such cells expressed significantly greater levels of ST2, IL17BR, TSLPR and KLRG1 as compared to ILC2 present in the skin of healthy controls. Furthermore, IL-33, but not IL-25, induced significant migration and

production of type 2 cytokines and amphiregulin by skin-derived ILC2. Although TSLP could also induce cytokine production and migration, the levels were significantly lower than those observed with IL-33.

It is notable that amphiregulin has previously been implicated in barrier repair mechanisms in dermatitis [282] and evidence supports the role of innate immune responses in the pathogenesis of atopic dermatitis [53, 283, 284].

3.5.3 Cytokine expression and cell content of allergen challenged and unchallenged Atopic dermatitis skin compared to healthy controls

Epithelial barrier dysfunction reduced antimicrobial peptides and cytokine milieu present at the time of antigen exposure directs the immune system toward type 1 or type 2 or other immune responses. In AD lesions, reduced production of proinflammatory cytokines and high levels of IL-1 receptor antagonist along with increased concentration of epithelial cytokines IL-33 and TSLP promote type 2 cytokine production. Diminished ability to upregulate anti-inflammatory cytokine IL-10 associates with loss of regulatory function of immune system.

Whilst our data from lesional skin biopsies of AD patients would support a role for ILC2 in the inflammation associated with atopic dermatitis, it is possible that they represent a late event in established disease that does not contribute to the primary inflammation. In addition, some of our experiments required the *in vitro* culture of

ILC2s in order to obtain enough numbers for investigation. Although this technique is very useful, and has been successfully used in the mouse system to first discover and then investigate ILC2s [158], it may mean that some aspects of ILC2 biology are not truly reflected in *ex vivo* systems.

Therefore, in order to investigate ILC2s in the human, *in vivo*, and to look at the timing of their entry into allergic sites we characterised the associations of ILC2 infiltration after allergen challenge in the skin of allergic individuals. The ST2-expressing ILC2 population was enriched in the skin following intraepidermal delivery of house dust mite allergen. Allergic donors had immediate local urticarial responses with marked type 2 cytokine production within the skin blister in response to the intraepidermal administration of antigen. Indeed products of mast cell degranulation and epithelial cytokines such as IL-33, may contribute directly to ILC2 infiltration. It is noteworthy that in a recent study on murine skin, ILC2 were found to associate with mast cells [174]. The increase in ILC2 number over 26 hours is unlikely to be explained purely by local proliferation of ILC2 and suggests that ILC2 can be recruited from peripheral blood in response to allergen challenge. Indeed we also demonstrated that in mice, intradermal administration of HDM also induces infiltration ILC2 into the skin.

3.5.4 The role of KLRG1 on ILC2

Atopic dermatitis lesions in humans are associated with down-regulation of E-cadherin expression. We investigated ILC2 activity in the presence and absence of E-cadherin, an adhesion protein pivotal for maintaining the integrity of epithelia. Activated skin-resident ILC2 express high levels of the inhibitory KLRG1 receptor that, upon interaction with E-cadherin, down regulates expression of transcription factors and production of IL-13, IL-5 and amphiregulin. In the inflamed skin lesions of patients with atopic dermatitis, cleavage of E-cadherin [113] and production of epithelial cytokines may result in discontinuation of the inhibitory signal and potentially allow engagement of activating receptors. This may lead to the unrestricted release of type 2 cytokines and to the over-production of wound healing regulators by ILC2. Thus this KLRG1-E-cadherin model of ILC2 regulation may represent a novel mechanism for barrier sensing in the skin. It is of interest that E-cadherin promotes DC maturation and E-cadherin-stimulated DC can induce T regulatory cells in mice [285], whereas E-cadherin expressing DC promote Th17 responses and disease in a colitis model [286]. These findings are consistent with E-cadherin having broader relevance beyond a purely physical function, but having a critical role in signalling epithelial events to resident and recruited immune cells.

Taken together, these data show that ILC2 are resident in human skin but the frequencies of ILC2 are elevated in the setting of AD and expanded after allergen challenge. Skin ILC2 can be isolated and expanded *in vitro* for further functional

assays. *Ex vivo* skin ILC2 retain responsiveness to IL-33, with IL-33 promoting ILC2 migration and type 2 cytokine production. KLRG1-E-cadherin interactions exert an inhibitory effect on ILC2 activation, suggesting that settings associated with low E-cadherin expression may promote ILC2 activation. Barrier dysfunction is a key early event in the pathogenesis of atopic dermatitis which is further compounded by type 2 cytokine mediated inflammation. Therefore the current findings implicate a novel mechanism of barrier sensing. Although a population of ILC2-like cells has recently been reported in human and mouse skin, the role of IL-33 has been questioned. We now demonstrate that human skin-derived ILC2 express ST2 (IL-33R) and respond to IL-33 by producing type 2 cytokines.

In collaboration with Andrew McKenzie's group (LMB, Cambridge) and Padraic Fallon's group (Trinity College, Dublin), we have investigated the requirement of ILC2 in a mouse model of dermatitis. This work was undertaken in their laboratories and so is not presented here in detail. However the data showed that calcipotriol-induced dermatitis was dependent on ILC2 using two models of ILC2 depletion, namely CD90 antibody-based depletion on a RAG knockout background and RORa-deficient bone marrow chimaeras [198]. Furthermore, our experiments in pathway deficient mice indicate that IL-25R, IL-33R and TSLP-R are all important for the development of calcipotriol-induced skin inflammation. Taken together, it is likely that IL-25, IL-33 and TSLP are all involved in the regulation of ILC2 and allergic skin inflammation and such redundancy may have important therapeutic implications [197, 287-290].

Chapter 4: The role of CRTH2 in group 2 innate lymphoid cells

4.1 Introduction

4.1.1 Prostaglandins synthesis and receptors

Prostanoids are members of the eicosanoids family and include prostaglandins (PGs) and thromboxanes (TXs). Prostaglandins consist of PGE2, PGD2, PGF2 and PGI2. Prostaglandins are potent bioactive lipid mediators that derive from cell membrane phospholipids. Phospholipase A2 (PLA2) enzyme activity converts membrane phospholipids into arachidonic acid (AA). Sequential actions of cyclo-oxygenase (COX)-1 and COX-2 (also termed prostaglandin H synthase; PGSH) on AA give rise to PGH2 substrate [291]. PGH2 is then converted into bioactive prostaglandins by terminal synthases. PGDS converts PGH2 to PGD2 and 2 types of it has been identified; glutathione-independent, lipocalin-type PGDS (brain PGDS) and glutathione-dependent PGDS (hPGDS) that is expressed mainly in mast cells and macrophages [292] (Figure 4.1).

Prostanoids are released from several tissues and act as endogenous regulators to maintain local homeostasis. Prostanoids mediate a wide variety of reaction in the body including contraction relaxation of muscles and platelet activation. PGE2

induces fever and dilation of blood vessels, while PGF2 mediates vasoconstriction. PGI2 alters blood pressure, promotes and inhibits blood clotting [293, 294]. PGD2 can induce sleep while PGE2 promotes wakefulness [295]. Prostanoids exert their actions via eight specific receptors. They belong to G-protein coupled transmembrane receptors (GPCR) with 7 domains [296] and classified into five types; termed DP, EP, FP, IP, TP that bind to PGD2, PGE2, PGF2, PGI2 and TXA2, respectively. EP receptors are subdivided into four receptors based on their response to agonists and antagonists; termed EP1, EP2, EP3 and EP4.

Two receptors have been identified for PGD2. DP1 was the first receptor recognised and is coupled to Gαs and increases intracellular cAMP levels. PGD2 also mediates its action through binding to CRTH2 (chemoattractant receptor homologous molecule expressed by T helper 2 cells) also called DP2 and GPR44 [297, 298].

PGD2 is a selective marker of mast cell activation and is a main prostanoid produced by mast cells [299, 300]. PGD2 is released rapidly by mast cells upon cross linking of surface IgE within minutes. Allergen challenge induces PGD2 production in airways of asthmatic patients [301], nasal mucosa of patients with allergic rhinitis [302, 303] and skin of AD patients [304]. Moreover, a subpopulation of TH2 lymphocytes can express hPGDS and produce PGD2 in response to anti-CD3/CD28 stimulation in physiologically significant quantities. Interestingly PGD2 production by TH2 cells occurs in temporally delayed phase of inflammation and in chronic allergic setting compared to mast cell responses [292]. The production of PGD2 by dendritic cells is controversial. Dendritic

cells incubated with TSLP promote expansion of $CRTH2^+$ $CD4^+$ TH2 effector memory cells which in turn up-regulate PGDS and polarize further TH2 differentiation [305].

PGD2 has a pivotal role in physiological and pathological processes in a variety of tissues [291]. It mediates sleep induction, perception of pain, anti-coagulation, vasodilation and broncho-constriction. It is a major mediator in allergy and inflammatory processes [291]. PGD2 alters blood flow, promotes eosinophil and TH2 lymphocyte recruitment and increases production of type 2 cytokines. hPGD2 synthase has an opposing effect on neutrophilic [306], TH1 [307] and TH17 mediated inflammation. Carrageenin-induced pleurisy in rats increased production of COX-2 at 2-hour and 48-hour time points. While the first peak was associated with an increase in neutrophils and PGE2, the second peak induced production of PGD2, PGJ2 and was associated with resolution of inflammation [306]. Furthermore, hPGD2S-/- mice showed prolonged exaggerated delayed hypersensitivity reactions induced by methylated BSA (mBSA) which was mediated through terminal 15d-PGJ_2 [307].

PGD2 binding to DP1 mediates mast cell maturation in the skin. Mast cell progenitors produce type III PLA2 (PLA2G3) that assists production of PGD2 with the help of lipocalin type synthase (L-PGDS) from fibroblasts. PGD2 binds to DP1 receptor on immature mast cells and stimulates maturation [308]. Impaired mast cell maturation and anaphylactic reactions were observed in mice deficient in PLA2G3, DP1 and L-PGDS [308]. Furthermore, neutralizing antibodies of PLA2G3, inhibitors of L-PGDS and antagonists of DP1 reduce histamine production by mast cells when cultured with fibroblasts. These findings support a role for prostanoids in skin inflammation [309].

Intravenous infusion of PGD2 induced intense facial flushing and nasal congestion with no effect on blood pressure and lung function [310] - this was thought to be mediated by the DP1 receptor as oral administration of a selective DP1 agonist, BW245C, caused similar symptoms [311]. Using BW245C has shown that DP1-PGD2 interaction also mediates relaxation of vascular smooth muscle [312].

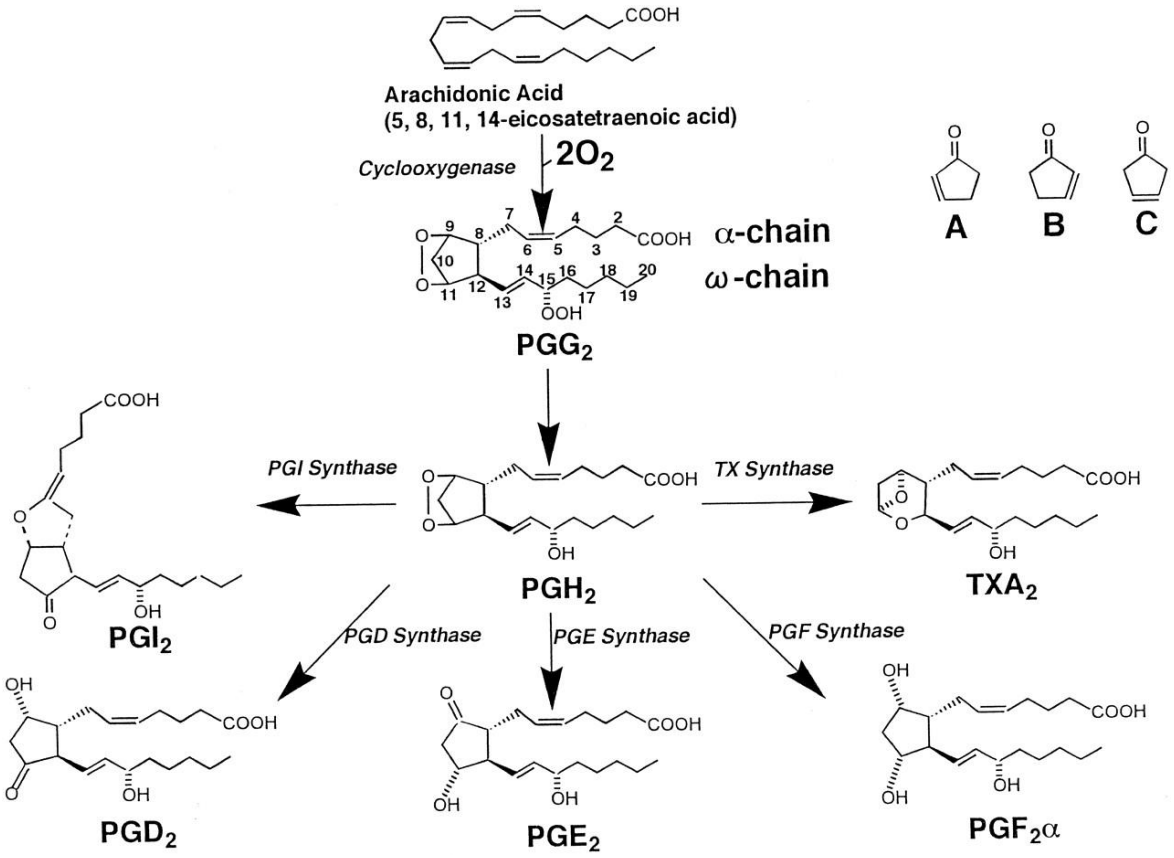


Figure 4.1 Biosynthetic pathways of prostanoids. Arachidonic acid is first converted to PGG2 and PGH2 by cyclooxygenase. PGH2 is converted to series 2 prostaglandins (PGD2, PGE2, PGF2, PGH2, PGI2) and TXA2 is catalyzed by respective synthase as shown. Adapted from reference [296]

4.1.2 CRTH2

CRTH2, originally termed GPR44, is a GPCR receptor for prostaglandin D 2 (PGD2), a major mediator released from activated mast cells [313]. Before the discovery of ILC2 cells, CRTH2 was already known to be highly expressed on eosinophils, basophils and Th2 cells. The expression of CRTH2 is more promiscuous than DP1. While CRTH2 and DP1 both bind PGD2, their structure is completely different. CRTH2 is closely related to chemotactic receptors such as the leukotriene B_4 receptors BLT1 and BLT2, the complement C5a receptor and the formyl peptide receptors. CRTH2 functionally couples with G-protein $G_{\alpha i/o}$ and reduces intracellular cAMP [314] which activates PI3K dependent phosphorylation of GSK3β and reduction in phosphorylated NFAT [315].

Emerging evidence suggests that the activation of CRTH2 leads to many pro-inflammatory responses in leukocytes including chemotaxis of eosinophils, basophils and Th2 cells [313, 316, 317], Th2 cytokine production [318] even in the absence of TCR-triggering or co-stimulation [319], and pro-inflammatory protein expression by eosinophils and Th2 cells [318, 320]. Previous studies also demonstrated that the signalling of CRTH2 suppresses Th2 cell apoptosis [321], a process which is likely to impede the resolution of allergic inflammation. The type 2 cytokine production mediated by CRTH2 is markedly enhanced by another group of mast cell mediators cysteinyl leukotrienes [322]. Allergic responses mediated by IgE, mast cells, Th2 cells and eosinophils are dramatically reduced in mice where CRTH2 is genetically ablated

or by small molecule CRTH2 antagonists [323-325]. Antagonism of CRTH2 is currently being considered as a potentially useful approach for the treatment of allergic diseases, including asthma, rhinitis and atopic dermatitis [326].

Intra-tracheal administration of PGD2 and its metabolite 15-deoxy-Delta12,14-PGJ2 induced pulmonary eosinophilia in Brown Norway rats. This effect was mimicked by DP2 agonists, 15R-methyl-PGD2 and 13-14-dihydro-15-keto-PGD2, which suggested that PGD2 attracts eosinophils via interaction with CRTH2 [327]. Furthermore, CRTH2 antagonist Ramatroban can inhibit eosinophilia [328]. PGD2 exacerbated inflammation and eosinophil infiltration in the skin of mouse model of AD [329]. PGD2 can induce production of type 2 cytokines in a dose dependent manner by TH2 cells without need for co-stimulation. The CRTH2 agonist, 13,14-dihydro-15-keto-PGD2, showed a similar effect [319].

4.2 Aim

We sought to determine the role of PGD_2 and CRTH2 in human ILC2s and compare it with the established ILC2 activators IL-25 and IL-33.

4.3 Results

4.3.1 CRTH2 mediates chemotaxis of human ILC2 cells

To understand the role of CRTH2 in human ILC2 cells, the effect of PGD2 with that of IL-33 and IL-25 on ILC2 migration was compared. Lineage negative (i.e. CD3, CD4, CD8, CD14, CD19, CD56, CD11c, CD11b, FcεRI, TCRγδ, TCRαβ and CD123 negative), $CD45^{high}$, $CD127^{+}$ and $CRTH2^{+}$ ILC2 cells were isolated from human skin biopsies and peripheral blood of healthy adult donors (Figure 4.2), and tested with dose titrations of PGD2, IL-33 and IL-25 in chemotaxis assays (Figure 4.3). Both PGD2 and IL-33 caused ILC2 migration in a dose-dependent manner peaking at approximately 100 nM for PGD2 and 30 ng/ml for IL-33. The chemoattractive effect of IL-25 on ILC2 cells was very weak. The maximum response achieved by PGD2 was 4.75 fold higher than that achieved by IL-33.

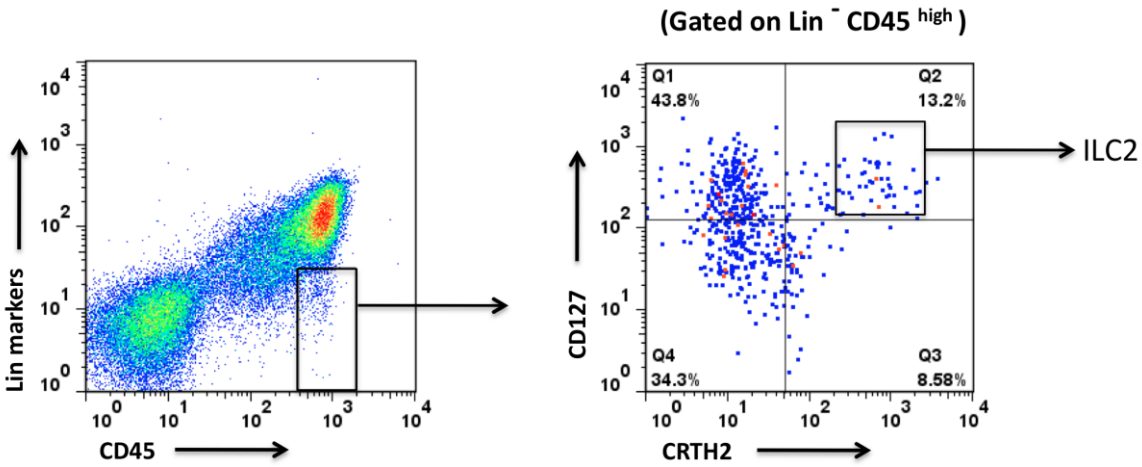


Figure 4.2 ILC2 cell isolation. ILC2s isolated from the human skin were lineage marker negative (CD3, CD4, CD8, CD14, CD19, CD56, CD11c, CD11b, FceRI, TCRgδ, TCRαβ and CD123), CD45high, IL-7Rα positive and CRTH2 positive.

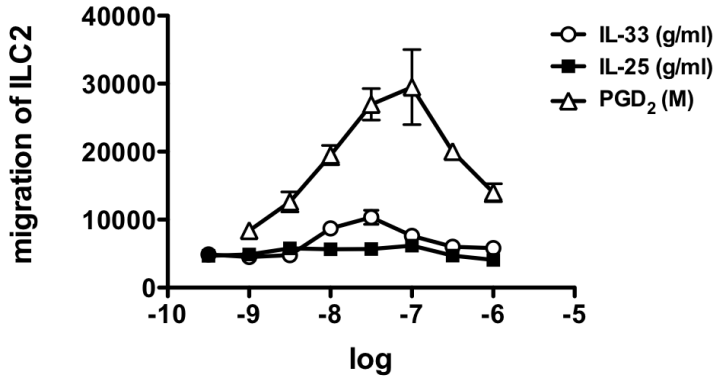


Figure 4.3 Migration of ILC2 cells to PGD2 is mediated by CRTH2. ILC2 cells were stimulated with various concentration of IL-25, IL-33 or PGD2 and the cell migration was examined with a chemotaxis assay.

To confirm the receptor mediating ILC2 migration induced by PGD2 was indeed CRTH2, the selective CRTH2 antagonist TM30089 was used. In chemotaxis assays triggered by PGD2 (30 nM), ILC2 migration was completely inhibited by TM30089 (1 mM) (Figure 4.4).

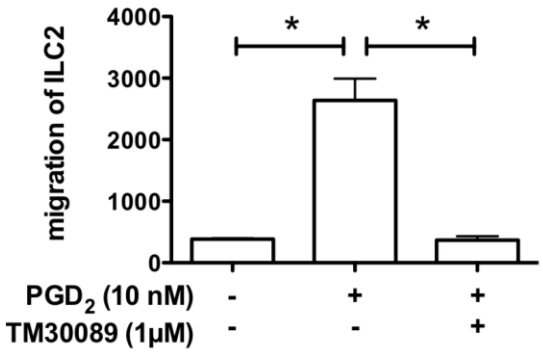


Figure 4.4 ILC2 cells were exposed to the gradient against 10 nM PGD2 in the absence or presence of 1 µM TM30089 in a chemotaxis assay. CRTH2 antagonist TM30089 completely blocked chemotactic effect of PGD2. Data are representatives of 3 independent experiments and expressed as mean±SEM of triplicate samples.

To further elucidate the potential contribution and interaction of PGD2, IL-33 and IL-25 on ILC2 migration, the effects of different combinations of these stimulators were examined (Figure 4.5). To avoid saturation of the response during the assay, low concentrations of the stimulators below the peak in their dose curves (5nM for PGD2,

10ng/ml for IL-33 and IL-25) were used for these combination tests. No additive effect was detected when the stimulators were combined. The cell migration in response to the combinations of PGD2 and IL-33 or PGD2, IL-33 and IL-25 appears to be mainly mediated by CRTH2 as the responses were largely inhibited by TM30089.

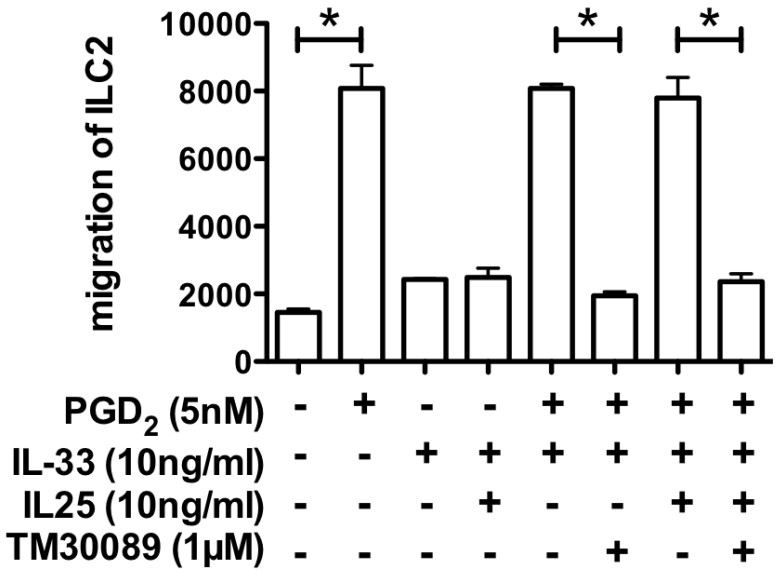


Figure 4.5 ILC2 cells were treated with the gradient against PGD2 (5 nM), IL-33 (10ng/ml), IL-25 (10ng/ml) alone or their different combinations in the presence or absence of TM30089 (1 μM) and the cell migration was determined with chemotaxis assays. Data are representatives of 3 independent experiments and expressed as mean±SEM of triplicate samples.

4.3.2 Activation of CRTH2 induced type 2 cytokine productions in human ILC2 cells

One of the most striking features of ILC2 cells is their ability to produce the type 2 cytokines IL-4, IL-5, and IL-13 [158]. To investigate the role of CRTH2 in type 2 cytokine production in human ILC2 cells, the cells were stimulated with increasing concentrations of PGD2 for 2.5 h for mRNA analysis or for 4 h for protein analysis (Figure 4.6). The treatment caused increased cytokine expression at both mRNA levels in the cells and protein levels released in the culture medium in a dose-dependent manner (Figure 4.6). The EC_{50} of PGD2 for IL-4, IL-5, and IL-13 production at the mRNA level was 88, 178, and 111 nM respectively, and at the protein level was 195, 118, and 82.6 nM, respectively.

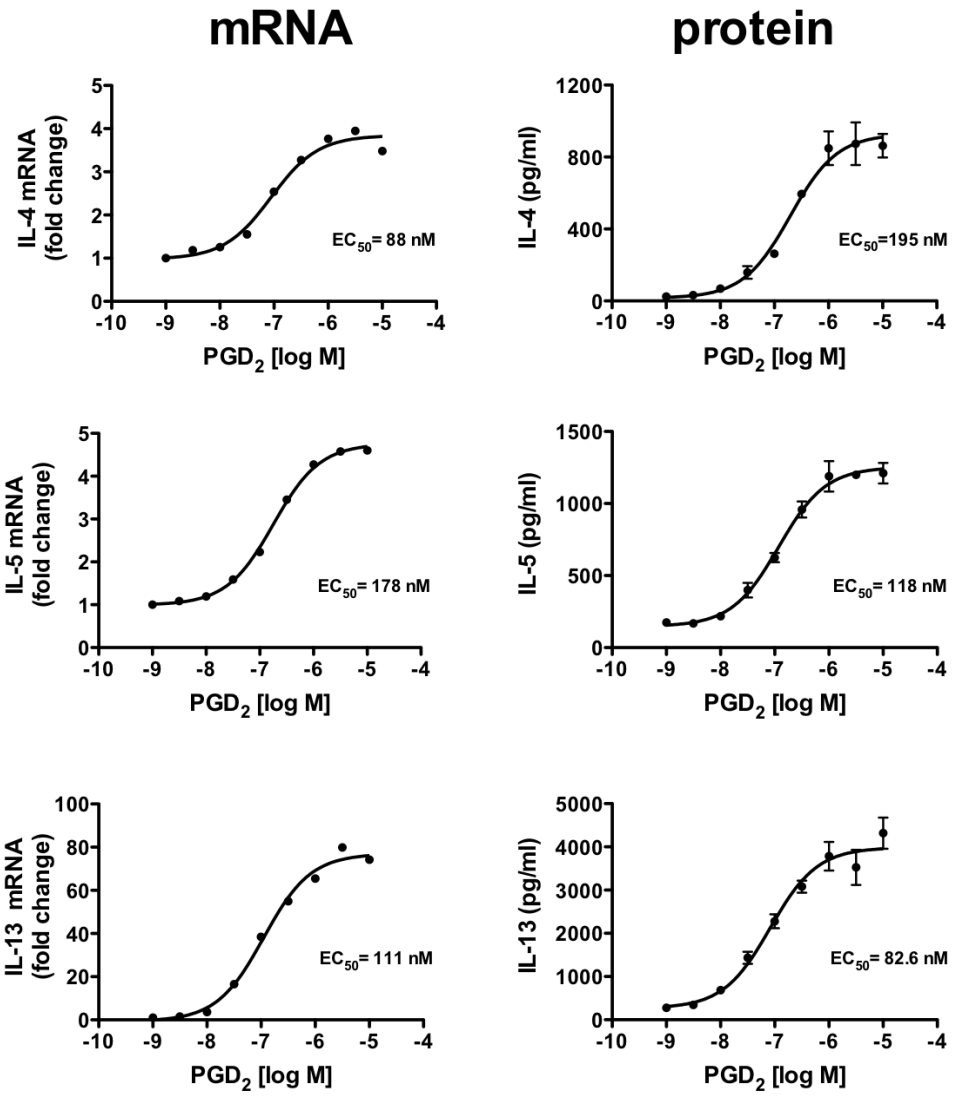


Figure 4.6 CRTH2 mediates type 2 cytokine production in ILC2 cells in response to PGD2. ILC2 cells were incubated with various concentration of PGD2 for 2.5 h (mRNA) or 4 h (protein). Total RNA was extracted from the cell pellets for q-PCR, and the mRNA level of cytokines was measured to monitor the changes in gene expression. The mRNA levels in untreated cells were treated as 1 fold. The supernatant was collected, and cytokine concentrations were determined by Luminex assay. Data are representatives of 3 independent experiments.

To elucidate the receptor mediating the effects of PGD2 on the cytokine production, the effects of the CRTH2-specific inhibitor TM30089 were examined (Figure 4.7). The type 2 cytokine production induced by 100 nM PGD2 was completely blocked by 1 μM TM30089.

It has been reported that IL-33 and IL-25 are important mediators in promoting type 2 cytokine production from ILC2 cells [330-332]. To define the effect of the combination of PGD2, IL-33 and IL-25 on type 2 cytokine production in ILC2 cells, the cells were treated with PGD2, IL-33, or IL-25 alone (at concentrations close to their relative EC_{50}) or in combination as indicated in Figure 4.7 for 4 h (Figure 4.7). Both IL-33 and IL-25 did evoke type 2 cytokine production from ILC2s. The efficacy of both IL-33 and IL-25 was, however, much weaker than that of PGD2. Interestingly, the combination of IL-33 and IL-25 did not enhance the stimulation compared with either IL-33 or IL-25 alone; however, the combination of these cytokines, particularly IL-25 with PGD2, enhanced cytokine production with an apparent synergistic effect. The contribution of PGD2 in these combination treatments was effectively blocked by TM30089.

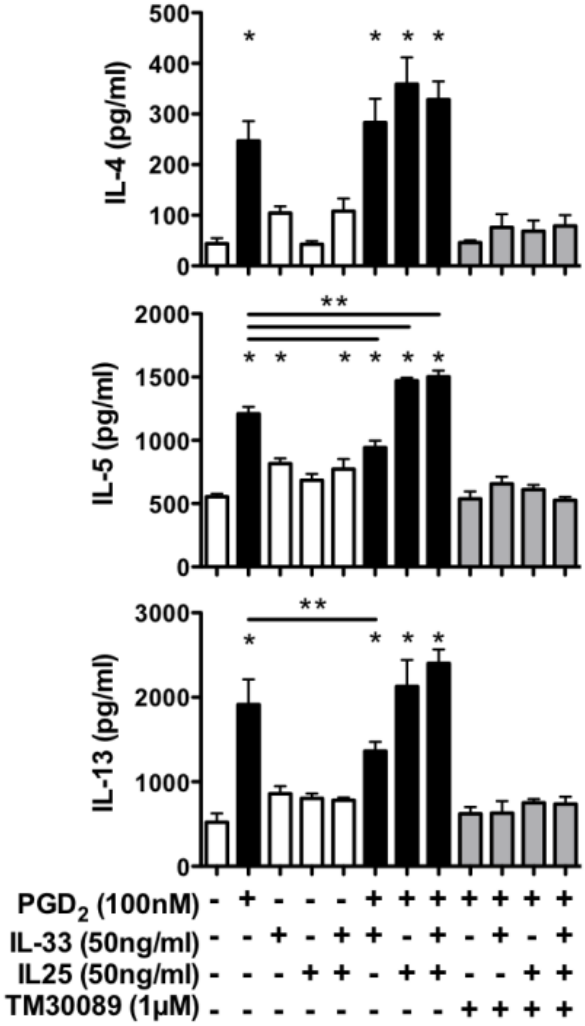


Figure 4.7 ILC2 cells were treated with or without PGD2 (100 nM), IL-33 (50 ng/ml), IL-25 (50 ng/ml) alone or their different combinations in the presence or absence of TM30089 (1 μM) for 4 h. The cell supernatant was collected, and the concentrations of released cytokines were measured by Luminex assay. Data are representatives of 3 independent experiments.

4.3.3 Activation of human ILC2 cells via CRTH2 regulated other cytokine productions

To further understand the potential pro-inflammatory role of CRTH2 in ILC2 cells, the effect of CRTH2 on other cytokine production was investigated (Figure 4.8). The cells were incubated with increasing concentration of PGD2 for 4 h, and the protein levels of IL-3, IL-8, IL-9, IL-17A, IL-17F, IL-21, GM-CSF, CSF-1, and IFNγ were measured. PGD2 induced the production of IL-3, IL-8, IL-9, IL-21, GM-CSF, and CSF-1 in a dose-dependent manner (Figure 4.8). The EC_{50} of PGD_2 for IL-3, IL-8, IL-9, IL-21, GM-CSF, and CSF-1 were 79.8, 65.7, 47.4, 43, 132.5, and 29.2 nM respectively. No IL-17A, IL-17F or IFNγ was detected.

The production of IL-3, IL-8, IL-9, IL-21, GM-CSF and CSF-1 induced by PGD2, both at mRNA and protein levels, was enhanced by combination with IL-33 and IL-25. This enhancement was particularly significant for IL-8, IL-9 and GM-CSF production (Figure 4.9). In contrast, the mRNA level of IFNγ was down-regulated by PGD2 at nM concentrations (Figure 4.10). As expected, the regulatory effects of PGD2 on these cytokines, whether activating or inhibitory, were reversed by TM30089 (1 μM) (Figure 4.9 and 4.10).

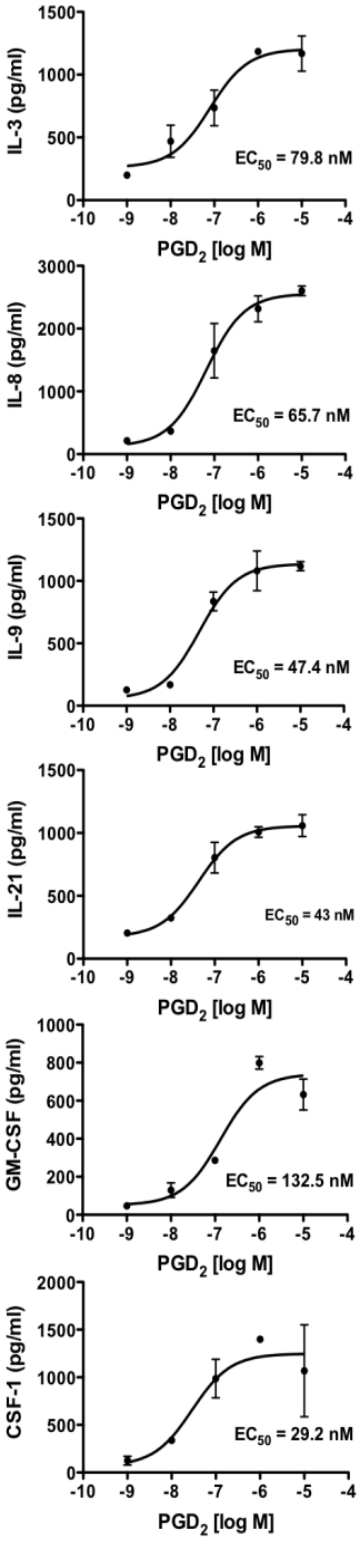


Figure 4.8 Activation of CRTH2 evokes diverse pro-inflammatory cytokine production in ILC2 cells. ILC2 cells were stimulated with various concentration of PGD2 for 4 h. The cytokine concentrations in the supernatant were determined by luminex assay

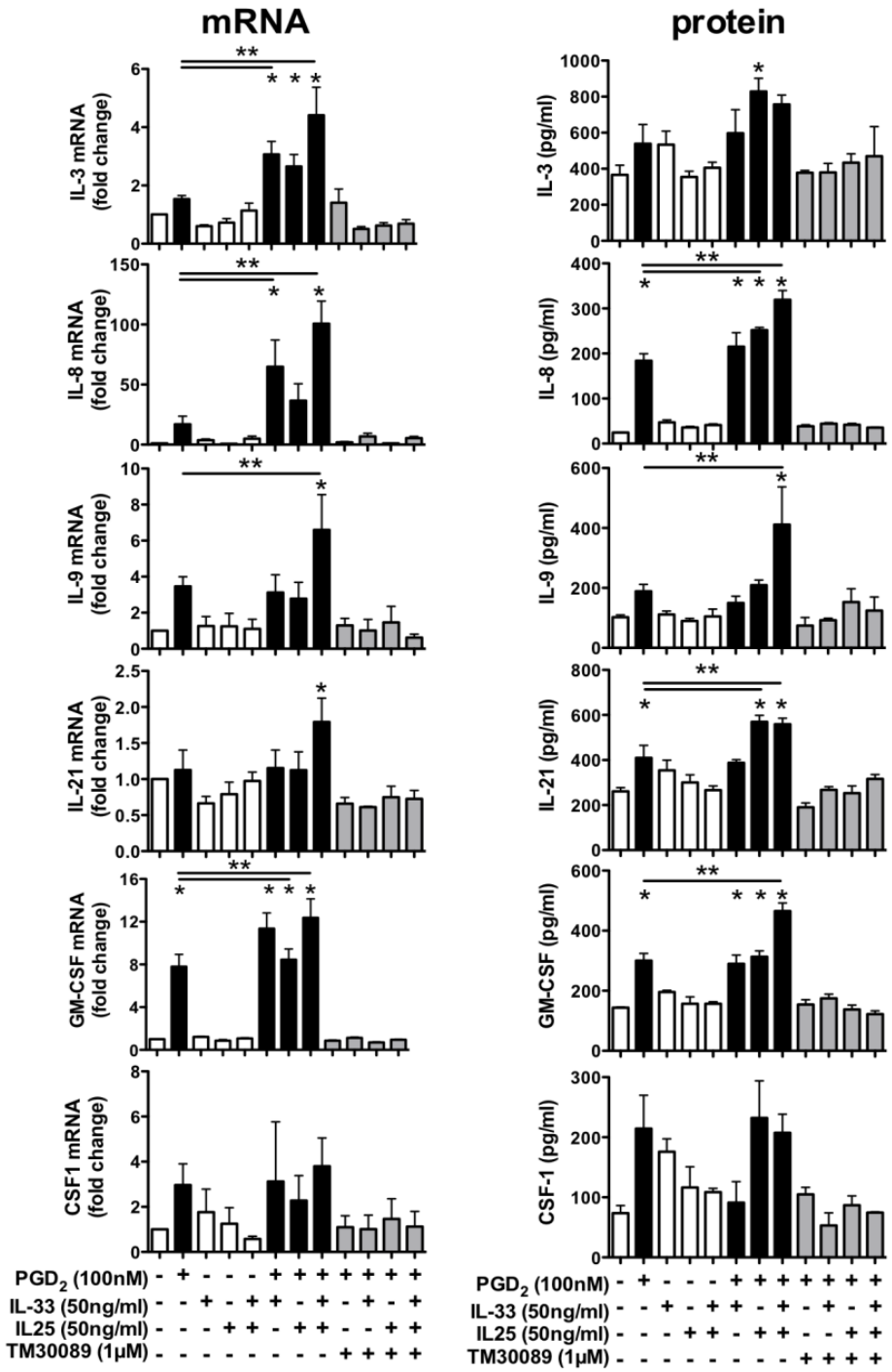


Figure 4.9 ILC2 cells were treated with or without PGD2 (100 nM), IL-33 (50 ng/ml), IL-25 (50 ng/ml) alone or their different combinations in the presence or absence of TM30089 (1 µM) for 2.5 h (mRNA) or 4 h (protein). The mRNA level of cytokines in the cell pellets was measured by Quantigene assay, and the concentrations of released cytokines in the cell supernatant were measured by luminex assay

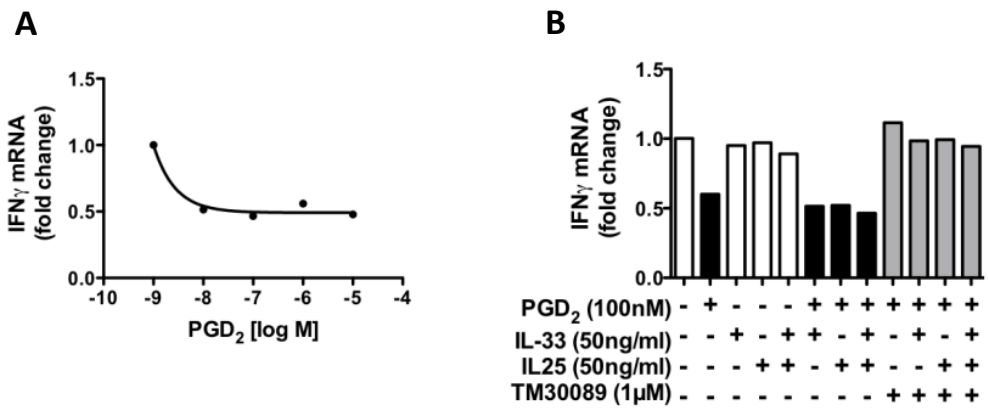


Figure 4.10 ILC2 cells were treated with increasing concentration of PGD2 (A) or PGD2 (100 nM), IL-33 (50 ng/ml), IL-25 (50 ng/ml) alone or their different combinations in the presence or absence of TM30089 (1 μM) for 2.5 h (B) and mRNA level of IFNγ in the cell pellets was determined by q-PCR. The mRNA levels in the cells incubated with medium alone were treated as 1 fold.

4.3.4 Activation of CRTH2 up-regulated expression of IL33 receptor but down-regulated expression of CRTH2 in human ILC2 cells

First the expression of IL-33 receptor, ST2, was examined on lin$^-$ IL-7Rα$^+$ CRTH2$^+$ and lin$^-$ IL-7Rα$^+$ CRTH2$^-$ cells. Interestingly ST2 was mainly expressed on CRTH2 positive population (Figure 4.11). Next the effect of IL-33 cytokine stimulation on lin$^-$ IL-7Rα$^+$ CRTH2$^+$ and lin$^-$ IL-7Rα$^+$ CRTH2$^-$ populations was compared. While IL-33 had little effect on lin$^-$ IL-7Rα$^+$ CRTH2$^-$ cells, it induced production of IL-13 in lin$^-$ IL-7Rα$^+$ CRTH2$^+$ population (Figure 4.12).

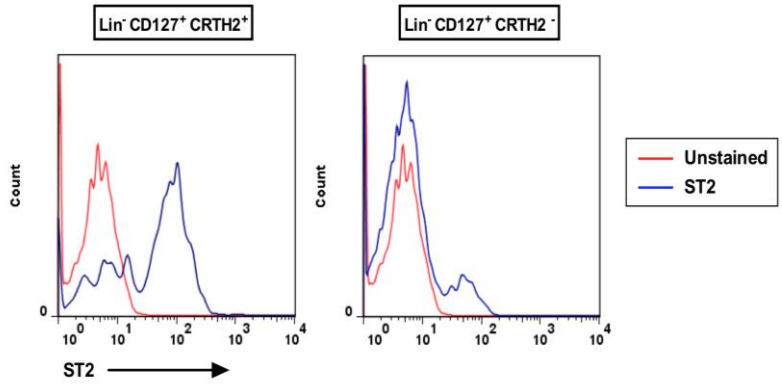


Figure 4.11 Expression of ST2 on lin$^-$ IL-7Rα$^+$ CRTH2$^-$ and lin$^-$ IL-7Rα$^+$ CRTH2$^+$ cells was compared. CRTH2$^+$ cells were the main population of lin$^-$ IL-7Rα$^+$ cells expressing ST2.

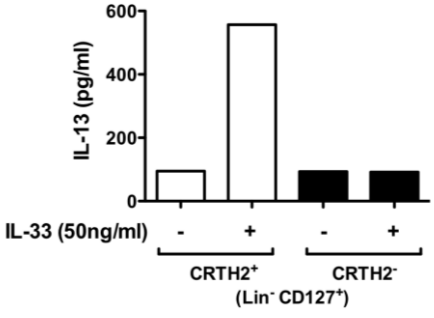


Figure 4.12 Lin$^-$ IL-7Ra$^+$ CRTH2$^+$ and lin$^-$ IL-7Ra$^+$ CRTH2$^-$ cells stimulated with IL-33 (50ng/ml) and expression of IL-13 was measured by ELISA. IL-33 induced production of IL-13 in Lin$^-$ IL-7Ra$^+$ CRTH2$^+$

To further explore potential interaction between IL-33/IL-25-mediated and PGD2-mediated immune responses, the effect of these activators on the expression of their receptors in ILC2 cells was examined. After stimulation with 100 nM PGD2 for 2.5 h, the mRNA for IL-33 receptor ST2 was increased significantly, the mRNA for IL-25 receptor IL17RA was also up-regulated slightly, while the mRNA for CRTH2 was reduced markedly (Figure 4.13). The effect on the level of mRNA of the IL-25 receptor component IL17RB was minor. Treatment with IL-33 or IL-25 alone had no significant effect on the mRNA levels of these receptors; however, the combination of PGD2, IL-33 and IL-25 enhanced the up-regulation of ST2 mRNA (Figure 4.13). The CRTH2-dependent regulation of these receptors was inhibited by TM30089.

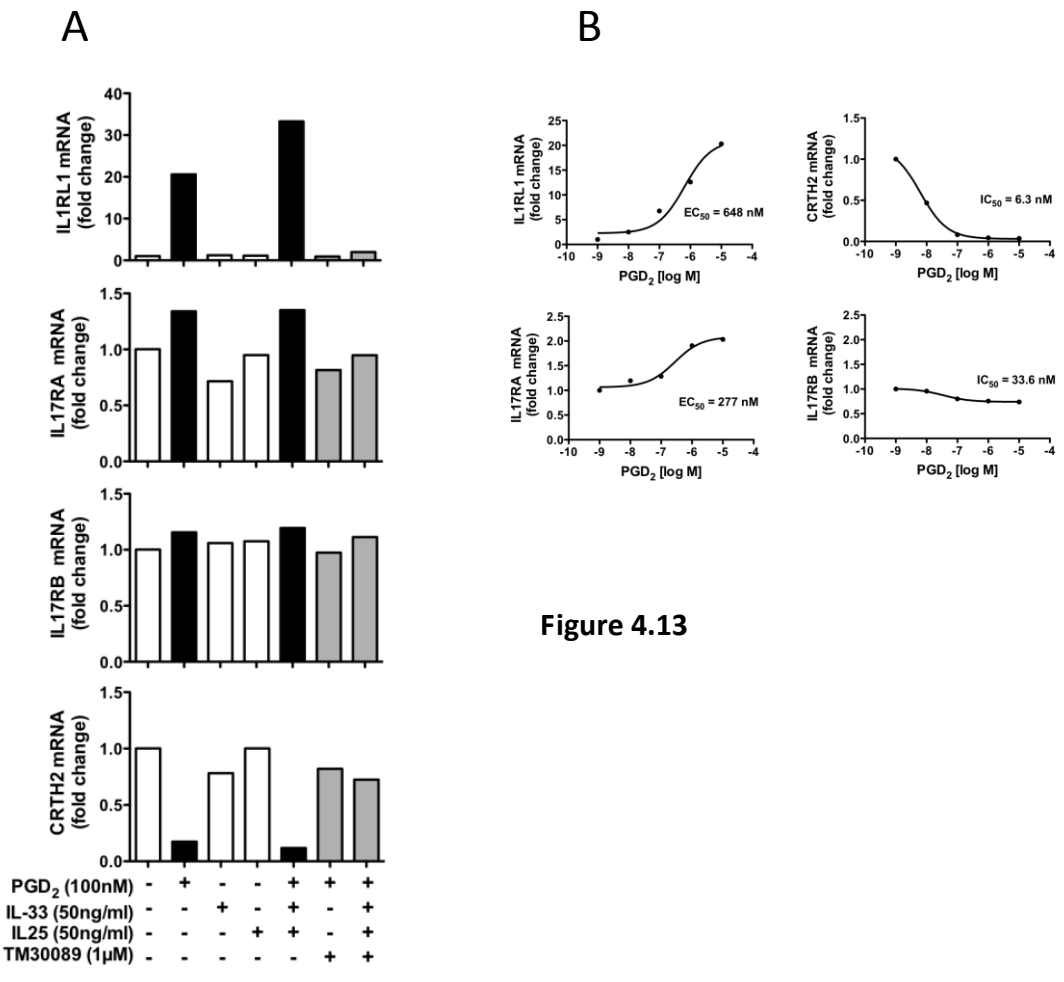


Figure 4.13

Figure 4.13 Activation of CRTH2 up-regulates the expression of IL-33 receptor and down-regulates the expression of CRTH2 in ILC2 cells. (A) The cells were treated with or without PGD2 (100 nM), IL-33 (50 ng/ml), IL-25 (50 ng/ml) alone or their combination in the presence or absence of TM30089 (1 μM) , (B) or with increasing concentration of PGD2 for 2.5 h. The mRNA level of receptor genes (IL1RL1, IL17RA, IL17RB and CRTH2) in the cell pellets was measured by q-PCR. The mRNA levels in the untreated cells were treated as 1 fold. Data are representatives of 3 independent experiments

To verify the regulation of these receptors at a protein level, the expression of ST2 and CRTH2 on the cell surface of ILC2 cells was analysed by flow cytometry after treatment with PGD2 (150 nM) in the presence or absence of TM30089 (1 μM). A greater than 10% increase in ST2 positive cells (from 14.3% to 25.2%) was detected after 4 h treatment with PGD2, and this increase was inhibited by TM30089 (Figure 4.14). Decreased expression of CRTH2 was detected after 6 h treatment with PGD2, and the blockade of CRTH2 activity with TM30089 reversed this down-regulation (Figure 4.15).

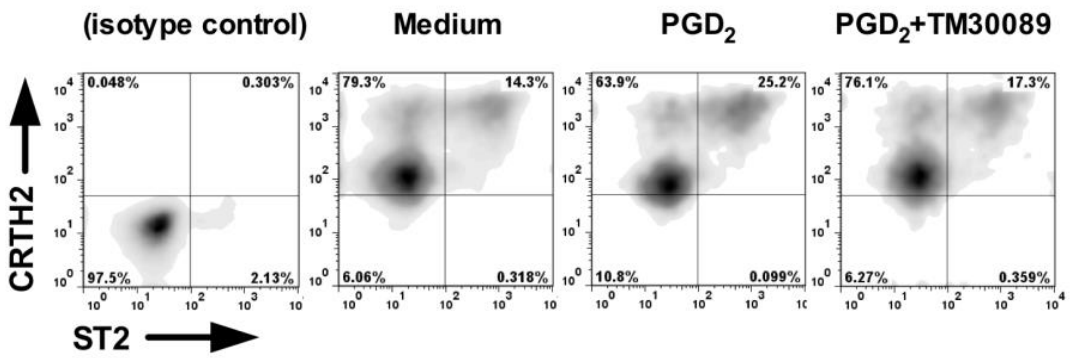


Figure 4.14 ILC2 cells were incubated with medium or PGD2 (150 nM) in the presence or absence of TM30089 compound (1 µM) for 4 h. The expression of IL1RL1 (ST2) was determined with flow cytometry analysis

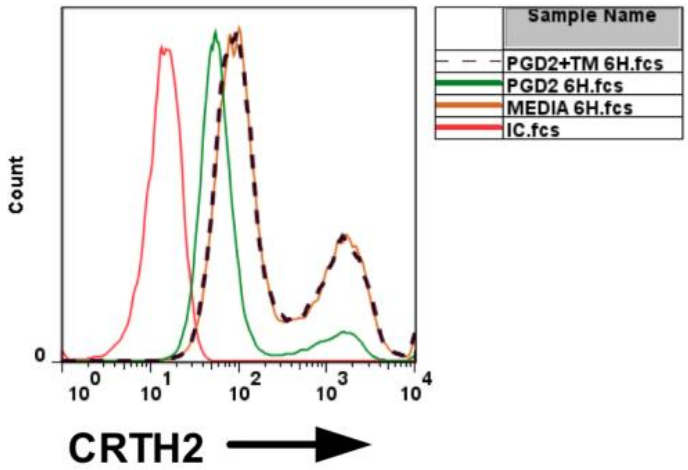


Figure 4.15 ILC2 cells were incubated with medium or PGD2 (150 nM) in the presence or absence of TM30089 compound (1 µM) for 6 h. The expression of CRTH2 was determined by flow cytometry

4.3.5 CRTH2 mediated triggering of ILC2 cells by endogenous PGD2 from human mast cells

Mast cells are the major source of PGD2 during allergic responses [299, 333]. To confirm the activation of CRTH2 in ILC2 cells under physiological conditions, the impact of endogenously synthesized PGD2 from activated human mast cells on human ILC2 cells were examined. Only low levels of PGD2 (<0.1 ng/2x10^6 cell/ml) were detectable in the supernatant from resting mast cells. After 1 h activation with IgE followed by anti-IgE antibody, mast cell cultures produced high levels of PGD2 (>11 ng/2x10^6 cell/ml). Co-treatment of IgE/anti-IgE–activated mast cells with diclofenac (10 µM), an inhibitor of COX2, during the period of anti-IgE stimulation abolished PGD2 production (<0.2 ng/2x10^6 cell/ml) (Figure 4.16). Only very low levels of IL-13 (<200 pg/2x10^6 cell/ml) could be detected in any of these mast cell supernatants. The supernatants of these mast cell treatments were used to test the effects of endogenous PGD2 in human ILC2 cells.

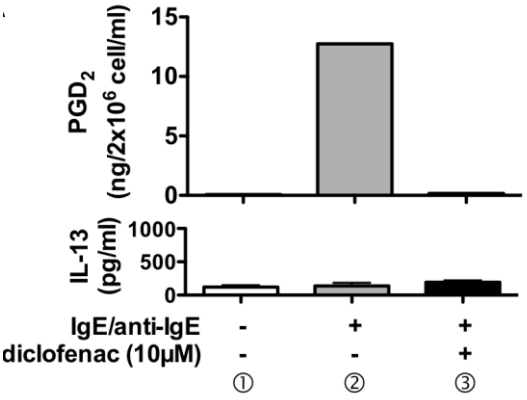


Figure 4.16 Human mast cells were treated with medium (white) or IgE and anti-IgE antibody in the presence (black) or absence (gray) of 10 µM diclofenac for 1 h, and the supernatants were collected and assigned as mast cell supernatants ① to ③.

As expected, the capacities of the supernatants to activate ILC2 cells were dependent on the levels of PGD2 contained in the supernatants. The supernatant containing high levels of PGD2 (supernatant 2) but not the supernatant derived from the resting mast cells (supernatant 1) induced strong cell migration (Figure 4.17).

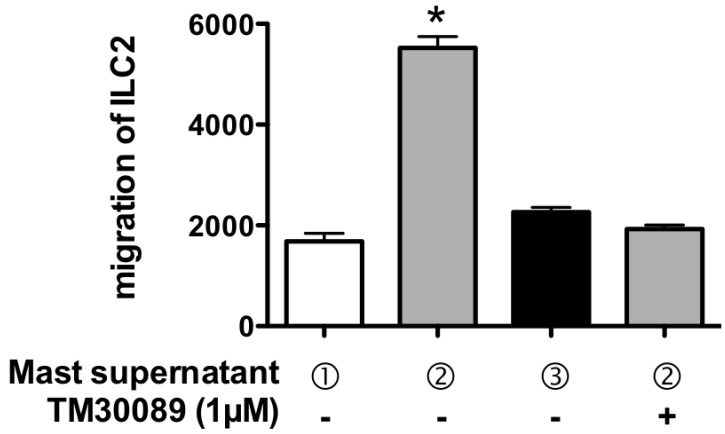


Figure 4.17 ILC2 cells were exposed to a gradient against different mast cell supernatants in the presence or absence of 1 μM TM30089. The number of cell migration was measured with chemotaxis assay

Treatment of ILC2 cells with supernatant 2 also caused the production of type 2 cytokines (Figure 4.18). Blockade of PGD2 synthesis with diclofenac (supernatant 3) removed most of the capacity to produce type 2 cytokines by ILC2 cells (Figure 4.18). These ILC2 responses to supernatant 2 were blocked by TM30089 (Figure 4.18).

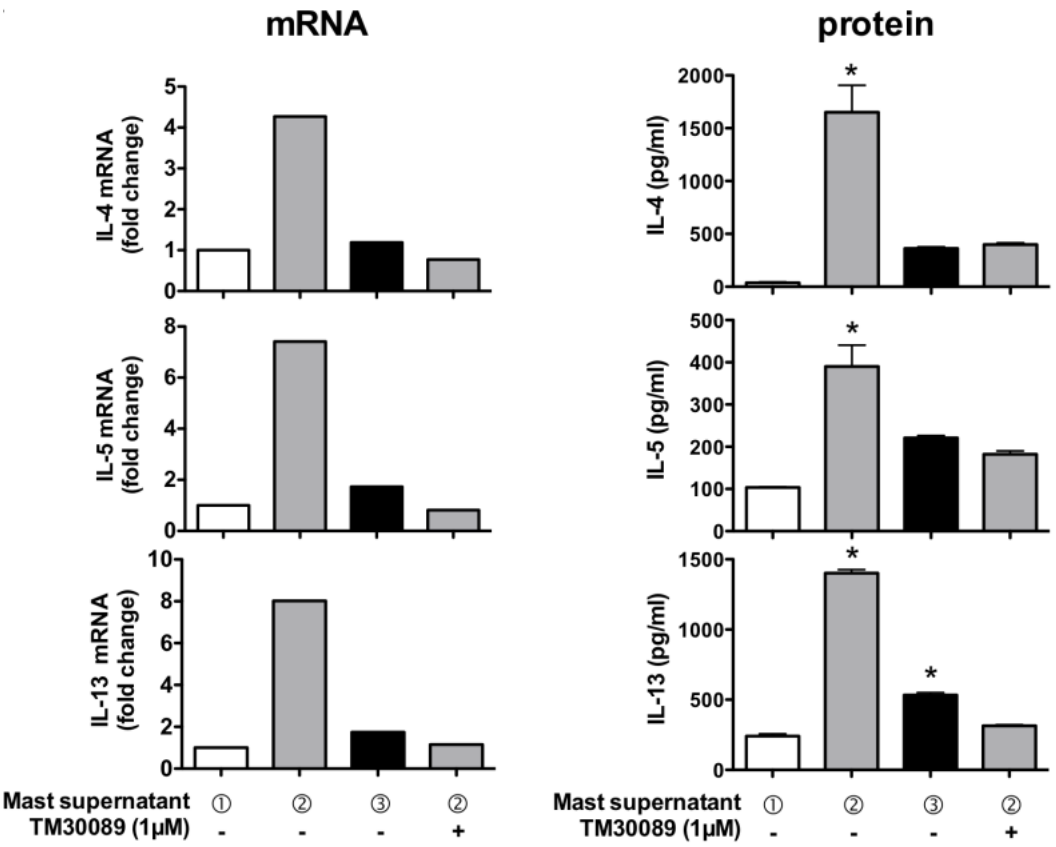


Figure 4.18 ILC2 cells were incubated with 1:1.5 diluted mast cell supernatants in the presence or absence of TM30089 (1 μM) for 3 h. Total RNA was extracted from the cell pellets, and the mRNA level of type 2 cytokines (IL-4, IL-5 and IL-13) was measured with q-PCR. Concentrations of type 2 cytokines were determined by Luminex assay.

Treatment of ILC2 cells with supernatant 2 increased production of other pro-inflammatory cytokines (IL-3, IL-8, IL-9, IL-21, GM-CSF, and CSF-1) although the effect of diclofenac on production of IL-3, IL-9 and CSF-1 was not significant (Figure 4.19).

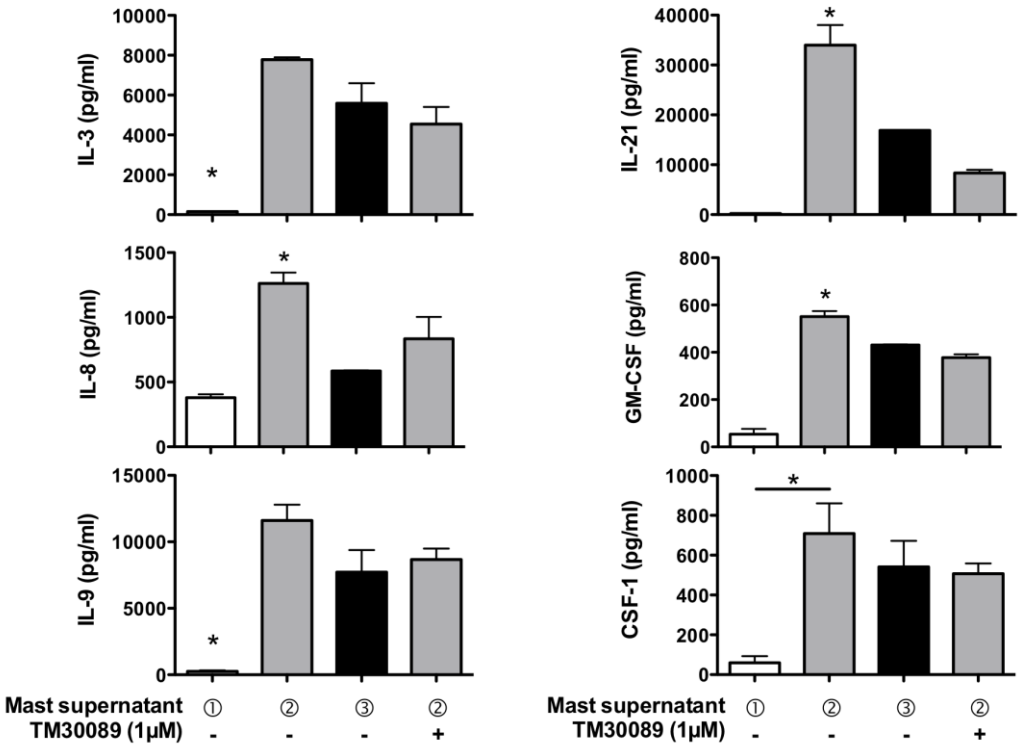


Figure 4.19 ILC2 cells were incubated with 1:1.5 diluted mast cell supernatants in the presence or absence of TM30089 (1 μM) for 3 h. Concentrations of IL-3, IL-8, IL-9, IL-21, GM-CSF and CSF-1 cytokines were determined by Luminex assay.

Similar to the results from experiments using exogenous PGD2, the supernatant from activated mast cells up-regulated the mRNA of ST2 significantly, IL17RA weakly, and down-regulated the mRNA of CRTH2 in ILC2 cells (Figure 4.20). These effects were also inhibited by TM30089.

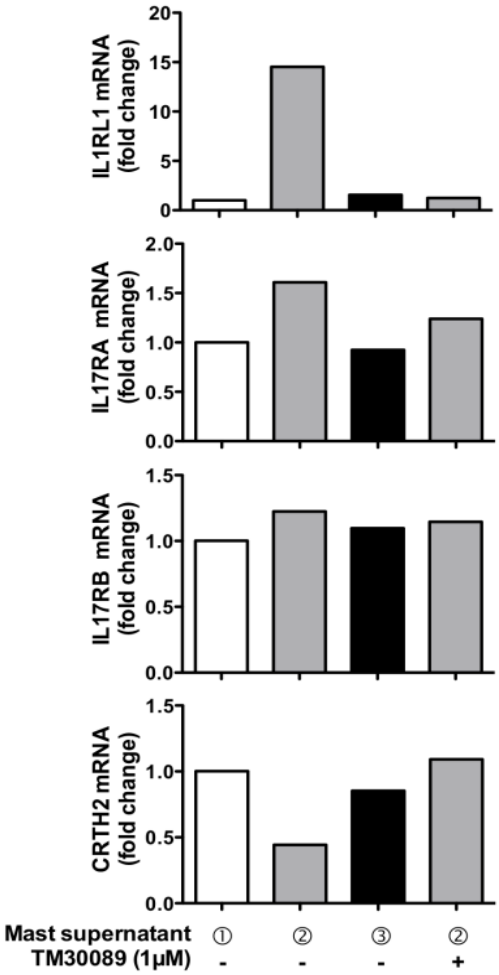


Figure 4.20 ILC2 cells were incubated with 1:1.5 diluted mast cell supernatants in the presence or absence of TM30089 (1 μM) for 3 h. The mRNA level of receptors (IL1RL1, IL17RA, IL17RB and CRTH2) was measured by Quantigene assay.

4.4 Discussion

Activation of group 2 ILC cells leads to the production of classical type 2 cytokines IL-4, IL-5 and IL-13, thus promoting type 2 immunity. Increased numbers of ILC2 cells have been observed in inflamed tissues, such as the allergic lung in mice [175, 191] and nasal polyps in humans [173] and human skin [197]. It has been revealed recently that CRTH2, a receptor for PGD2, is expressed in human ILC2s [173]. In this chapter the effect of PGD2 on ILC2 was studied. It elicits many strong pro-inflammatory responses in ex-vivo isolated ILC2 cells from human skin and peripheral blood. PGD2 induced migration of the cells, promoted production of type 2 cytokines (IL-4, IL-5, and IL-13) and many other pro-inflammatory cytokines (IL-3, IL-8, IL-9, IL-21, GM-CSF, and CSF-1). The stimulatory effect of PGD2 in ILC2s was mediated by CRTH2 as it was inhibited completely by a specific CRTH2 antagonist TM30089 [321]. These pro-inflammatory roles of CRTH2 in ILC2 cells could be confirmed under pathophysiological conditions by utilising endogenous synthesized PGD2 from human mast cells activated with IgE and anti-IgE antibody. Our findings therefore reveal a novel and potent mechanism for ILC2 activation in type 2 immunity.

A number of studies have recently identified the epithelium-derived cytokines, IL-25 and IL-33, as critical activators of ILC2-mediated innate immunity against parasite infection and responses to allergen challenge [121, 334, 335]. Lack of IL-25 and IL-33 signalling delays the onset of type-2 responses mediated by ILC2 cells in mouse models [158, 336-338]. In our studies of human ILC2 cells, administration of IL-33

initiated cell migration and type 2 cytokine productions. IL-25 also induced cytokine production although the effect on chemotaxis was marginal. However, the efficacy of IL-25 and IL33 on ILC2 cell activation was much weaker than that of PGD2 in short incubation periods suggesting that PGD2 could be another important activator of ILC2 cells. Combination treatment with PGD2, IL-33 and IL-25 enhanced the cytokine production of ILC2 cells, although no synergistic effect was detected in chemotaxis assays. Interestingly, activation of CRTH2 strongly upregulated the expression of IL-33 receptor ST2, and moderately up-regulated the IL-25 receptor IL-17A in ILC2 cells. Therefore IL-25, IL-33 and PGD2 could act in concert in ILC2-mediated immune responses.

ILC2 are enriched at the site of inflammation after parasitic infection or allergic challenge [173-175, 191], but the mechanism involved in their recruitment of the cells remains obscure. IL-33 caused ILC2 migration in a dose-dependent manner, but the efficacy of IL-33 was much weaker than that of PGD2. The migration of ILC2 toward PGD2 was completely inhibited by a CRTH2 antagonist implying that CRTH2 is an important chemoattractant receptor in human ILC2. Both IL-25 and IL-33 did not potentiate the migration of ILC2 in response to PGD2, suggesting that, if the three activators co-existed in inflamed tissue, PGD2 could serve as a dominant contributor to the recruitment cascade of ILC2s. It has been well established that activation of group 2 ILC population is characterized by the production of high levels of type 2 cytokines including IL-4, IL-5, IL-9 and IL-13 that in turn affect antibody class switching, recruitment of inflammatory effector cells such as eosinophils, basophils and mast cells, and goblet cell hyperplasia leading to mucus production, all of which

constitute the immune responses to parasite infection, allergen challenge and forms of tissue damage [332, 339, 340]. Furthermore, ILC2 are capable of producing many other pro-inflammatory cytokines after activation, including IL-3, IL-8, IL-21, GM-CSF and CSF-1. These cytokines could also play important roles in orchestrating ILC2 mediated immune responses. IL-3 can be critical for the growth and differentiation of $CD34^+$ progenitor cells into basophils and mast cells, and monocytes into dendritic cells [341, 342]. IL-8 is a potent chemokine for neutrophils [343, 344], a cell type that is associated with severe asthma [345, 346]. IL-21 can induce inflammation in mice through regulation of recruitment of neutrophil and monocyte populations [347], and has been also found to be involved in the pathogenesis of allergic disorders and autoimmune diseases (including inflammatory bowel diseases, rheumatoid arthritis, psoriasis and systemic lupus erythematosus) by controlling the growth, survival, differentiation, and function of both T and B cells [348-351]. GM-CSF and CSF-1 also contribute to allergic and autoimmune diseases [352, 353]. GM-CSF is critical for eosinophil and neutrophil survival, and enhances their activities [354, 355]. Over-expression of GM-CSF in mice enhances allergic sensitization, airway inflammation and fibrosis [356, 357]. Anti-GM-CSF antibodies administered during allergen challenge of sensitized mice inhibited airway inflammation and mucus production [358]. It is interesting that the production of IL-3 and GM-CSF are induced at same time as IL-4, IL-5, IL-9, and IL-13 since the genes for these cytokines are located at a same chromosome locus 5q31-33, a major susceptibility locus for asthma and high IgE values [359]. In contrast to the cytokines above, the activation of CRTH2 down-regulated gene transcription level of IFNγ in ILC2 cells, although the protein level was

undetectable, suggesting that CRTH2 signalling could potentially favour viral infection. In fact, an unexpected efficacy in reduction of viral infection by one CRTH2 drug has been observed in clinical trials [360]. Therefore, through activation of CRTH2, ILC2 cells might be involved in other, as yet unrecognized immune responses.

PGD2 is the major arachidonic acid metabolite released from mast cells during an allergic response [299, 333, 361]. High concentrations of PGD2 have been detected in the airways of asthmatics challenged with allergen [301]. In order to determine whether CRTH2 in ILC2s was functional under physiological conditions, the effect of endogenously synthesized PGD2 from human mast cells on ILC2s was examined. The ILC2 cell responses to mast cell supernatants were very similar to those seen in response to exogenously synthesized PGD2. The only difference was that some responses to the mast cell supernatants could not be completely blocked by the CRTH2 antagonist or by inhibition of PGD2 synthesis. This could be caused by the presence of active mediators (other than PGD2) released from activated mast cells in the supernatant, which drive production of specific cytokines. Mast cells are found mainly in epithelial barriers such as skin and mucosal tissues, including the lungs and gastrointestinal tract, and increase in number after exposure to allergens [362]. A recent paper has demonstrated that, in mouse skin, ILC2 specifically migrated to and interacted with skin-resident mast cells [174]. Therefore, ILC2 can also contribute to mast cell-mediated type 2 immunity [363]. Although multiple stored or de novo-synthesized inflammatory mediators are released from the activated mast cells (data not shown), given that ILC2 migration and type 2 cytokine production in response to mast cell supernatant can be inhibited almost completely by CRTH2 antagonism, it is

likely that PGD2/CRTH2 serves as the a dominant link between activated mast cells and the activation of ILC2. It is widely known that mast cells orchestrate adaptive type 2 immunity to helminths or allergen through IgE/FcεRI dependent activation [364]. However, mast cells can also be non-specifically activated in an IgE/FcεRI independent way by substances such as peptides, basic compounds, anaphylatoxins, dextrans and cytokines [364-366]. Many studies have revealed the critical role of PGD2/CRTH2 in adaptive type 2 immunity, particularly in mast cell mediated activation of Th2 cells and eosinophils [313, 316, 318-320]. Here we further extend their role to the activation of ILC2 cells. Type 2 cytokine production in human Th2 cells mediated by CRTH2 was markedly enhanced by another group of mast cell mediators cysteinyl leukotrienes (cysLTs) [322]. A recent report has described that type 2 ILCs in lung of mice express cysteinyl leukotriene receptor 1, which regulates type 2 cytokine production [194]. This suggests a possibility that CRTH2 and leukotriene receptors could also have synergistic interaction in mast cell mediated human ILC2 activation.

In conclusion, this chapter highlights the important pro-inflammatory role of CRTH2 and its ligand PGD2 in human group 2 ILCs, and potential role of human ILC2 cells in mast cell-mediated adaptive immune system. In addition to IL-25 and IL-33, PGD2 is clearly another important, potent driving force in the activation of ILC2 cells. It can directly stimulate ILC2s via CRTH2 in an adaptive way, and can also potentiate IL-25/IL-33-mediated innate responses. Through mast cell interaction, ILC2 cells can contribute to both innate and adaptive type 2 immunity, and CRTH2 plays a pivotal role in bridging innate and adaptive pathways in ILC2 cells.

Chapter 5. Type 2 innate lymphoid cells express functional NKP30 receptor

5.1 Introduction

5.1.1 Natural cytotoxicity receptors

Natural cytotoxicity receptors (NCR) were discovered in the 1990s in the search for MHC-independent activating receptors on NK cells [367]. The NCR family in humans includes NKp46 (NCR1, CD335), NKp44 (NCR2, CD336) and NKp30 (NCR3, CD337). While mice express functional NKp46, they lack NKp44 and NKp30. Although these receptors are grouped together, they share limited amino-acid sequence and structure. NCRs in humans are crucial in protection against various tumours (carcinoma, neuroblastoma and leukemia) and viral infections (influenza, hepatitis and Ebola) [368].

Besides NK cells, NCRs can be induced on a subset of IL-15 exposed umbilical cord blood T cells (UCB T cells) [369]. $V\delta1^{+}$ peripheral blood T cells up-regulate NCR expression in the presence of AKT-dependent signalling provided by IL-2, IL-15 and TCR stimulation [370]. During late secretory phase of menstrual cycle exposure to progesterone induces NKp30 expression on endometrial epithelial cells [371]. Several studies have evaluated the expression of natural cytotoxicity receptors (NCRs), NKP44 (NCR2 and CD336), NKP46 (NCR1 and CD335) and NKP30 (NCR3 and CD337) on group

one and three innate lymphoid cells in human but the expression of these markers has not yet been evaluated on group two ILCs [372].

NKP30 is an activating type I immunoglobulin like transmembrane receptor found mainly on human NK cells [373]. It has one Ig-like extracellular domain that connects to the transmembrane region by a short 6-amino acid stem. It is encoded in the extremely polymorphic telomeric end of class III region (class IV) of the human MHC locus [374, 375]. The intracellular domain has no signalling motifs and charged arginine residues in the transmembrane domain can couple with both CD3ζ homodimers and CD3ζ/FcRγ heterodimers [374, 376] (Figure 5.1).

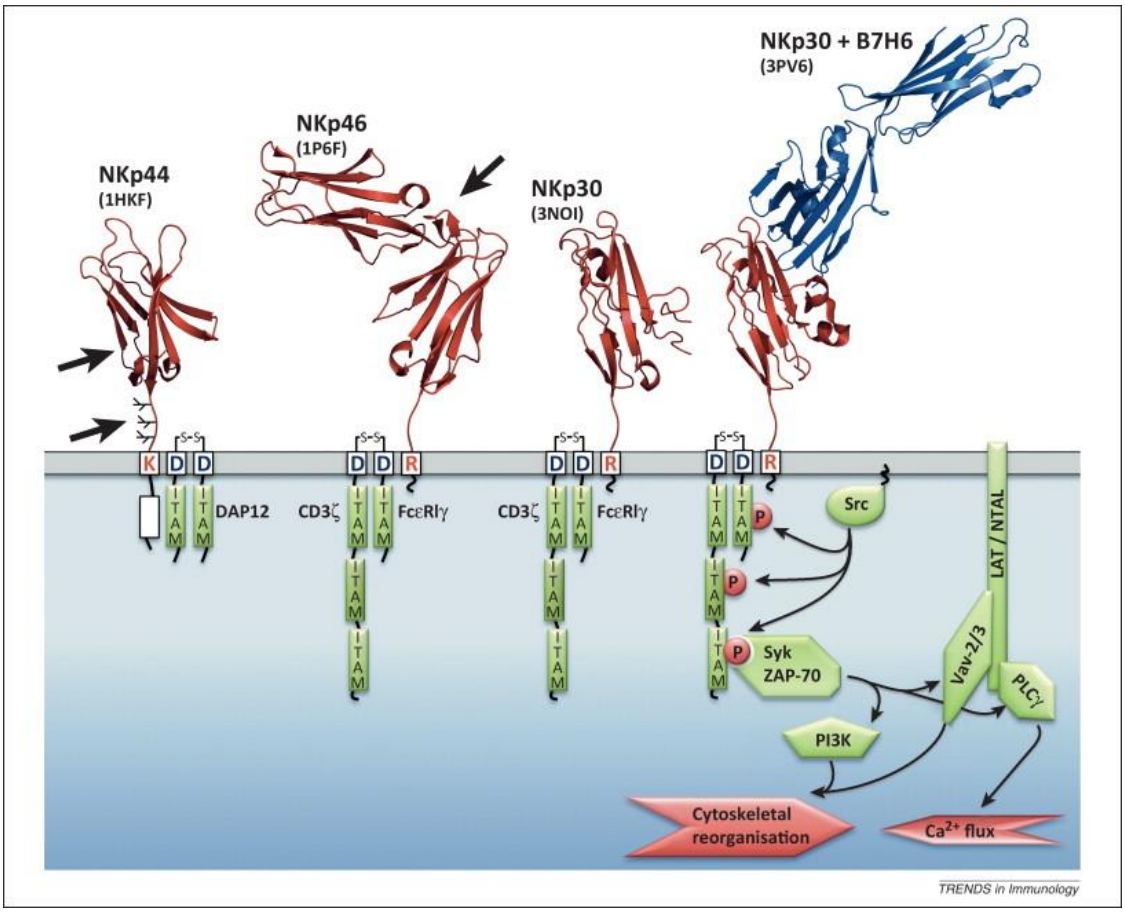


Figure 5.1 All NCRs are type I membrane proteins comprised of an ectodomain with one (NKp30 and NKp44) or two (NKp46) immunoglobulin-like domains connected to a transmembrane-spanning α-helix via a short stalk domain. For signalling, the NCRs associate with adaptor proteins via an opposing charge contact within the corresponding transmembrane segments. The intracellular immunoreceptor tyrosine based activation motifs (ITAMs) of the adaptor molecules are shown as boxes. Reference [377]

Alternative splicing of exon 4 gives rise to six distinct isoforms. Three isoforms (a-c) have extracellular V-type Ig domain while isoforms (d-f) encode C-type domains (Figure 5.2). V-type expressing variants are more prevalent than C-type Ig expressing

isoforms. Isoforms NKP30a, NKP30b and NKP30c have 36, 12 and 25 amino acid residues, respectively. Upon interaction with respective ligands, isoforms a and b convey cytotoxicity responses, release of IFN-ɣ and TNF-α in NK cells and trigger dendritic cell maturation [375, 378, 379] whereas splice variant NKP30c reduces IFN-ɣ production, cytotoxicity and increases the production of anti-inflammatory cytokine IL-10 [375]. These differences are due to the fact that crosslinking of NKp30 receptor results in reduced association of intracellular domain with CD3ζ [380].

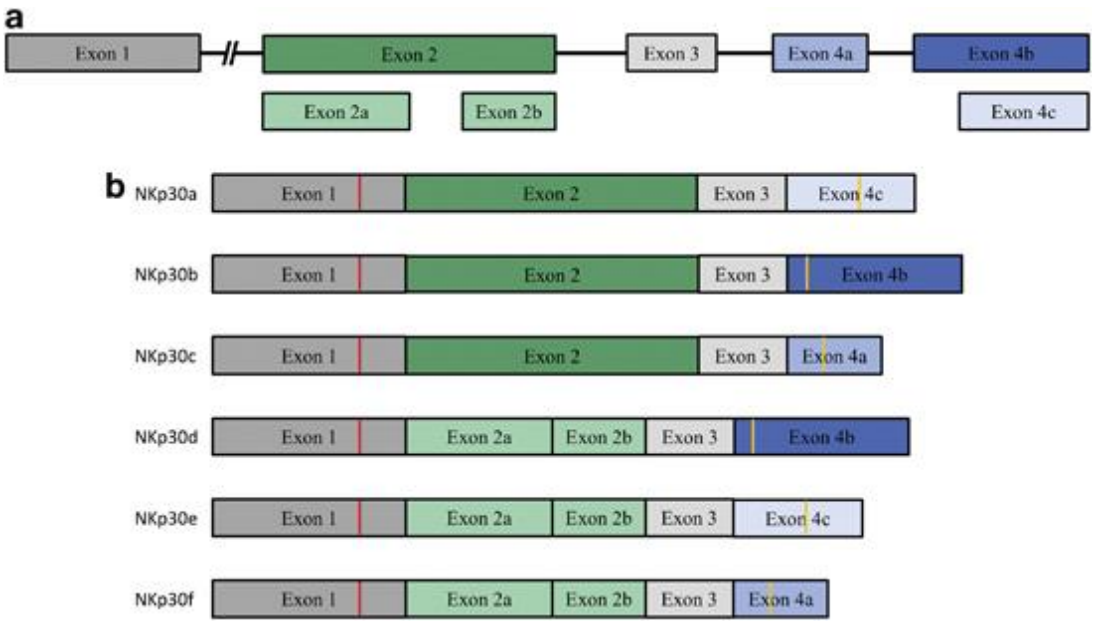


Figure 5.2 NKp30 splice variants. (a) Exonic organization of the different Exons ofNKp30 gene. (b) Exon 1 and 3 are present in all splice variants. NKp30a–c are coded by exon 2 resulting in a V-type Ig domain whereas NKp30d–f are coded by exons 2a and 2b resulting in a C-type domain. NKp30a and NKpp30e are using exon 4c, NKp30b and NKp30d are using exon 4b and NKp30c and NKp30f are using exon 4a. The start codons are indicated by a red bar and the stop codons are indicated by an orange bar.

5.1.2 NKp30 ligands

Several physiological, tumour and viral markers have been identified as NKP30 ligands (Figure 5.3).

5.1.2.1 Viral and parasite derived ligands

Cells infected with vaccinia virus are susceptible to NK cell mediated lysis but vaccinia virus does not induce MHC down-regulation nor cause increase expression of NKG2D ligands. Interestingly, hemagglutinin antigens of vaccinia virus and ectromelia virus can activate NKp30 on NK cells [381]. CMV tegument protein pp65 [382] is another ligand for NKp30. NKp30-Fc fusion protein interacts with intracellular pp65 in infected cells. Direct NKp30 pp65 interaction inhibits NK cell cytotoxicity by dissociating the intracellular domain with adaptor CD3ζ [382]. Infection with parasite *Plasmodium falciparum* induces expression of parasite derived ligands on the erythrocytes membrane. NKp30 and NKp46 can bind to erythrocytes infected with *P.falciparum*. *P. falciparum* erythrocyte membrane protein-1 consists of 2–7 Duffy binding-like domains. NKp30 specifically binds to the first Duffy binding–like (DBL)—1α domain of *Plasmodium falciparum* erythrocyte membrane protein—1 (PfEMP–1) [383].

5.1.2.2 Cellular ligands

5.1.2.2.1 HLA-B associated transcript 3 (BAT3) [384] also termed BCL2-associated athanogene 6 (BAG-6) has been shown to bind NKp30 [385]. BAT3 is involved in controlling cellular stress and DNA damage through interaction with P53 [386].

Canonical Caspase-3 mediated cleavage of C-terminal region of BAT-3 by Ricin, type II ribosomal inactivating protein, induces apoptosis [387]. BAT3 regulates apoptosis by increasing stability and reducing degradation of the cell survival protein, YWK-II/APLP2 [388]. BAT3 is expressed in the nucleus but it can be shuttled to the cytoplasm and cell surface in tumour cells and apoptotic cells [388]. Soluble BAT3 can be released from tumour cells and bind directly to NKp30 triggering cytotoxicity, release of IFN-γ, TNF-α and rejection of tumour [389]. Interestingly, the A sub-domain at the C-terminal end of BAG-6(686-936) has a role in tumour escape mechanisms and inhibits NKp30 mediated NK cell activation [385]. N-linked glycosylation at amino acid position 68 influences binding to BAT3 ligand [390].

5.1.2.2.2 B7H6 is the principle cell surface ligand of NKp30 and like other members of B7 family have 2 immunoglobulin (Ig) extracellular binding domains [391]. The crystal structure of B7H6 NKp30 interaction has been identified. The flexible stalk region of NKp30 is important in ligand binding. The front β-sheet of B7H6 binds to front and back β-sheets of NKp30. N-linked glycosylations at amino acid positions 42, 68 and 121 (N42Q/N68Q/N121Q) are essential in ligand binding and triggering signalling pathways. Positions 42 and 68 are located outside the ligand binding domain (LBD) and are important in conformational changes and shaping the LBD pockets [390].

Although B7H6 is not expressed on normal tissues in the steady state, recently it has been reported that under inflammatory conditions $CD16^+$ $CD14^+$ monocytes in patients with sepsis express B7H6 which was correlated with mortality rates. In patients with Gram negative sepsis soluble B7H6 was also detected. *In vitro*

stimulation of monocytes with proinflammatory cytokines IL-1β, TNF-α and TLR ligands upregulated B7H6 expression and produced soluble B7H6 [392]. Moreover, in Primary Sjögren's syndrome (pSS) there is a higher frequency of NKp30 expressing NK cells which is correlated with NKp30 dependent IFN-γ production levels. Salivary gland epithelial cells can express B7H6 and infiltration of NK cells in these glands associated with severity of exocrinopathy [393].

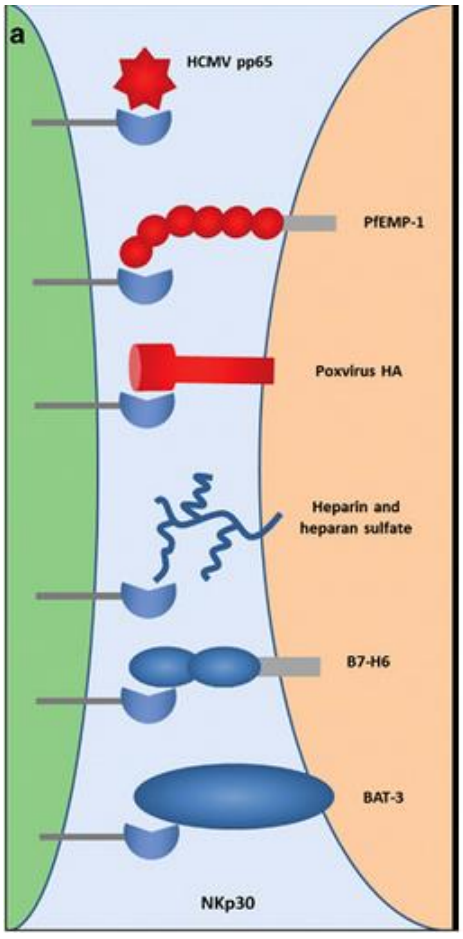


Figure 5.3 NKp30 interactions with its known ligands. Cellular ligands are shown in blue and pathogen-derived ligands are shown in red. Reference [380].

5.2 Results

5.2.1Type 2 innate lymphoid cells express natural cytotoxicity receptor, NKP30

Innate lymphoid cell family members do not possess an antigen-specific receptor and are thought to rely on a combination of activating and inhibitory signals for their effector functions, but the mechanisms are unclear [394]. NK cells are the prototype of this family and one of the earliest and best characterized populations in this group. Major stimulating receptors in human NK cells are NKG2D, NKp46, NKp44, NKp30 and inhibitory receptors belong to the KIR family receptors. In chapter 3, I have shown that human type 2 innate lymphoid cells do not express KIR2DL1/S1, KIR3DL1/S1, and KIR2DL2/S2/L3/S4, the KLR family receptors, NKG2A and NKG2C but they rely on cytokine and lipid receptors ST-2, IL-17RB, TSLP and CRTH2 and can be activated by IL-33, IL-25, TSLPR and PGD2 respectively [198, 280]. This effect can also be inhibited by interaction of KLRG1 with its ubiquitously expressed ligand E-Cadherin on epithelial cells [198]. The KLRG1-Ecadherin interaction is believed to be one of the mechanisms of barrier sensing by ILC2. Novel activating natural cytotoxicity receptor 3 (NCR3, NKp30) was first thought to be specific to the NK cell population but was later reported on the group three ILC family. To further investigate recognition strategies that ILC2 employ to sense the micro environment, the expression of NKp30 on type 2 innate lymphoid cells was examined and first cultured ILC2 lines were stained for NKp30 (NCR3) which showed high level of NKp30 expression (Figure 5.4).

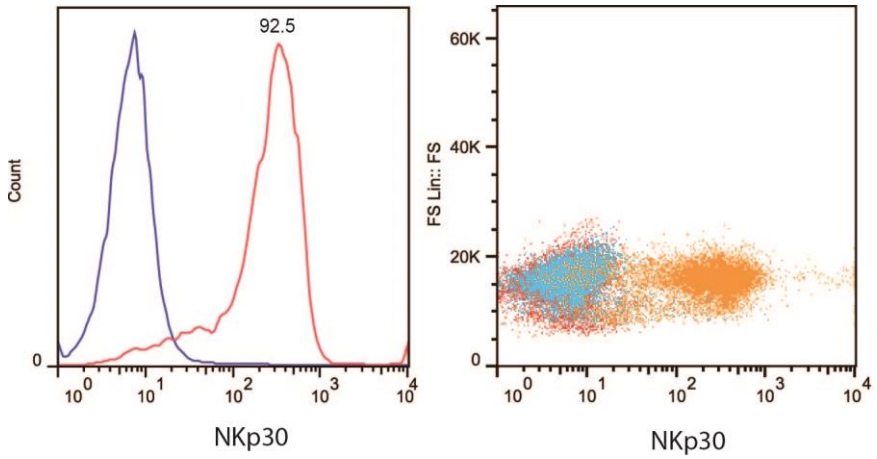


Figure 5.4 Cultured ILC2 express natural cytotoxicity receptor, NKP30. Representative FACS plot of NKp30 expression (red line) on cultured ILC2 compared to isotype control staining (blue line) (n=5).

However, IL-2 is known to induce up-regulation of NKp30 in NK cells and its presence in the culture media potentially induced up-regulation of NKp30 by ILC2 [395, 396]. Therefore to evaluate the expression of this receptor ex-vivo lineage negative (CD3, CD4, CD8, CD14, CD19, CD56, IL3R, FcεRI, CD11b, CD11c) cells that express CD45, CD127 (IL-7Rα), CRTH2 from peripheral blood of healthy donors were selected. Approximately 17±1.53% of human ILC2 express NKp30 on their surface *ex-vivo* (n=4) (Figure 5.5).

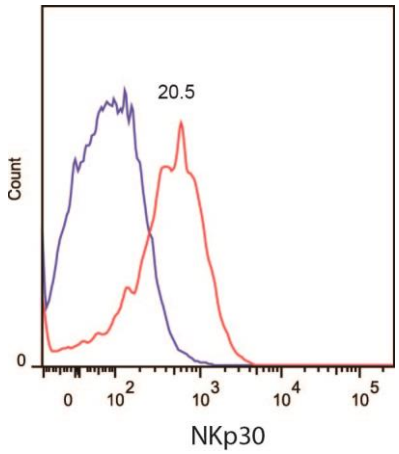


Figure 5.5: Freshly isolated ILC2 express natural cytotoxicity receptor, NKP30. NKp30 expression (red line) on surface of freshly isolated ILC2 (n=4) was measured by flow cytometry (blue line: unstained control). ILC2 were gated as lineage negative (CD3, CD4, CD8, CD14, CD19, CD56, CD11b, CD11c, IL-3R, FcεRI), $CD45^{+}$, IL-$7R\alpha^{+}$, $CRTH2^{+}$.

5.2.2 ILC2 express isoform c of NKp30

NKp30 protein is encoded by genes located in the highly polymorphic telomeric end of the class III region. This section is now designated class IV as it encodes proteins mainly involved in immune and inflammatory responses such as TNF (tumour necrosis factor) family cytokines, AIF1 (Allograft inflammatory factor 1), lymphotoxin-α (LTα), LTβ, BAT-1 (D6S81E), LST1 (leucocyte-specific transcript 1) and HSP70 (heat shock protein 70) [397]. NKp30 encodes 6 different alternatively spliced variants depending on the parts of the exon 4 they utilize. Isoforms a, b and c use exon 4III, 4II and 4I respectively and form V-type immunoglobulin extracellular domains, whereas isoforms d, e and f create a C-type Ig domain [397, 398]. Splice variants a, b and c are highly expressed in tissues and comprise 36, 12 and 25 amino acid residues, respectively. Each isoform has distinct cytoplasmic signalling structure and elicits different signals. To investigate the function of NKp30 isoforms Delahaye et al transfected NKL cell lines with different splice variants [375]. Upon cross linking, NKp30a and b isoforms showed immune-stimulatory function and produced large amounts of IFN-γ and TNF-α while NKp30c transfected cells released significant amount of the immune suppressive cytokine, IL-10. Moreover, NKp30c engagement associated with reduced production of IFN-γ and TNF-α by NK cells. NKp30a expressing cells mediated strong cytotoxicity responses and expression of CD107a on the cell surface upon release of granules [375].

To determine the expression of NKp30 isoforms on type 2 innate lymphoid cells, quantitative RT-PCR was performed using NKp30a, b and c isoform specific primers.

The data were normalized to the ubiquitously expressed GAPDH housekeeping gene. ILC2 mainly expressed isoform c of NKp30 protein (Figure 5.6).

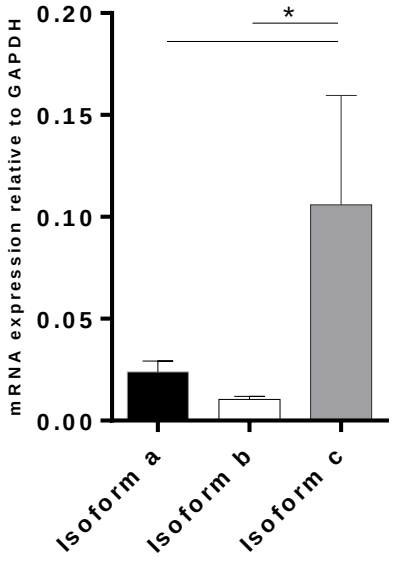


Figure 5.6 Expression of different isoforms of NKp30 on type 2 innate lymphoid cells were measured by quantitative RT-PCR. mRNA levels were normalized to the house keeping gene GAPDH (n=9).

5.2.3 Plate bound B7H6 and cell lines expressing B7H6 induce production of type 2 cytokines by ILC2

Multiple pathogen-derived and self-ligands have been identified to bind the NKp30 receptor. Vaccinia virus HA glycoprotein and a component of HCMV tegument protein, pp65 are pathogen derived ligands that inhibit NK cell effector functions by dissociating NKp30 from CD3ζ adaptor protein required for downstream signalling [382]. The Duffy binding-like 1a domain of the Plasmodium falciparum erythrocyte binding protein is an activating pathogen derived NKp30 ligand that induce cytotoxicity and IFN-γ, TNFα production [374, 378, 398, 399]. Among NKp30 self-ligands is nuclear factor Human Leukocyte Antigen-B-Associated Transcript 3 (BAT3). BAT3 is an intracellular ligand that interacts with P53 and induces apoptosis after DNA damage or endoplasmic reticulum stress. In response to heat shock BAT3 is expressed on the cell surface and becomes accessible to NKp30 [384, 400]. It can also be secreted by iDC [400]. Interestingly BAT3 binding does not affect simultaneous pp65 recognition which confirms the presence of several epitopes on NKp30 [378]. B7H6 is another self-ligand that is expressed on the surface of tumour cell lines. It belongs to the B7 family of receptors that encode ligands for CD28 and CTLA-4 (B7-1 and B7-2), but unlike other members of the B7 family it is selective for NKp30 and does not bind other CD28 or NCR receptors. It is composed of 2 immunoglobulin extracellular domains (a distal V like and a proximal C like domains) with an adjacent phase 1 intron. Both the front and back β sheets of NKp30 binds to V-like domain of B7H6 via hydrophobic interactions [374, 401]. To investigate whether the NKp30 receptor expressed on ILC2 mediates effector functions, ILC2s were activated using

recombinant human B7H6 Fc chimera protein. Cross linking NKp30 receptor on ILC2 by plate bound B7H6 protein induced production of high amounts of IL-13 (Figure 5.7).

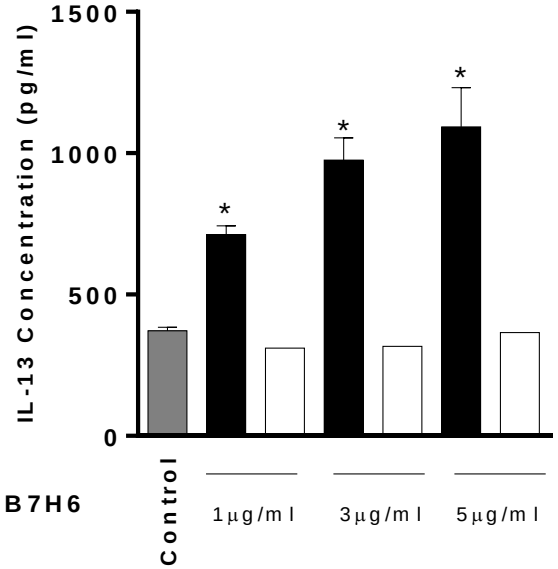


Figure 5.7 Plate bound B7H6 induce production of type 2 cytokines by ILC2. IL-13 expression by ILC2 after 24 hours of culture with increasing concentration of rhB7H6 or isotype control (n=5) - Control ILC2 (grey bar), ILC2 incubated with indicated concentrations of isotype control (white bars) or rhB7H6 protein (Black bars).

Next we screened a panel of tumour cell lines for their ability to activate type 2 innate lymphoid cells. K562 (myelogenous leukemia line), THP-1 (monocytic leukemia line) and Jurkat (T cell leukemia line) tumour cell lines induced IL-13 expression by ILC2 (Figure 5.8).

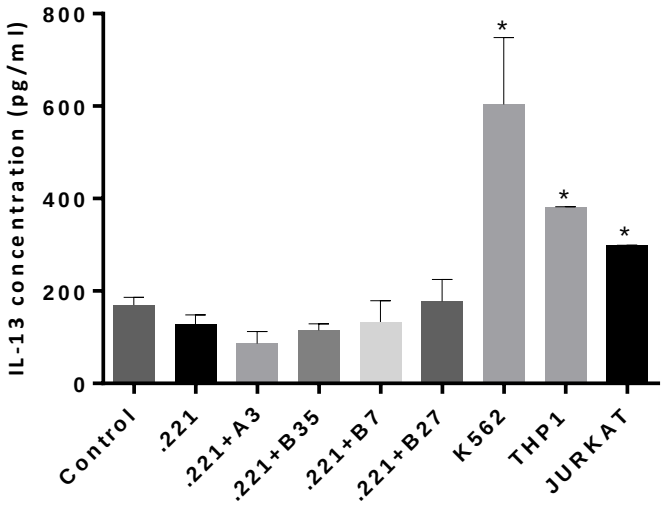


Figure 5.8 ILC2s were cultured with various tumour cell lines for 24 hours and production of IL-13 cytokine were measured by ELISA. K562, Jurkat and THP-1 cell lines triggered production of IL-13 by ILC2s

Further analysis showed that these cell lines can express B7H6 on their cell surface (Figure 5.9). There was no expression of BAT3 on the surface of tumour cell lines (Figure 5.9).

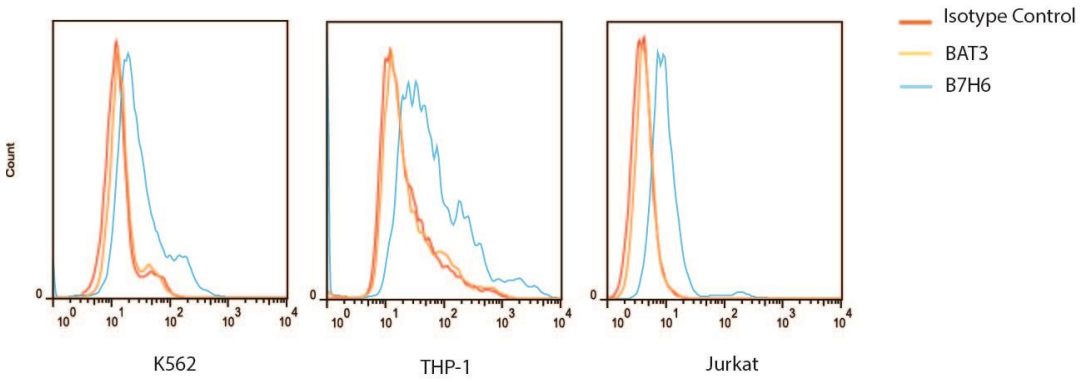


Figure 5.9 Flow cytometry analysis of B7H6 and BAT3 expression on the surface of K562, THP-1 and Jurkat tumour cell lines.

Comparing NKp30 mediated activation of ILC2 with PGD2 and IL-33 activation showed that NKp30 crosslinking is as potent as PGD2 and IL-33 in activating ILC2 cell lines (Figure 5.10).

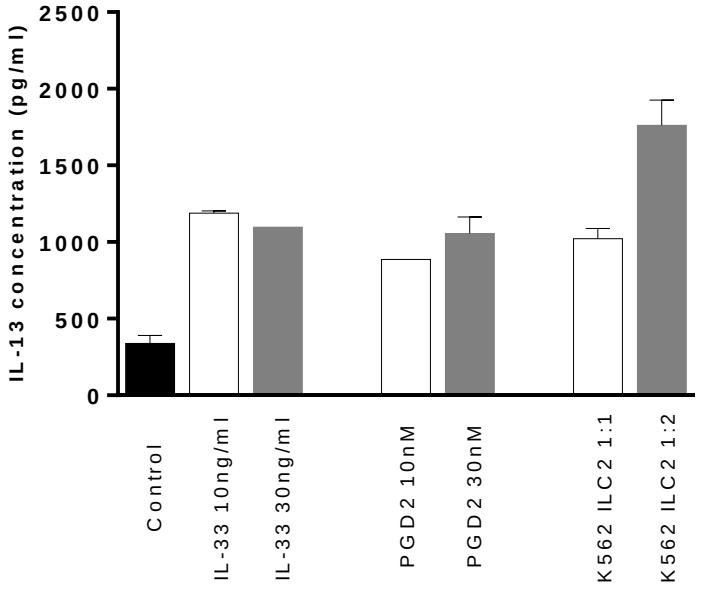


Figure 5.10 The concentration of IL-13 produced by ILC2 upon stimulation with 10 ng/ml and 30ng/ml IL-33, 10nM and 30nM PGD2 and 1:1, 2:1 ILC2: K562 for 4 hours were compared using IL-13 ELISA assay. NKp30 mediated activation of ILC2 was comparable to IL-33 and PGD2 activation.

To test whether NKp30 crosslinking can induce expression of other cytokines we used multiplex cytokine analysis. Activation of ILC2 by K562 cells can induce production of IL-2, IL-3, IL-4, IL-5, IL-13, IL-8 and GM-CSF (Figure 5.11).

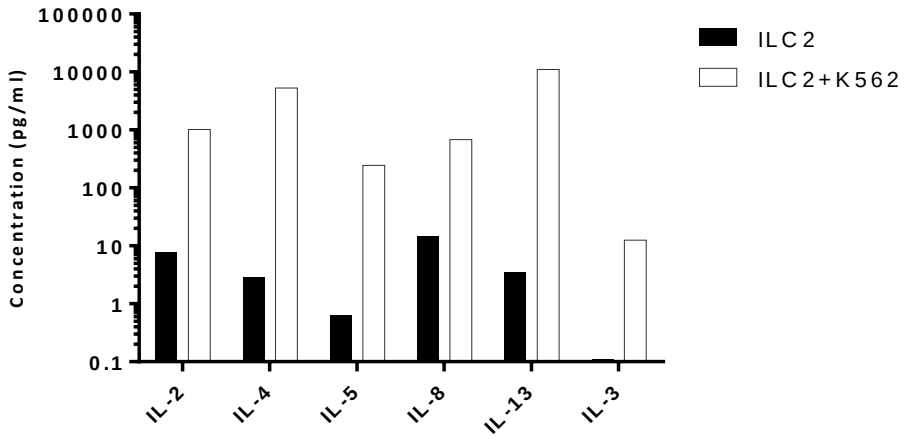


Figure 5.11 Luminex analysis of ILC2 supernatant following 4 hour incubation with K562 tumour cell lines. NKp30 mediated activation of ILC2 increased production of IL-2, IL-4, IL-5, IL-8, IL-13 and IL-3

To confirm that the ILC2 activation is mediated by NKp30 receptor, we incubated the cells with increasing concentration of NKp30 blocking antibody for 1 hour before culture with the K562 cell line. Blocking NKp30 reduced the expression of IL-2, IL-3, IL-4, IL-5, IL-13, IL-8 and GM-CSF in a dose dependent manner (Figure 5.12). NKp30 mediated activation of ILC2 is contact dependent as supernatant from cultured tumour cell lines could not activate ILC2 (Figure 5.13) and indeed 24-plex analysis of K562 supernatant did not reveal any ILC2 stimulating cytokine or chemokines (data not shown). To confirm the NKp30 mediated effector function of ILC2 *ex-vivo*, we incubated freshly isolated innate lymphoid cells from the blood of healthy donors with K562 tumour cell line and showed that K562 can activate ILC2 and induce production of IL-13 (Figure 5.14).

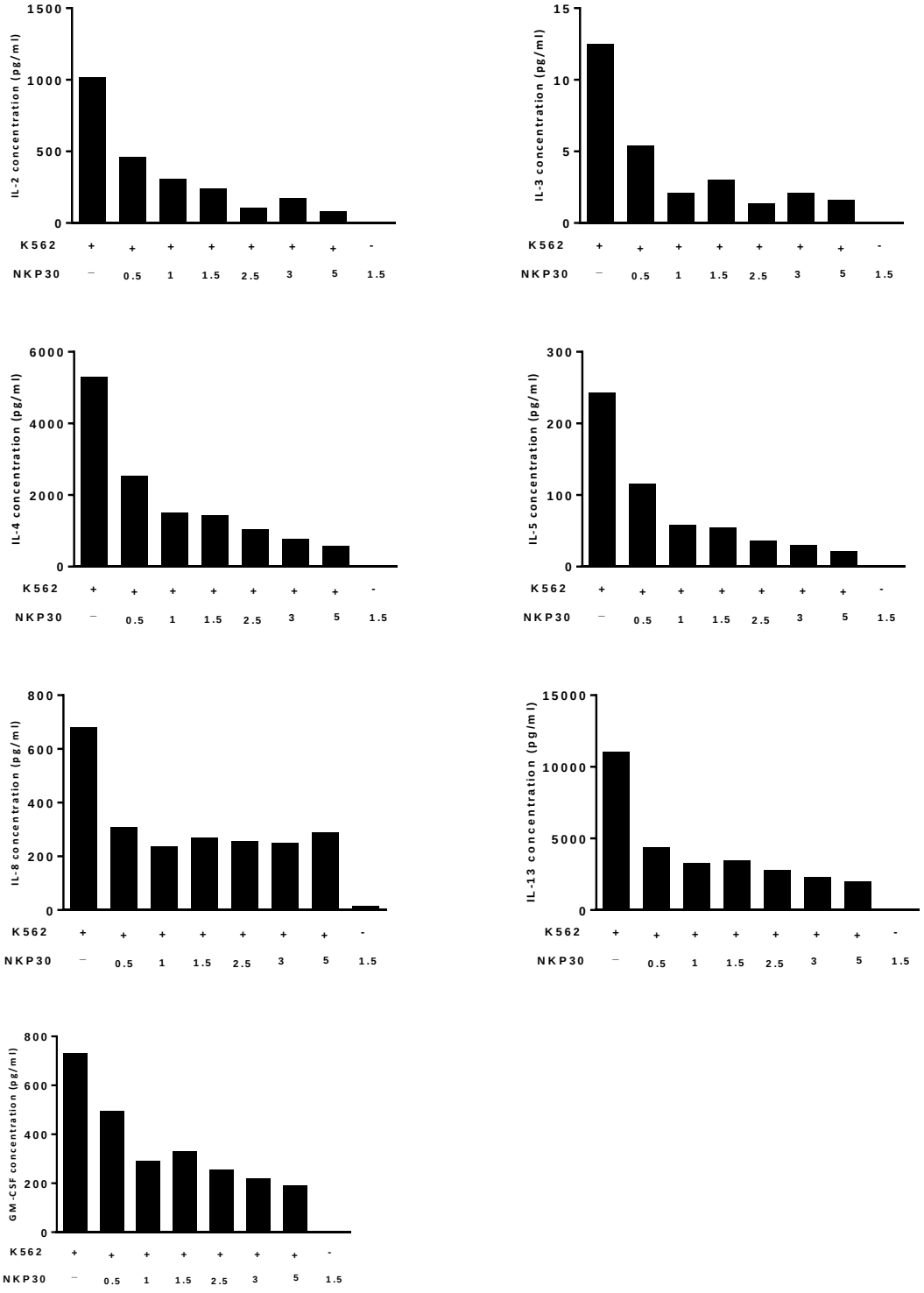


Figure 5.12 ILC2s were incubated with increasing amount of NKp30 blocking antibody before co culture with K562 cell line that reduced the production of IL-2, IL-3, IL-4, IL-5, IL-8, IL-13 and GM-CSF.

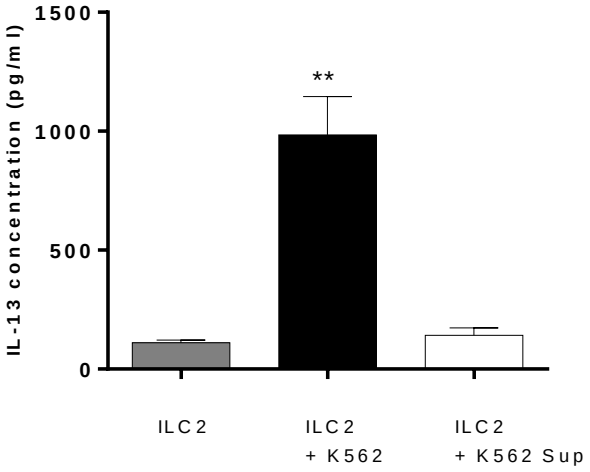


Figure 5.13 The capacity of K562 and K562 supernatant to evoke IL-13 production by ILC2s were measured by ELISA assay. K562 supernatant did not activate ILC2.

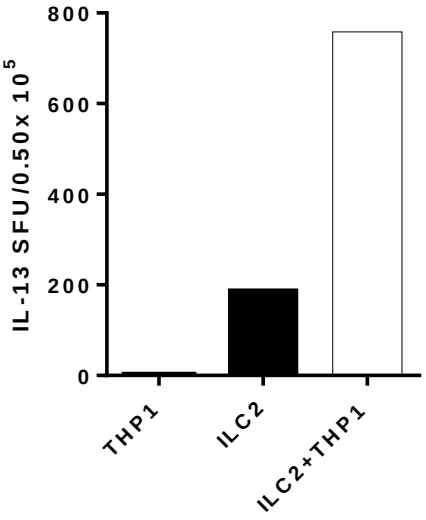


Figure 5.14 Freshly isolated ILC2 were cultured with K562 tumour cell line for 24 hours and IL-13 cytokine production was measured by ELISPOT (figure representative of 3 donors with similar result).

5.2.4 B7H6 is expressed on basal epidermis in normal tissue and suprabasal epidermis in lesional skin biopsies of atopic dermatitis patients

Several studies have reported the expression of B7H6 on tumour cell lines. To investigate whether B7H6 is also expressed in normal tissue, tissue sections of healthy skin and lesional skin biopsies of patients with atopic dermatitis were evaluated. Interestingly, we found expression of B7H6 protein in healthy skin tissue which was confined to the basal layer of epidermis. Comparing with lesional skin biopsies of adult patients with atopic dermatitis showed B7H6 expression throughout suprabasal layers of the epidermis and dermis (Figure 5.15).

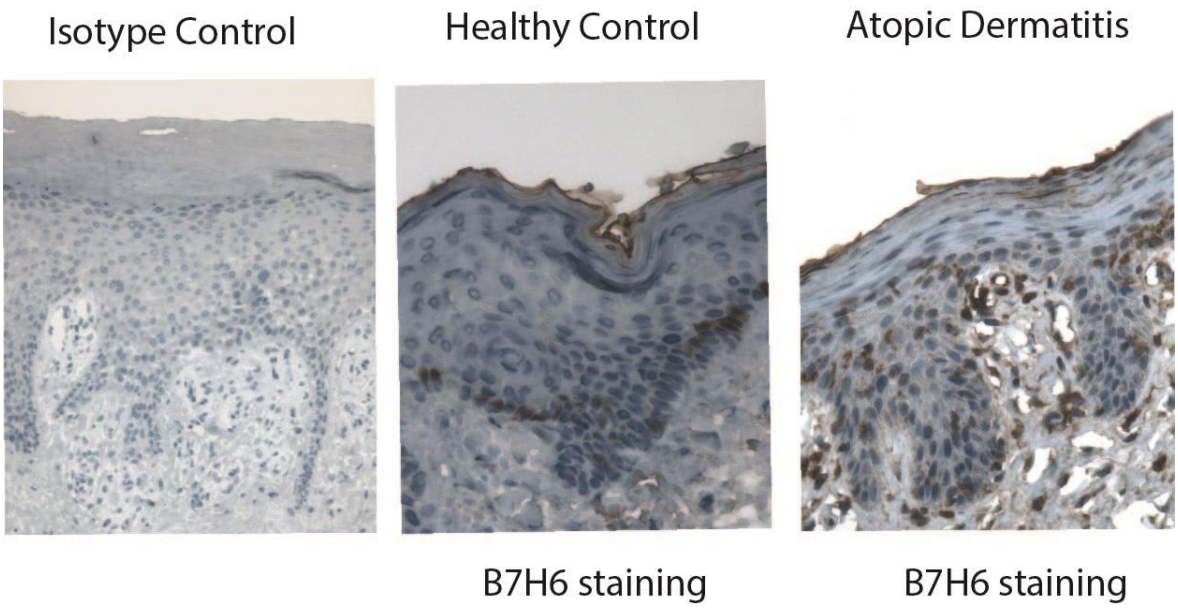


Figure 5.15 IHC analysis of NKp30 ligand, B7H6, expression in healthy control and atopic dermatitis lesions. B7H6 is expressed at basal epidermis in healthy control skin sections and throughout suprabasal epidermis in AD lesions.

It is therefore plausible to speculate that widespread expression of B7H6 in the skin of patients with atopic dermatitis can lead to NKp30-mediated activation of ILC2. This finding is compatible with our earlier observation that the ILC2 resident in the skin of patients with atopic dermatitis show an activated phenotype [198]. To further investigate our hypothesis, the immortalized human keratinocyte cell line (HaCaT) was used. Relatively low expression of B7H6 (Fig 3b) was observed in HaCaTs. B7H6 is known to be up-regulated under inflammatory conditions [402]. Therefore to mimic the inflammatory conditions of atopic lesions, we incubated HaCaT cell line with combination of IFN-γ, TNF-α, epithelial cytokines, mast cell products and type 2 cytokines. IFNγ was used at the concentration of 300 U/mL, the minimum concentration described in previous studies which used up to 1000 U/mL [403-406]. All other cytokines were used at 100ng/ml concentration [407].

Consistent with earlier reports Proinflammatory cytokines IFN-γ and TNF-α induced 5.4 and 1.4 fold upregulation of B7H6, respectively. Interestingly, type 2 cytokines IL-13 and IL-4 also significantly increased the expression of B7H6, 2 and 1.4 fold respectively, whereas epithelial cytokines and mast cell derivative PGD2 reduced cell surface expression of B7H6 on HaCaT cell lines (Figure 5.16).

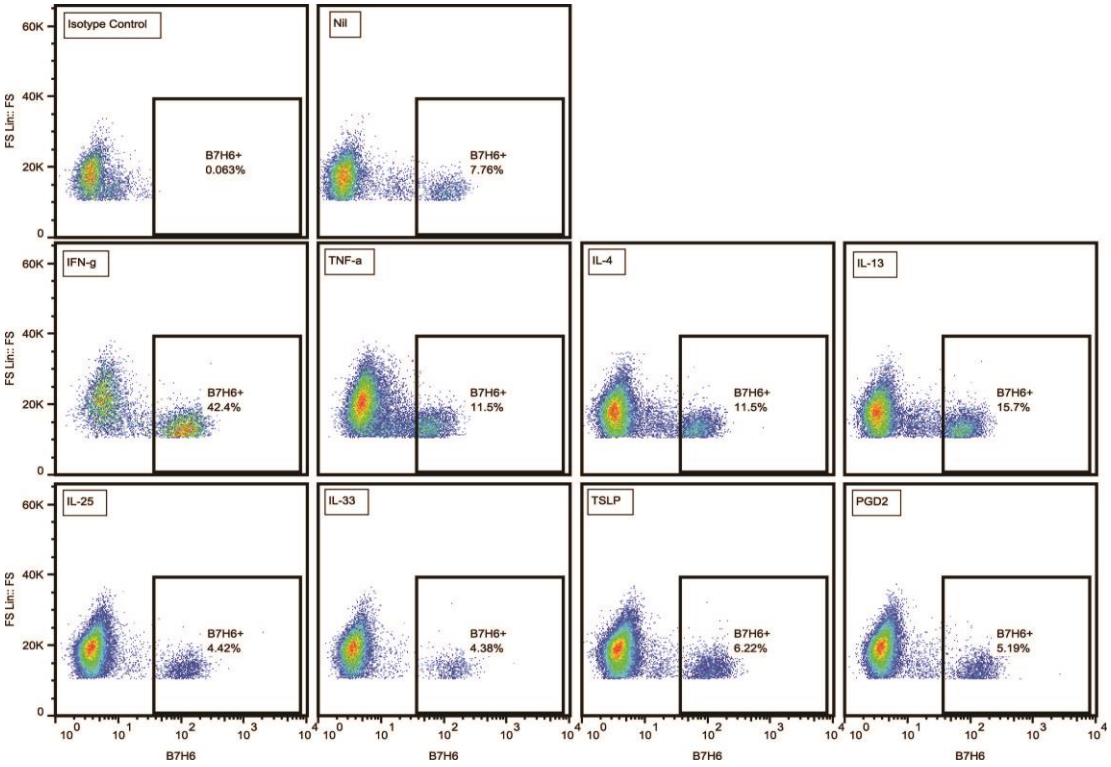


Figure 5.16 The expression of B7H6 on HaCaTs was evaluated after 72 hours of incubation with IFN-γ (300U/ml), TNF-α (100ng/ml), IL-4 (100ng/ml), IL-13 (100ng/ml), IL-25 (100ng/ml), IL-33 (100ng/ml), TSLP (100ng/ml), PGD2 (100nM) by flow cytometry and compared with isotype control staining.

5.2.5 NKp30 mediated activation of ILC2 triggers the canonical pathway of NFkB activation

All NKp30 isoform monomers have arginine charged residues in their transmembrane regions that couple with aspartate amino acids in CD3ζ homodimers or CD3ζ/FcRγ heterodimers by a salt bridge [408]. CD3ζ is an ITAM bearing adaptor molecule essential for transmitting downstream signalling [374]. Isoform b is constantly associated with CD3ζ while isoform c shows a weaker interaction. NKp30 cross linking triggers canonical pathway of nuclear factor κB (NFκB) activation. NFκB dimers are bound to the inhibitory protein IκB. IκB kinase complex (IKK) which consists of IKKα, IKKB and regulatory protein NEMO phosphorylates IκB. Phosphorylated IκB undergoes proteasomal degradation that releases NFκB to translocate to the nucleus [409].

To study the signalling pathway of NKp30 activation, we first monitored the temporal kinetics of cytokine release by ILC2. Production of IL-13 was significantly increased within 1 hour of NKp30 cross linking and peaked at 2-4 hours (Figure 5.17 representative figure of 3 experiments). FACS analysis of ILC2s incubated with B7H6 bearing cell line K562 for 4 hours confirmed increase in phosphorylation of IκB (Figure 5.18).

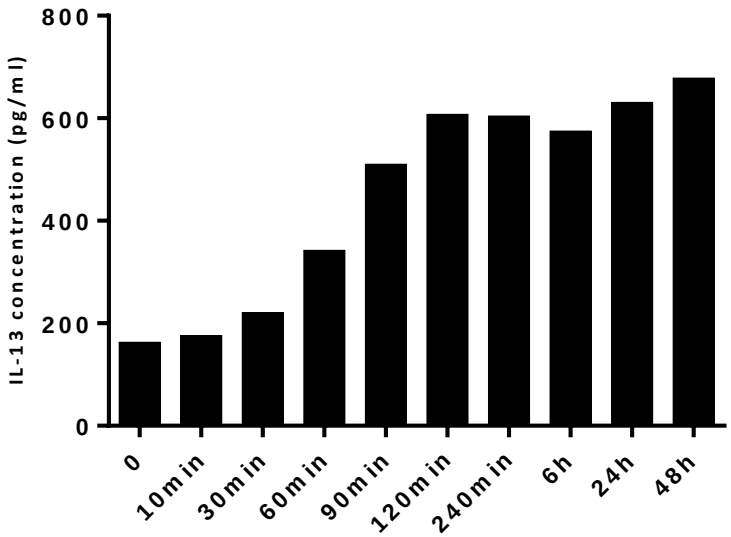


Figure 5.17 ILC2s were cultured with ligand expressing cell lines at 2:1 ratio for 10, 30, 60, 90, 120, 240 minutes, 6 hours, 24 hours and 48 hours. The expression of IL-13 was measured by ELISA (data representative of 3 experiments with similar results).

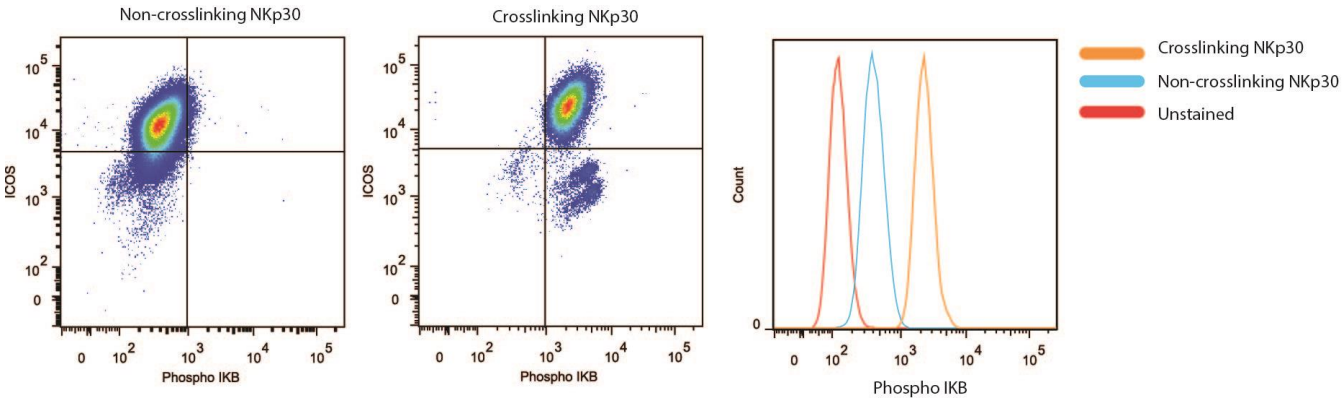


Figure 5.18 The expression of phosphorylated IKB in ILC2s was evaluated by flow cytometry after 4 hours of culture with ligand expressing cell lines ELISA (data representative of 4 similar experiments).

5.3 Discussion

In 1999 Moretta *et al.* discovered natural cytotoxicity receptors with activating properties that can trigger an immune response on recognition of cognate cellular and viral ligands and therefore play an important role in NK cell anti-tumour and anti-viral cytotoxicity [410]. A decade later the expression of these markers have been shown on a subset of cord blood and peripheral blood T cell lymphocytes in response to stimulation with IL-15 [369, 370]. Although NCRs have been detected on type 1 and type 3 innate lymphoid cells, their specific functions have not yet been fully elucidated [411, 412]. Here we identified and characterized NKp30 expression on type 2 innate lymphoid cells, a novel activating receptor that plays an important role in both freshly isolated and cultured human ILC2s thus representing an additional marker for ILC2 identification.

Alternative splicing exon 4 of NCR3 gene gives rise to 6 isoforms; of which 3 are more prevalent. Crosslinking of each receptor induces distinct signalling pathway and different pattern of cytokine production. NKp30a and NKp30b isoforms are classified as immune-stimulatory whereas NKp30c is immune-regulatory [375]. NK cell sub-populations can express all three isoforms and relative contribution of each receptor depends on the expression levels on the cell surface. Interestingly, ILC2 showed predominant expression of immune-modulatory splice variant, NKp30c. Although it remains possible that ILC2s may express isoforms a and b under different conditions. Further investigation of regulating processes is required to determine conditions for upregulation of each splice variant. Moreover, the prevalent expression of isoform c

on NK cells has been seen in patients with gastrointestinal stromal tumour (GIST) and associated with reduced survival due to defective IFN-γ, TNF-α and IL-12 production, defective NK DC dialog and increased immunosuppression and IL-10 production [375].

Studying NKp30 receptor is hampered as among natural cytotoxicity receptors mice can only express functional NKp46 - they lack the genes encoding NKp44, and NKp30 is a non-functional genomic sequence (pseudogene) [374]. Therefore there is no suitable mouse model to study the function of this molecule *in vivo*. NKp30 is widely considered to be a cytotoxic receptor on NK cells triggering tumour cell lysis and production of IFNγ and TNF-α. Interestingly NKp30 engagement on ILC2 augments the production of type 2 cytokines IL-4, IL-5, IL-13 and GM-CSF as well as other inflammatory cytokines such as IL-2, IL-3 and IL-8. As previously discussed, these cytokines have a crucial role in allergic and inflammatory conditions and genes encoding these cytokines are located at the same chromosome locus 5q31-33 [359] therefore it is credible that these cytokines produced simultaneously. Proliferation and differentiation of $CD34^+$ progenitor cells into basophils and mast cells are highly dependent on IL-3. It also regulates dendritic cell differentiation from monocytes [341, 342]. IL-8 is a potent neutrophil chemo-attractant [343, 344] and contributes to allergy and severe asthma [345, 346]. GM-CSF has recently been appreciated as a type cytokine [352, 353] and a neutrophil and eosinophil survival factor [354, 355].

Another difficulty that limits our understanding of the role of NKp30 is poor characterisation of its ligands and their distribution on normal tissues under haemostasis and inflammatory condition. Two self-ligands have been identified to

bind to NKp30; a novel member of B7 family receptors, B7H6, and intracellular protein BAT3 [374, 391, 398, 401]. BAT3 is a nuclear protein and not usually expressed on the surface of the cells. Consistent with activation of NKp30 expressing cells through B7H6, our data demonstrated that plate bound recombinant human B7H6 can induce production of IL-13 by ILC2 cells. Moreover, responsiveness of ILC2s correlated with the concentration of plate bound recombinant B7H6 protein. Interestingly incubation of multiple tumour cell lines with ILC2 showed that cell lines that activated ILC2s and induced production of IL-13 such as THP1, K562 and Jurkats, lacked cell surface staining of BAT3 while maintained B7H6 expression; suggesting that B7H6 is the main NKp30 ligand being identified on the experimented tumour cell lines. Indeed IL-13 production was diminished upon prior incubation of ILC2s with increasing concentration of NKp30 blocking antibody. We showed that the IL-13 production occurs rapidly, consistent with an early innate immune function to respond during the initial phases of an immune response.

Until recently B7H6 was believed to be absent on normal tissues and restricted to tumour and transformed cells [398]. In 2013 Matta and colleagues revealed that B7H6 can be induced on non-transformed cells in various conditions of cell stress such as infections and inflammation [392]. B7H6 was selectively upregulated on $CD14^+$ $CD16^+$ proinflammatory monocytes and neutrophils when stimulated with TNF-α and IL-1β *in vitro* and in septic conditions *in vivo* [392]. These data have guided us to propose some molecular bases for the direct interaction and cross-talk between NKp30 positive ILC2 and ligand expressing tissues. In fact staining of formalin fixed, paraffin-embedded sections of normal human skin revealed low level of B7H6

expression in the basal layer of epidermis whereas higher frequency of B7H6 expressing cells were detected throughout the epidermis and in the dermis in lesional tissue sections of patients with atopic dermatitis. Further investigation is required to precisely identify the dermal populations expressing B7H6 although based on available data proinflammatory monocytes and neutrophils are plausible candidates.

Testing unstimulated keratinocyte cell line, HaCaTs, confirmed similar low level of B7H6 expression. Proinflammatory cytokines such as TNF-α and IFN-γ and type 2 cytokines IL-13 and IL-4 increased expression of B7H6. It has been well established that type 2 cytokines found in high concentrations in lesions of AD patients [115, 116, 146, 243]. Our data in chapter 3 on contents of HDM allergen challenged skin blister fluid also confirmed this finding. Type 1 cytokines IFN-γ and TNF-α have also been found in higher levels during chronic stage of AD. These cytokines upregulate MHC I and MHC II on keratinocytes and increase antigen presentation and T cell activation [89].

The expression of B7H6 by keratinocytes provides a novel perspective on the link between inflammation and NKp30 mediated activation of ILC2s. Keratinocytes in AD lesions not only activate ILC2s by producing epithelial cytokines but also direct interaction via B7H6 can trigger production of type 2 cytokines. In chapter 3 I have shown interactions between keratinocytes and ILC2s under inflammatory conditions via production of cytokines. We proposed that in patients with atopic dermatitis, increased release of epithelial cytokines by keratinocytes stimulates ILC2s to produce type 2 cytokines. These cytokines subsequently increase expression of B7H6 on

keratinocytes which then directly interact with ILC2s and leads to further activation of these cells in AD lesions, particularly in the context of reduced E-cadherin expression.

Another implication of our finding that deserves further investigation is NKp30 mediated anti-viral activation of ILC2s. Viral hemagglutinin is another well-established ligand for NKp30. Several studies have shown the role of ILC2 in the context of influenza infection [172, 193].

Chapter 6. Discussion

Over the past few years a previously unappreciated family of innate effector cells have been identified. Their comprehensive functional capabilities range from lymphoid organogenesis, tissue remodelling, wound healing, immune protection and homeostasis to contribution in inflammation and allergic responses. Owing to their striking similarities to T cell lymphocytes, outstanding advances have been made in relatively short period of time. Distinct subsets have been phenotypically and functionally described, developmental lineage relationships and transcription factors identified and contribution to human pathology is under active investigation. Nevertheless, we still face many challenges in this field.

ILC subgroups produce a similar array of cytokines and have comparable effector functions to subsets of T cell lymphocytes. Group 1 ILCs mirror T helper 1 cells and produce type 1 cytokines, IFN-γ and TNF-α. Similarly group 2 ILC2 produce type 2 cytokines, IL-4, IL-5, IL-13 and mimic T helper 2 lymphocytes. Group 3 ILCs release IL-17 and IL22 corresponding to T helper 17 and T helper 22 subsets (Figure 6.1). Furthermore, transcription factors that control development and effector functions of T helper subsets also regulate cytokine production profile of ILCs. Group 1 ILCs and TH1 cells are controlled by T-bet and EOMES, while group 2 ILCs and TH2 lymphocytes depend on GATA-3 transcription factor and require Notch signalling. Similarly Group 3 ILCs, TH17 and TH22 express RORγt and Aryl Hydrocarbon receptor (AHR) (Figure 6.1).

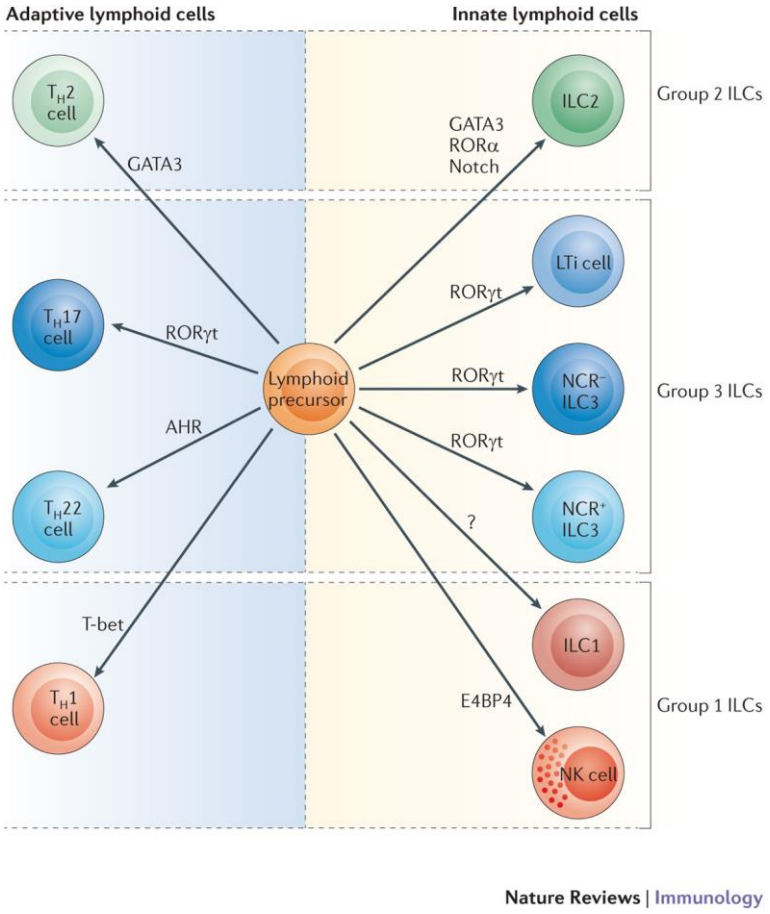


Figure 6.1 ILC subgroups express similar cytokines and transcription factors as subsets of T cell lymphocytes. Group 1 ILCs and TH1 cells produce IFN-γ and TNF-α and express T-bet and EOMES. ILC2 and TH2 are regulated by GATA-3 and express IL-13, IL-4 and IL-5. ILC3 are dependent on RORγt and produce IL-22 and IL-17 similar to TH17 and TH22. Adapted from reference [228].

It is likely that during evolution various environmental factors especially infections exerted selective pressure on shaping of the immune system. As a result, a population of innate cells with a protective role against infections developed to mediate effector functions similar to that of the adaptive immune system. However it has been suggested that ILCs might be primordial T cell precursors [228]. ILCs develop independently of the thymus, and their existence can be traced back to ancient

vertebrates [413]. Homologous cytokines to those produced by the ILC family were described in sea urchins, sea squirts and oysters. However transcription factors required for development of this family, RORA and RORC, have not been defined in organisms lower than vertebrates [228]. Lymphotoxin-β (LT-β) is another essential factor for formation of ILCs. Avian species lack this receptor and it has been reported first in mammals. It has been proposed that NCR^- ILC3 were possibly the first ILCs to appear and through interaction with stromal organizer cells formed lymph nodes [340]. Although the evolutionary origin of ILCs has not yet been defined, the rapid pace of discovery in this field and studying of lower vertebrates will soon identify whether they have a role in development of the adaptive immune system.

Nonetheless, there is a convincing body of evidence indicating initial signalling by ILCs influences adaptive responses. Unlike T cells, innate immune responses do not require previous encounter with antigen and can develop rapidly to protect the host at early stages of infection. Furthermore, polarized cytokines produced by innate lymphoid cells can bias the adaptive immune system towards suitable responses. The initial signal can be subsequently maintained by the adaptive immune system following clonal expansion of differentiated T cell lymphocytes [340]. Examples that give credibility to this idea are the studies by Neil [158], Moro [169], Price [168]and Saenz [170]. Following *Nippostrongylus brasiliensis* helminth infection, ILC2 are the predominant early source of IL-13 production before the initiation of adaptive immune responses. IL-$17RB^{-/-}$ IL-$1RL1^{-/-}$ mice that are severely deficient in ILC2 showed considerable delay in worm expulsion. Furthermore, ILC2s enhanced IL-13 production by TH2 lymphocytes [158, 168-170].

Further evidence on the role of ILCs in the crosstalk between the innate and adaptive immune system originate from their anatomical location. ILCs reside in the interface of T and B cell zones in the splenic follicles of mice and can express co-stimulatory molecules essential for T cell priming and survival including TNF ligands- OX40 ligand (OX40L; TNFSF4) and CD30 ligand (CD30L; TNFSF8) [414-416]. Interestingly in foetal and neonatal stages, ILCs lack expression of these markers and antigen encounter induces tolerance in T cells rather than immune responses [256]. OX40L- CD30L-mediated interactions of ILC and T cells induce survival of TH2 [417] and memory T cells [418]. Other crucial anatomical locations are gut and skin which are two of the main antigen encounter sites in the body and ILCs provide a constant immunosurveilance function. Following formation of isolated lymphoid follicles in the intestine, ILCs contribute to homeostatic conditions by interaction with commensal bacteria. They induce production of active TGF-β which helps in class switching to IgA [179].

Contrasting to T cell lymphocytes that have intrinsic ability to expand in response to specific antigen encounter, ILCs are believed to be short lived populations and their half-life at least in intestinal lamina properia and mesenteric lymph nodes of mice is around 22 to 26 days. They are constantly replaced by newly regenerated cells from the bone marrow. Their maintenance is regulated by cytokines that are produced either by innate populations like epithelial cells and fibroblasts or adaptive T cell subsets. Interestingly development of various ILC populations is dependent on age rather than inherent ability of these cells to proliferate [157].

‘So, ILCs — how did we miss them? Well, we had just gated them out, assuming that we already knew all the players. Who knows what other unknowns wait to be discovered within the LIN$^-$population’ [228]

When I started my thesis, there were few data about ILC2 and approaches to their study. There was debate about the best markers to use to identify them, and there were no data about their role in disease. I aimed to test the hypothesis that ILC2 are present in human skin and contribute to the pathogenesis of dermatitis. Furthermore I aimed to investigate the associated underlying mechanisms. I have discussed my findings through answering a number of questions, as follows.

6.1 Are ILC2 resident in human skin?

ILC2s have been discovered in various organs. Studies in mice have reported ILC2s in the lung, mesenteric lymph nodes (mLN), bone marrow, liver, spleen, intestine and fat associated lymphoid clusters (FALC) [158, 168, 169, 190, 193]. Similarly in humans ILC2s were found in the blood, nasal polyps, sinus epithelium, foetal and adult gut [172, 173]. Their wide distribution suggests that they may have a role in tissue homeostasis and organ specific inflammatory responses [419]. Furthermore, epithelial barrier surfaces are continuously exposed to myriad of environmental stimuli such as infectious pathogens, commensal bacteria, toxins and allergens. Precise regulation of innate and adaptive immune responses is required to maintain homeostasis at these sites and avoid local and systemic inflammation. ILC2 have been shown to have an essential protective role in barrier integrity in lungs and intestine. In lungs ILC2 promote tissue repair and epithelial barrier regeneration following H1N1 PR8 influenza A infection. In $RAG^{-/-}$ mice infected with influenza virus, depletion of ILC2 cause significant barrier impairment which was restored upon adoptive transfer of ILC2. ILC2 mediate epithelial repair in this model by producing a wound healing regulating protein, amphiregulin [172]. Moreover, intranasal allergen challenge with *alternaria alternata* increased production of amphiregulin and induced epithelial repair [189]. In the gut ILC2s have a protective role against helminth infections. Following *Nippostrongylus brasiliensis* infection, IL-13 produced by ILC2 augments

physiological responses for worm expulsion such as goblet cell hyperplasia, increased mucus production and increased smooth muscle contractions. ILC2 depletion considerably prolonged worm expulsion in this mouse model [158, 168, 169].

Considering ILC2 role in protection against infections, wound healing and tissue repair in epithelial surfaces of lung and intestine, **we hypothesised that ILC2 exists in the skin**. To investigate this, I first assessed established methods of isolating cells from skin biopsies. The current methods resulted in isolation of limited number of cells that were far from the predicted frequency of cells in the skin. Furthermore, due to scarcity of ILCs, evaluation of these populations using current methods was cumbersome. Effective isolation of lymphoid cells was achieved by using collagenase P enzymatic treatment. The isolated cells expressed similar phenotypic markers such as skin homing receptors, CD3, CD8 and CD45RA reported by previous studies using established methods. The functional integrity of isolated cells was confirmed by rapid production of IFN-ɣ, IL-13, IL-22 and IL-17 *ex-vivo* after stimulation suggesting that collagenase P treatment is a reliable method of isolating intact cells from the skin.

Using phenotypical markers previously described in identification of ILC2 in humans, we detected a population of lineage negative (CD3, CD4, CD8, CD14, CD19, CD56, CD11c, CD11b, FcεRI, TCRγδ, TCRαβ and CD123), $CD45^{+}$, $CD127^{+}$ (IL-7Rα) and $CRTH2^{+}$ cells in the skin with the frequency of 0.04 - 2.94% of lymphoid population. This population lacked the expression of KIR receptor family (KIR2DL1/S1, KIR3DL1/S1, and KIR2DL2/S2/L3/S4) and KLR family receptors, NKG2A and NKG2C which shows that they are distinct from NK cells. We gated out CD56 expressing cells to minimise the

inclusion of NK cells, but it is possible that this stringent approach underestimates the numbers of ILC2 in human skin.

Skin resident ILC2s expressed GATA-3 and RORα transcription factors but lack the expression of RORγt, similar to ILC2s isolated from other organs. A significant proportion of isolated ILC2 expressed skin homing markers, CLA, CCR10 and CCR4 which would support that these cells traffic to the skin in inflammatory conditions. To test their role in epithelial repair, we evaluated their ability to produce amphiregulin after stimulation. ILC2 isolated from the skin produced significantly greater amount of amphiregulin when compared to blood derived ILC2s.

6.2 Do ILC2 contribute to the pathogenesis of atopic dermatitis?

Allergic reactions are characterised by aberrant hyperreactivity of the immune system following exposure to innocuous environmental antigens. These responses include increased production of type 2 cytokines that lead to eosinophilia, goblet cell hyperplasia, increased mucus production, mast cell degranulation, smooth muscle contraction and increased serum IgE levels. Previously TH2 cells were considered the sole producers of type 2 cytokines but the discovery of ILC2 challenged this notion.

There is a convincing body of evidence that even though TH2 cells are still the major producers of type 2 cytokines, ILC2 also contribute to the pathogenesis of allergic reactions. In experimental mouse models of asthma ILC2 represent a main source of

type 2 cytokines in early stages of disease. ILC2s induce airway hyperreactivity following infection with H3N1 strain of influenza A and intranasal challenge with *Alternaria*, papain, house dust mite and OVA [175, 189, 193, 281, 331]. In humans, ILC2 were discovered in healthy lung parenchyma and broncho-alveolar lavage (BAL) fluid of patients receiving a lung transplant as lineage negative cells (CD3, TCRαβ, CD11c, CD11b, CD56, CD19) that express IL-7Rα and ST2 subunit of the IL-33 receptor [172]. An increased frequency of ILC2 has been reported in nasal polyps [173] and sinonasal mucosa [420] of patients with chronic rhinosinositis (CRS), another allergic inflammatory condition. Epithelial cells in patients with CRS with polyps showed increased expression of IL-33 and TSLP [420]. Ulcerative colitis is an inflammatory bowel disease in which increased type 2 cytokines, IL-4, IL-5 and IL-13 correlates with intestinal pathology. Enrichment of ILC2 was observed in oxazolone induced mouse model of ulcerative colitis. ILCs were localised to the lamina propria of gut mucosa. Furthermore, blocking IL-25 either by neutralizing IL-25 or blocking its receptor IL-17RB improved intestinal pathology [228].

Production of type 2 cytokines and contribution to the allergic reactions made ILC2 a suitable member of 'type 2 franchise' which includes TH2 lymphocytes, mast cells, eosinophils and basophils [159]. Therefore, **we sought to investigate whether ILC2 contribute to pathogenesis of skin allergic inflammation of patients with atopic dermatitis**. Examination of lesional skin biopsies of AD patients confirmed higher frequency of infiltrating ILC2 (0.28-4.36% of lymphoid cells) compared to normal skin (0.04 - 2.94%). We observed similar concentration of circulating ILC2 in the blood of patients and healthy controls. Increased expression of cytokine receptors IL-17RB,

ST2 and TSLP-R was detected on ILC2 in dermatitis lesions probably due to activated phenotype of the cells in these lesions.

Although the presence of ILC2 in AD lesions suggested their involvement in pathogenesis of this disease, it did not demonstrate the kinetics of their contribution. In particular it was not known whether the cells infiltrate the skin or proliferate in situ. Therefore we investigated their role in initiating allergic inflammation of the skin using the suction blister technique. Sampling the skin with and without intraepidermal HDM challenge indicated enrichment of ST2 expressing ILC2s 26 hours following HDM challenge. This timecourse is probably due to recruitment of ILC2 from peripheral blood rather than mere expansion of a resident population.

The presence of ILC2 in atopic dermatitis lesions does not prove causality. In collaboration with Andrew McKenzie and Padraic Fallon, we therefore used a mouse model of dermatitis to define the role of ILC2 in this system. Using two systems of ILC2 deficiency, we found that ILC2 were critical for the development of calcipotriol-induced dermatitis and that this was dependent on IL-25R, IL-33R and TSLP-R pathways. As discussed below, these findings were compatible with our human data in vitro and ex vivo, and therefore support a role for ILC2 in the pathogenesis of dermatitis.

6.3 What cytokines and lipid mediators activate ILC2?

The ILC2 population is now considered a novel target for treatment strategies of allergic diseases. Hence, it seems crucial to acquire detailed information on ILC2 activation and regulation processes in allergic reactions. **Our next aim was to study cytokines and lipid mediators that activate ILC2 in inflammatory conditions**. One of the most important characteristics of ILC2 is their ability to respond to epithelial cytokines, IL-25, IL-33 and TSLP. Furthermore, higher expression of these cytokines was observed in the skin of atopic patients. Therefore we stimulated the skin resident cells with the combination of these cytokines and evaluated their response using multiplex bead array. IL-33 stimulation induced production of type 2 cytokines, IL-13 and IL-5 but not IL-17A and IL-22 in ILC2 isolated from skin biopsies. This was enhanced when ILC2 were stimulated with a combination of IL-33, IL-25 and TSLP. Upregulation in the gene expression levels correlated with increase in protein release suggesting that this was not simply due to the release of pre-formed cytokines. Interestingly the higher levels of type 2 cytokines were detected in unchallenged skin of atopic patients compared to healthy individuals. Following intraepidermal delivery of HDM extracts, a further increase in the level of type 2 cytokines was observed which was correlated with disease severity and serum IgE levels suggesting that these cytokines have a central role in pathogenesis of atopic dermatitis. Consistent with activation in response to epithelial cytokines, ILC2 express cytokine receptors IL-17RB (IL-25R), ST-2 (IL-33R) and TSLPR. Stimulation with IL-33 and TSLP alone or in combination with IL-25 upregulated expression of these receptors. Accordingly, ILC2s

isolated *ex-vivo* from dermatitis lesions of atopic patients showed higher expression indicating an activated phenotype.

Another important inflammatory mediator is prostaglandin D2 (PGD2). PGD2 is produced mainly by mast cells and binds to CRTH2 receptor on ILC2. The role of PGD2 in asthma is well established. Scratching behaviour in mice increased PGD2 levels in the skin. PGD2 contribution to the skin inflammation following epicutaneous OVA antigen exposure in sensitized mice was demonstrated using $CRTH2^{-/-}$ mice [421]. Furthermore, hapten specific IgE induced 35-55% less ear swelling in $CRTH2^{-/-}$ mice than wild type littermates. It was also associated with decreased infiltration of lymphocytes, basophils and eosinophils and reduced production of chemokine RANTES in the inflammation site. Lower serum IgE level was also noted. A similar result was achieved by using CRTH2 antagonist, ramatroban or PGD2 blocking agent, HQL-79 [323]. In a murine model of FITC-induced contact hypersensitivity the levels of CRTH2 and PGD2 in lesions correlated with infiltration of eosinophils and neutrophils similar to AD lesions and was abolished by CRTH2 antagonist [422]. Therefore, I was interested to investigate the effect of this mediator on ILC2. Similar to its effect on eosinophils and TH2 lymphocytes, PGD2 significantly enhanced chemotactic activity of ILC2. Although IL-33 could also attract the cells to the site of inflammation, PGD2 was a more potent chemoattractant. Stimulation of ILC2 with PGD2 not only increased production of type 2 cytokines, IL-4, IL-5 and IL-13 at the protein and gene level, it also induced release of IL-3, IL-8, IL-9, IL-21, GM-CSF, and CSF-1 in a dose-dependent manner. Interestingly PGD2 enhanced responsiveness of ILC2 to IL-33 and IL-25 by upregulating relevant receptors, ST2 and IL-17RA subunits

of IL-33 and IL-25 respectively. Similar results were observed using supernatant from IgE activated mast cells. Endogenously synthesized PGD2 induced chemotaxis, increased type 2 cytokine production and upregulated ST2 and IL-17RA. Using CRTH2 antagonist (TM) and PGD2 synthesis inhibitor (Diclofenac) confirmed that these effects were mediated by PGD2. These findings are being further investigated through a clinical trial of a CRTH2 antagonist.

6.4 What signals do ILC2 employ to sense epithelial integrity?

There are likely to be many systems in place for the detection of barrier compromise. Given that ILC2 are resident in human skin and would be well placed to act early in response to barrier compromise, we next aimed to determine how the activity of ILC2 is regulated and what mechanisms they employ to sense epidermal integrity. Defective epidermal barrier, increased permeability and subsequent susceptibility to secondary infections are major characteristics of atopic dermatitis lesions. E-cadherin is a crucial adhesion protein in maintaining the integrity of epithelium. As explained in detail in chapter 3, AD lesions in humans are associated with down-regulation of E-cadherin expression. Furthermore, previous studies have shown high expression levels of E-cadherin ligand- KLRG1 on ILC2 in mice [230]. KLRG1 is an inhibitory receptor that upon interaction inhibits proliferation and cytokine production in the cells expressing its cognate ligand.

ILC2 in humans also demonstrated expression of KLRG1 which was upregulated upon activation of the cells with epithelial cytokines. Investigating the activity of stimulated ILC2 in the presence E-cadherin showed significant reduction in expression of transcription factors RORα and GATA-3, diminished production of type 2 cytokines IL-13, IL-5, IL-4 and amphiregulin. Accordingly, we proposed a model of regulation of ILC2 activity in inflamed lesions of AD patients. Reduction in E-cadherin expression and increased production of epithelial cytokines allow engagement of activating receptor without transduction of inhibitory signals from KLRG1, thus allowing overproduction of cytokines and amphiregulin. This KLRG1–E-cadherin model of ILC2 regulation may represent a novel mechanism for barrier sensing in the skin.

6.5 What is the role of NKp30 on ILC2?

ILC2 lack rearranged antigen-specific receptors, but cell:cell contact mechanisms underlying their activation are unknown. We identified the expression of an activating receptor, NKp30, on cultured and freshly isolated ILC2s. Real-time PCR experiments showed the predominant expression of NKp30c splice variant on tested ILC2 lines which is an immunomodulatory isoform. Similar to NK cells, it remains possible that ILC2 can upregulate the expression of other isoforms under certain conditions which require further investigation. Crosslinking of NKp30 on ILC2 either following incubation with recombinant human B7H6 or cell lines that express this ligand, induced expression of type 2 cytokines IL-4, IL-5, IL-13 and GM-CSF as well as

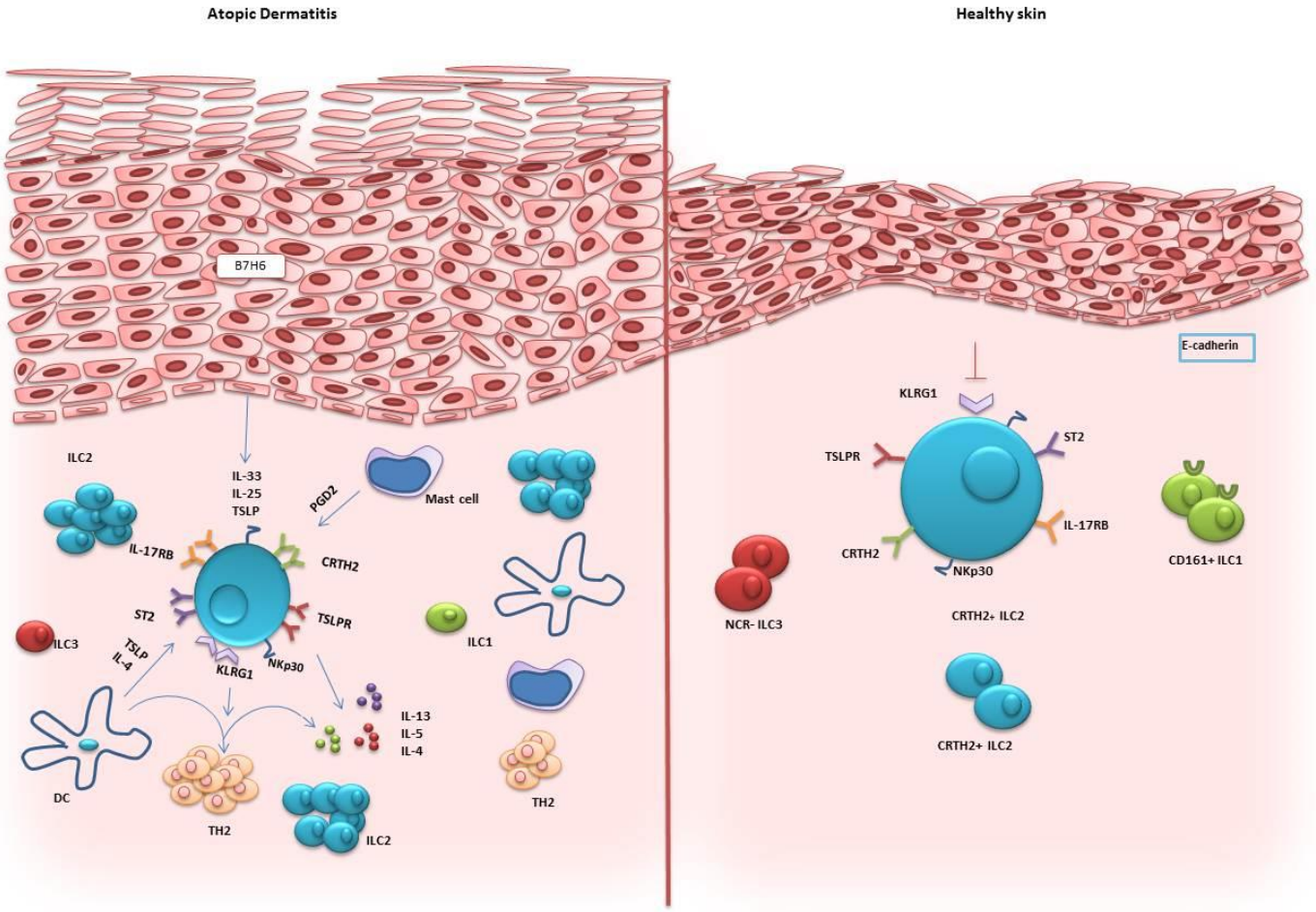
other inflammatory cytokines such as IL-2, IL-3 and IL-8. While, low levels of B7H6 expression were observed on basal epidermis on tissue sections of healthy donors, lesional biopsies of patients with atopic dermatitis revealed high expression throughout the suprabasal epidermis. Similarly, the keratinocyte cell line, HaCaT, expressed low levels of B7H6 that were upregulated following incubation with IFN-γ, TNF-α, IL-4 and IL-13. Our data provide a novel perspective on the link between inflammation and NKp30 mediated activation of ILC2 and identify the first cell:cell surface activating molecule for ILC2.

6.6 Disease model and role of ILC2

Atopic dermatitis is a chronic inflammatory relapsing skin disease with complex genetic and environmental susceptibility factors. Several gene mutations, including filaggrin null mutations, result in defective skin barrier. The innate and adaptive immune system contributes to the pathogenesis of this disease, with roles for many cells including keratinocytes, eosinophils, basophils, dendritic cells, mast cells and TH2 lymphocytes. In the skin, innate lymphoid cells comprise ILC1, ILC2 and ILC3 populations [200, 201]. A higher frequency of ILC2 with an activated phenotype was observed in the lesional skin biopsies of patients with atopic dermatitis and established mouse models of atopic dermatitis supporting their contribution to the pathogenesis of this disease [197-199]. Under inflammatory conditions in AD lesions, keratinocytes produce high levels of epithelial cytokines, IL-33, IL-25 and TSLP.

Binding of allergen specific IgE to the mast cell high affinity receptor (FcεRI) induces release of lipid mediators including PGD2. Combination of epithelial cytokines and lipid mediators activate resident ILC2, attract circulating ILC2 to site of inflammation and upregulate expression of cytokines receptors IL-17RB (IL-25R), ST2 (IL-33R) and TSLP-R. Activated ILC2s produce high levels of type 2 cytokines IL-13, IL-4, IL-5, IL-6, IL-9, GM-CSF and the wound healing regulator amphiregulin. Activation of ILC2 upregulates expression of inhibitory receptor KLRG1, and the cleavage of its ligand, E-cadherin, in lesions of AD patients potentially allows overproduction of cytokines. Consequently type 2 cytokines may increase expression of B7H6 on keratinocytes which then could directly interact with ILC2 and lead to further activation of these cells via NKp30 in AD lesions (summarised in Figure 6.2). Furthermore, ILC2 may present antigen to T cells which could amplify the subsequent adaptive immune response. Such a disease model is likely to reflect only part of the disease process, but does identify potential targets for therapeutic intervention. Indeed the group are currently participating in a clinical trial of a CRTH2 antagonist for the treatment of eczema.

Figure 6.2: Model of potential contribution to atopic dermatitis disease pathogenesis. ILC1, ILC2 and ILC3 are resident in the normal human skin. ILC1s express CD161. ILC2s express IL-17RB, ST2, CRTH2, TSLPR, an inhibitory receptor KLRG1 and activating receptor NKp30. In homeostatic conditions, the expression of adhesion molecule E-cadherin on normal human keratinocytes and low expression of B7H6 inhibit the activation of ILC2s. ILC2s are enriched in atopic dermatitis lesions and show higher expression of ST2, IL-17RB and TSLP-R, probably an activated phenotype. They express IL-13, IL-5, and IL-4 in response to IL-33, IL-25 and TSLP produced by keratinocytes and PGD2 released by mast cells. Furthermore, higher expression of B7H6 contributes to activation via NKp30 receptor. Concurrently, the diminished expression of E-cadherin on keratinocytes is a novel mechanism of sensing a dysfunctional barrier. The frequencies of ILC1 and ILC3 in AD lesions are similar to healthy skin.


Atopic Dermatitis
Healthy skin
B7H6
E-cadherin
ILC2
IL-33
IL-25
TSLP
PGD2
Mast cell
IL-17RB
CRTH2
ST2
TSLPR
ILC3
TSLP
IL-4
KLRG1
NKp30
ILC1
IL-13
IL-5
IL-4
DC
TH2
TH2
ILC2
KLRG1
TSLPR
ST2
IL-17RB
CRTH2
NKp30
CD161+ ILC1
NCR- ILC3
CRTH2+ ILC2
CRTH2+ ILC2

6.7 Future directions

Although research in the field of innate lymphoid cells moves at fast pace, many important questions regarding the role of ILCs in health and disease still remain unanswered. Detailed interactions of ILC2s with other cell types and surrounding environment including epithelial cells, keratinocytes, fibroblasts, cells of innate and adaptive immune systems especially T and B cell lymphocytes would provide a better understanding of the extent of their contribution to homeostatic conditions and disease pathogenesis. *In vitro* strategies include studying ILC2 survival and proliferation following culture with keratinocytes, fibroblasts and T cells with and without exogenous cytokines or on human skin models. Development of ILC2 reporter mouse strains will also enable us to identify and study ILC2 behaviour in the context of other cell types and during immune responses *in vivo*.

Like other members of the innate immune system, ILCs do not respond through rearranged specific antigen receptors and are activated through cytokines and other mechanisms. Therefore, it is important to systematically evaluate signals and mechanisms that regulate their activation during and after the onset of inflammation which would help us to identify specific targets for therapeutic intervention. Studying the role of ILC2 especially production of wound healing proteins during the recovery phase of immune responses in lungs and skin requires further investigation.

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