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The Phenotype and Function of Nasal Mucosal T cells in Health and Polyposis

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The Phenotype and Function of Nasal Mucosal T cells in Health and Polyposis

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A thesis submitted to King's College London for the degree of Doctor of Philosophy

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August 2015

I confirm that the work submitted in this thesis is my own.

Emily Puo San Lam

Abstract

Chronic rhinosinusitis with nasal polyposis (CRSwNP) in western countries is characterised by eosinophilia and IgE production. Type 2 innate lymphoid cells from polyps produce Th2 cytokines in response to IL-25 and IL-33 but the relevance of this axis to local mucosal T cell responses is unknown. Furthermore, the local T cell response in relation to the healthy versus inflamed nasal mucosa remains poorly characterised.

In this thesis, the nasal mucosal T cell phenotype in health and CRSwNP and the role of the IL-25/IL-33 axis in the adaptive Th2 response was examined. Normal nasal mucosal and nasal polyp tissues were cultured in a short-term explant model before analysis. Peripheral blood from the same subjects were analysed in parallel to determine the local versus peripheral response.

Polyp-derived cells contained a discrete population of IL-25 receptor (IL-25R) positive Th2 cells, which were absent in the periphery and healthy nasal mucosa. IL-25R$^+$CD4$^+$ polyp Th2 cells co-expressed ST2 and responded to IL-25 and IL-33 with enhanced IL-5 and IL-13 production. Within the IL-25R$^+$CD4$^+$ population, several identical TCR Vβ complementarity-determining region 3 (CDR3) sequences were detected in different subjects, suggestive of clonal expansion driven by common antigen(s). Th17 cells were abundant in healthy nasal and polyp explants but not in the periphery and represented the most preferentially expressed T cell subset in the nasal mucosa compared to the periphery. Furthermore, this was associated with a protective signature.

This study is the first demonstration of human IL-25R$^+$ Th2 cells co-expressing ST2. IL-25 and IL-33 may interact locally with IL-25R$^+$ST2$^+$ polyp T cells to augment Th2 responses in CRSwNP. Targeting the IL-25/IL-25R and IL-33/ST2 pathways may represent attractive therapeutic strategies. The strong Th17 signature in the normal nasal mucosa suggests Th17 may be present in a protective, rather than pathogenic, capacity for normal nasal mucosal immunity.

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Abbreviations

AERD	Aspirin-exacerbated respiratory disease
AFRS	Allergic fungal rhinosinusitis
AHR	Aryl hydrocarbon
AHR	Airway hyperresponsiveness
AMP	Antimicrobial peptide
AS	Aspirin sensitive
ASMC	Airway smooth muscle cell
AT	Aspirin tolerant
ATP	Adenosine triphosphate
BAL	Bronchoalveolar lavage
BEGM	Bronchial Epithelial Cell Growth Medium
BMT	Bone marrow transplanted
BRC	Biomedical research centre
CBA	Cytometric bead array
cDNA	Complementary DNA
CDR3	Complementarity-determining region 3
CFSE	Carboxyfluorescein diacetate, succinimidyl ester
CLA	Cutaneous lymphocyte antigen
COPD	Chronic obstructive pulmonary disease
COX	Cyclooxygenase
CRS	Chronic rhinosinusitis
CRSsNP	Chronic rhinosinusitis without nasal polyps
CRSwNP	Chronic rhinosinusitis with nasal polyps
CRTH2	Chemoattractant receptor-homologous molecule expressed on Th2 cells
CysLT	Cysteinyl leukotriene
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EAE	Experimental autoimmune encephalomyelitis
ECM	Extracellular matrix

ECP	Eosinophil cationic protein
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ESS	Endoscopic sinus surgery
FACS	Fluorescence-activated cell sorting
FcR	Fragment constant receptor
FCS	Foetal calf serum
FOXP3	Forkhead box P3
GM-CSF	Granulocyte-macrophage colony-stimulating factor
hBD	Human beta defensin
HBEC	Human bronchoepithelial cell
HBSS	Hank's Balanced Salt Solution
HDM	House dust mite
HIES	Hyper IgE syndrome
HLA	Human leukocyte antigen
HMGB-1	High mobility group box-1
hPGDS	Hematopoietic prostaglandin D synthase
IFNγ	Interferon gamma
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
ILC2	Type 2 innate lymphoid cell
IMDM	Iscove's Modified Dulbecco's Medium
iNKT	Invariant natural killer T cell
IRF4	Interferon regulatory factor 4
JAK	Janus kinase
M2 macrophage	Alternatively activated macrophage
MACS	Magnetic-activated cell sorting
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinases
MPO	Myeloperoxidase
N. brasiliensis	*Nippostrongylus brasiliensis*
NIHR	National Institute for Health Research
NK	Natural killer
NM bx	Normal nasal mucosal biopsy

NOD	Non-obese diabetic
NSAID	Non-steroidal anti-inflammatory drugs
OSM	Oncostatin M
OVA	Ovalbumin
OX40L	OX40 ligand
PAMP	Pathogen-associated molecular pattern
PAR2	Protease-activated receptor 2
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCA	Principal component analysis
PFA	Paraformaldehyde
PGD_2	Prostaglandin D_2
PHA	Phytohaemagglutinin
PMA	Phorbol 12-myristate 13-acetate
PRR	Pattern recognition receptor
PSGL1	P-selectin glycoprotein ligand 1
PTGS2	Prostaglandin-endoperoxide synthase 2
qRT-PCR	Quantitative real-time polymerase chain reaction
rh	Recombinant human
RNA	Ribonucleic acid
RORC	Retinoic acid-related orphan nuclear hormone receptor C
RPMI	Roswell Park Memorial Institute medium
rRNA	Ribosomal RNA
S. aureus	*Staphylococcus aureus*
SCID	Severe combined immunodeficient
SE	Staphylococcal enterotoxin
SpA	Staphylococcal Protein A
SPE-C	Staphylococcal pyrogenic exotoxin-C
SPINK5	Serine protease inhibitor kazal-type 5
SPLUNC1	Short palate, lung, and nasal epithelium clone 1
SPT	Skin prick test
sST2	Soluble ST2
STAT	Signal transducer and activator of transcription
TCDD	2,3,7,8-tetracholorodibenzo-p-dioxin
T_{CM}	Central memory T cell

TCR	T cell receptor
TCR Vβ	TCR variable β
T_{EM}	Effector memory T cell
TGFβ	Transforming growth factor β
Th	T helper
TIMP	Tissue inhibitor of matrix metalloproteinases
TLR	Toll like receptor
TM ST2	Transmembrane ST2
TNFα	Tumour necrosis factor α
tPA	Tissue plasminogen activator
Treg	Regulatory T cell
TSLP	Thymic stromal lymphopoietin
TSST-1	Toxic shock syndrome toxin-1
VEGF	Vascular endothelial growth factor
ZO-1	Zonula occludens-1
γc	Common-γ chain

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Chapter 1 Introduction

1.1 Chronic rhinosinusitis

Chronic rhinosinusitis (CRS) is a heterogeneous group of chronic inflammatory diseases characterised by inflammation of the nasal mucosal lining and paranasal sinuses. Symptoms associated with CRS persist beyond a 12-week duration and include nasal blockage, nasal congestion, rhinorrhea and hyposmia (Fokkens *et al.*, 2012). Interestingly, CRS is most commonly diagnosed in males and most frequently observed in middle-aged patients (40-69 years) in both genders (Tan *et al.*, 2013). Epidemiology studies on CRS have wide-ranging results and estimates for the prevalence rate vary from 1-15% of the general adult population (Pawankar, 2003, Bachert *et al.*, 2014). In the GA2LEN (Global Allergy and Asthma European Network project) study involving 12 European countries, Hastan *et al.* (2011) estimated that 10.9% of the European adult population is affected by CRS. In the US, 14.2% of the adult population is reportedly affected. This represents a considerable socioeconomic burden with associated healthcare costs estimated at $8.6 billion per annum in the US alone (Kern *et al.*, 2007, Bhattacharyya, 2011). CRS is also associated with a significantly reduced quality of life comparable to that for chronic obstructive pulmonary disease and congestive heart failure patients (Soler *et al.*, 2011, Hulse *et al.*, 2015).

1.1.1 Chronic rhinosinusitis with and without nasal polyps

CRS is a complex disease and can be broadly classified into two subtypes based on disease presentation with nasal polyps (CRSwNP) or without nasal polyps (CRSsNP). An estimated 20% of CRS patients present with nasal polyps (Hamilos, 2000). These are defined as abnormal, often recurrent, mucosal tissue growths in the sinonasal cavity and are associated with tissue oedema and inflammatory cell infiltration. Furthermore, although CRSwNP and CRSsNP share common features such as pseudocyst formation and albumin accumulation, CRSsNP and CRSwNP display distinct pathomechanisms

(Bachert *et al.*, 2000, Mygind *et al.*, 2000, Pawankar, 2003). CRSsNP is dominated by a T helper (Th) 1 response with high levels of interferon gamma (IFNγ) and transforming growth factor beta (TGFβ) detected in supernatants from nasal tissue cultures. In contrast, CRSwNP is dominated by a Th2 eosinophilic response with upregulated levels of eotaxin (CCL11), eosinophil cationic protein (ECP) and interleukin (IL)-5 detected (Van Zele *et al.*, 2006). Symptoms also vary with facial pain and swelling more commonly associated with CRSsNP and hyposmia more frequently detected in CRSwNP patients (Bachert *et al.*, 2014).

1.1.1.1 Tissue remodelling

Remodelling is observed in both CRSwNP and CRSsNP. However, fibrosis is a feature primarily associated with remodelling in CRSsNP. Selective expression of TGFβ in the CRSsNP nasal mucosa has been demonstrated, resulting in increased collagen deposition (Watelet *et al.*, 2004b, Van Zele *et al.*, 2006, Van Bruaene *et al.*, 2009). Furthermore, imbalances in the expression of matrix metalloproteinases (MMP) and tissue inhibitors of MMP (TIMP) may play a role in the fibrotic process in CRSsNP. Although significantly increased levels of MMP-7 and MMP-9 were observed in both CRSwNP and CRSsNP patients compared to healthy controls (Li *et al.*, 2010, Watelet *et al.*, 2004a), significantly elevated expression of MMP-7 was observed in CRSwNP vs. CRSsNP patients (Watelet *et al.*, 2004a). Moreover, Li *et al.* (2010) showed that TIMP-1 and TIMP-4 levels were significantly decreased in CRSwNP compared to CRSsNP. Thus, these studies show that remodelling in CRSsNP is associated with increased fibrosis with excessive collagen deposition and reduced extracellular matrix (ECM) degradation compared to CRSwNP.

Further studies in CRSwNP have suggested that the blood coagulation pathway may be involved in mediating nasal polyp growth via the retention of plasma proteins such as

albumin. Fibrin and factor XIII were both significantly increased in CRSwNP compared to healthy controls, resulting in increased fibrin deposition (Takabayashi *et al.*, 2013b). Moreover levels of tissue plasminogen activator (tPA), which is responsible for the breakdown of fibrin, were reduced in nasal polyps compared to healthy tissue. Furthermore, this was exacerbated in a Th2 environment with *in vitro* stimulation of epithelial cells with Th2 cytokines resulting in decreased tPA production (Takabayashi *et al.*, 2013a). Together these studies demonstrate that although similar symptoms are observed for both CRS disease subtypes, disparate pathomechanisms exist. Indeed, Van Zele *et al.* (2006) proposed that CRSwNP and CRSsNP are 'distinct disease entities within the group of chronic sinus diseases'. For the purpose of this thesis, only CRSwNP will be discussed further.

1.2 Chronic rhinosinusitis with nasal polyps

The phenotype of CRSwNP disease is affected by genetic factors. Numerous studies have shown that a strong neutrophilic Th1/Th17 signature is present in the CRSwNP inflammatory response of Asian patients, specifically the southern Chinese population. Conversely, a Th2 response associated with high levels of IL-4, IL-5 and thymic stromal lymphopoietin (TSLP) is observed in CRSwNP patients from western countries (Van Zele *et al.*, 2006, Zhang *et al.*, 2008, Peterson *et al.*, 2012). However, even within these populations, a diverse immunological response is observed. An estimated 55% of Chinese CRSwNP patients are 'key cytokine negative' with the absence of an IL-5, IL-17 or IFNγ-mediated inflammatory response (Ba *et al.*, 2011). Instead, patients mount a strong neutrophilic response with elevated levels of myeloperoxidase (MPO), IL-1β, IL-6 and IL-8 detected in nasal polyp homogenates. A recent study has also shown that the phenotypic differences in CRSwNP disease is maintained in second-generation Asian patients born and raised in the USA. Nasal polyps from second-generation Asian CRS patients were associated with significantly reduced eosinophil infiltration and levels of ECP compared to Caucasian, Hispanic and African-American patients residing in the same US city (Mahdavinia *et al.*, 2015). These studies highlight the heterogeneous and complex nature of CRSwNP and the associated difficulties in studying the disease.

1.2.1 Co-morbidities

1.2.1.1 Asthma

Many other Th2-mediated conditions co-exist with CRSwNP with asthma representing a major co-morbidity. Approximately 58% of asthma patients suffer from concurrent CRSwNP versus 25% of non-asthmatics (Pearlman *et al.*, 2009). Furthermore, a strong association exists between CRSwNP and asthma (OR: 4.25; 95% CI: 3.74–4.71) although this study used reported anosmia to distinguish between CRSwNP and

CRSsNP (Jarvis *et al.*, 2012). Given that the pathogenic mechanisms in asthma are similar to CRSwNP (dominant Th2 response, airway remodelling, elevated immunoglobulin (Ig) E levels and eosinophilia), it is perhaps unsurprising that the two diseases can occur concurrently in the same individual. Whether asthma precedes or follows the onset of CRS symptoms is unknown but the strong association of CRSwNP with asthma lends support to the unified airway hypothesis. This theorises that any disease affecting the upper airway is also likely to affect the lower airway and has been extensively reviewed (Lipworth *et al.*, 2000, Passalacqua *et al.*, 2001, Krouse *et al.*, 2007, Guilemany *et al.*, 2009).

1.2.1.2 Allergy

Although the comorbidity of asthma with CRSwNP is well established, the association with allergy is less clear. Nasal polyposis is more common in non-allergic asthma (13%) versus allergic asthma (5%) (Settipane, 1996). Furthermore, a study by Robinson *et al.* (2006) showed that atopy was more prevalent in CRSsNP compared to CRSwNP patients (32.3% versus 27.5% respectively). In addition, Lund-Mackay score denoting CRS symptom severity was higher in non-allergic asthma compared to allergic asthma (Pearlman *et al.*, 2009). These studies, along with the failure to identify an allergen involved in CRSwNP, suggest that the allergic component is not a major underlying pathomechanism of the disease.

1.2.1.3 Aspirin-exacerbated respiratory disease (AERD)

Aspirin-exacerbated respiratory disease (AERD) is typically defined as a triad of late onset non-allergic asthma and CRSwNP with disease exacerbation upon the ingestion of cyclooxygenase (COX)-1 inhibiting non-steroidal anti-inflammatory drugs (NSAID) and aspirin (Stevenson, 2009). AERD is considered a pseudoallergic disease as it is non-IgE mediated but overproduction of leukotrienes results in a type 1

hypersensitivity-like response (Laidlaw, 2015). Settipane (1996) estimated that approximately 50% of patients with aspirin and NSAID sensitivity co-present with nasal polyps. Furthermore, AERD patients display the most severe form of nasal polyposis and are ten times more likely to undergo functional endoscopic sinus surgery compared to non-AERD patients. Moreover, they are also at higher risk of nasal polyp reoccurrence following polypectomy (Kim *et al.*, 2007, Garcia Cruz *et al.*, 2012).

1.2.1.4 Allergic fungal rhinosinusitis

In the past an excessive non-IgE mediated host response to fungi, particularly *Alternaria*, was hypothesised to be the initiator of CRS disease (Shin *et al.*, 2004). However, subsequent studies suggest that the heightened response of peripheral blood mononuclear cells (PBMCs) to *Alternaria* may not be due to antigen-specific effects but due to proteases present in *Alternaria* that are able to activate protease-activated receptors (Tan *et al.*, 2010, Bachert *et al.*, 2014). Moreover the lack of clinical efficacy of amphotericin in the treatment of CRS, via both nasal lavage and topical routes, further suggests that fungi are not the major pathogenic initiators of CRS disease (Ebbens *et al.*, 2006, Ebbens *et al.*, 2009).

Recently however, allergic fungal rhinosinusitis (AFRS) has been described and it is now recognised as a distinct CRS disease (Glass *et al.*, 2011). AFRS is distinguished from CRSwNP due to the presence of allergic fungal mucin containing viable hyphae and patients show IgE-mediated responses to one or more fungi (Hutcheson *et al.*, 2010). As a distinct disease entity, AFRS will not be discussed further in this thesis.

1.2.2 Pathogenesis of chronic rhinosinusitis with nasal polyps

CRSwNP is associated with a Th2 response as summarised in Figure 1.1. The secretion of IL-5 by Th2 cells, mast cells and type 2 innate lymphoid cells (ILC2s) has been implicated in recruitment of eosinophils to the local nasal tissue and propagation of the

Th2 response through numerous mechanisms as reviewed by Spencer *et al.* (2010). IL-4 and IL-13 are able to induce B cell class switching to produce IgE antibodies, mediated through activation of Janus kinases (JAK) 1 and 3 and signal transducer and activator of transcription (STAT) 6 (Shum *et al.*, 2008). Following sensitisation, crosslinking of high affinity FcεRI receptors on mast cells and basophils bound by IgE results in cell degranulation and the release of preformed and *de novo* synthesised mediators including leukotrienes, prostaglandins and histamine. Histamine contributes to nasal congestion and mucus gland secretion, leading to rhinorrhea via H_1 receptor activation (Pawankar *et al.*, 2011). In addition, the presence of alternatively activated macrophages (M2) has been demonstrated in CRSwNP with numbers positively correlated with IL-5 and ECP levels (Spencer *et al.*, 2010). Furthermore, ILC2s also contribute to the Th2 response in CRSwNP (discussed in section 1.4.)

In addition to the exacerbated Th2 response, an altered regulatory T cell (Treg) response is observed in CRSwNP. A reduced Treg population has been described in polyps and was associated with a decrease in the expression of the Treg transcription factor forkhead box P3 (FOXP3) (Van Bruaene *et al.*, 2008). Moreover, higher levels of the pro-inflammatory cytokine IL-6 have also been detected and was demonstrated to free helper and effector T cells from the suppressive effects of IL-10 secreted by Treg (Pasare *et al.*, 2003, Kern *et al.*, 2008).

As the aetiology of CRSwNP is unknown, the pathomechanisms of the disease remain the subject of current debate. In the literature, two main hypotheses are generally accepted: the superantigen theory and the immune barrier dysfunction theory.

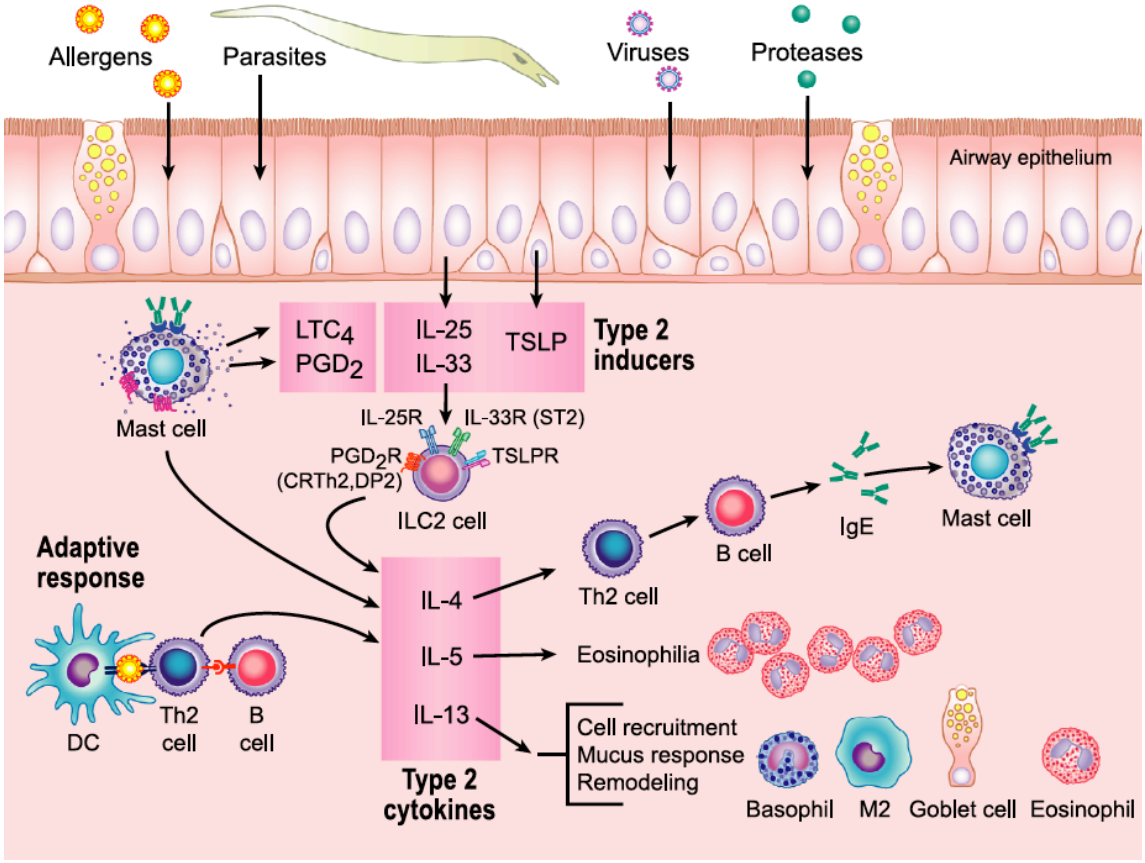


Figure 1.1 Overview of CRSwNP pathomechanisms

CRSwNP is associated with a Th2 response mediated by a number of immune cells including Th2 cells, B cells, ILC2s, mast cells, basophils, eosinophils and M2 macrophages. Epithelial-derived cytokines IL-25, IL-33 and TSLP are released in response to various stimuli in the nasal mucosa to potentiate the type 2 response. Image courtesy of Hulse *et al.* (2015).

1.2.2.1 Staphylococcal superantigen theory

CRSwNP patients are characterised by a high *Staphylococcus aureus* (*S. aureus*) colonisation rate compared to healthy controls. It is reported that 64% of CRSwNP patients and up to 80% of AERD patients are colonised with *S. aureus* compared to only 20-30% of healthy controls (Van Zele *et al.*, 2004). *S. aureus* has been found to exist in intracellular reservoirs in a number of cells including epithelial cells and myofibroblasts in the nasal mucosa of rhinosinusitis patients (Clement *et al.*, 2005). Sachse *et al.* (2010) have extended this observation further by showing that *S. aureus* is detected in

the epithelium of CRSwNP patients but not in CRSsNP. Biofilm formation has also been suggested as a reason for the persistent colonisation of the nasal mucosa by *S. aureus*. 60-70% of CRSwNP patients are biofilm positive and the presence of biofilm was found to be strongly associated with persistent nasal mucosal inflammation following surgery (Hochstim *et al.*, 2010, Wang *et al.*, 2014a).

In addition, S. aureus is able to produce superantigen microbial products such as staphylococcal enterotoxin (SE)-A, SEB and toxic shock syndrome toxin-1 (TSST-1) (Johnson *et al.*, 1991). In normal T cell activation, the T cell receptor (TCR) recognises foreign peptide presented by major histocompatibility (MHC) class II molecules in an antigen-specific manner. Superantigens are capable of interacting with T cells independently of the MHC peptide groove by crosslinking the variable β subunit of the TCR (TCR Vβ) with either or both of the αβ subunits of the MHC class II molecule (Figure 1.2). Antigen specificity is therefore not required and polyclonal activation of T cells is possible leading to substantial cytokine release and a strong inflammatory response (Johnson *et al.*, 1991, Brosnahan *et al.*, 2011).

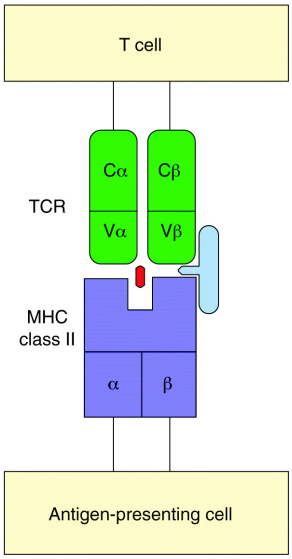


Figure 1.2 Schematic of superantigen binding to TCR and MHC Class II molecule.

Superantigen (light blue) is able to bind to the Vβ subunit of the TCR and crosslink with either or both of the αβ subunits of the MHC Class II molecule. This is independent of the peptide-binding grove required in conventional antigen presentation of peptides (red). Image courtesy of Papageorgiou *et al.* (2000).

The involvement of *S. aureus* in CRSwNP was first demonstrated with the detection of specific IgE to SEA and SEB in nasal polyp homogenates (Bachert *et al.*, 2001). A later study showed that increased levels of *S. aureus* enterotoxin specific-IgE in CRS patients with concomitant asthma or aspirin sensitivity were also associated with increased expression of ECP, total IgE and *S. aureus* colonisation which the authors concluded was indicative of a strong inflammatory reaction (Van Zele *et al.*, 2004). Furthermore, *S. aureus* superantigens (SEA, SEB, SEC, SED and TSST-1) were directly detected in polyp tissue using the enzyme-linked immunosorbent assay (ELISA) technique by Seiberling *et al.* (2005). Oligoclonal expansion of a larger number of TCR Vβ clones in polyps versus blood T cells has also been reported along with expansion of TCR Vβ domains associated with *S. aureus*, although this was observed in only 35% of patients examined (Conley *et al.*, 2006a, Conley *et al.*, 2006b).

A direct functional role of *S. aureus* in CRSwNP pathogenesis has been shown by Patou *et al.* (2008). The authors co-cultured nasal polyp tissue with SEB and another S. *aureus* virulence factor, Protein A (SpA). They were able to detect a relative increase in Th2 (IL-4, IL-5) and decrease in Treg cytokines (IL-10, TGFβ) upon culture of polyp tissue with SEB compared to control turbinate tissue. Furthermore, SpA co-culture led to increased levels of histamine and prostaglandin D2 (PGD_2) detected due to mast cell degranulation. Thus, superantigens are able to promote Th2 whilst supressing Treg responses in nasal polyps. Moreover, the Th2 environment also promotes the differentiation of $CD206^+$ M2 macrophages, which show reduced phagocytic activity of *S. aureus* in an *in vitro* phagocytosis assay (Krysko *et al.*, 2011).

Increased numbers of $CD19^+$ naïve B cells and $CD138^+$ plasma cells have been detected in CRSwNP compared to CRSsNP and control nasal tissues. Numbers of $CD20^+$ mature B cells were not however, concomitantly increased (Van Zele *et al.*, 2007). This was postulated to be due to the continuous presence of *S. aureus* acting as a stimulus for increased differentiation of $CD20^+$ B cells into plasma cells, resulting in the high IgE levels observed in CRSwNP. This was further supported by a later study with increased expression of class switch recombination-associated molecules (germline ε transcript, ε-mRNA, and IgE) detected in nasal polyps compared to allergic rhinitis or healthy controls (Gevaert *et al.*, 2013b).

In summary, polyclonal activation of Th2 cells by *S. aureus* superantigens can promote inflammation and eosinophilia in nasal polyposis through the release of cytokines and chemokines. Mast cells are abundant in nasal polyps and the presence of both *S. aureus* enterotoxin-specific and IgE has the potential to contribute to the constant degranulation of mast cells to further promote the inflammatory response (Zhang *et al.*, 2011).

1.2.2.2 Immune barrier dysfunction theory

A wealth of evidence, as discussed above, suggests that *S. aureus* superantigens play a role in driving CRSwNP disease. However, since only approximately 50% of CRSwNP patients express superantigen-specific IgE and colonisation of the nasal mucosa by *S. aureus* is observed in 20-30% of healthy people, it has been hypothesised that the aetiology of CRSwNP must be due to an inherent defect in the epithelium, either immunological or mechanical (Kern *et al.*, 2008). Along this line, Meng *et al.* (2013) have shown that the epithelial cell tight junction proteins; E-cadherin, zonula occludens-1 (ZO-1) and occludin, are expressed at lower levels in mature CRSwNP polyps compared to in the healthy nasal mucosa (Figure 1.3). The impairment of the epithelial barrier function in polyps is further supported by the heightened levels of oncostatin M (OSM) expressed in polyps compared to control uncinate tissue (Pothoven *et al.*, 2015).

OSM is a member of the IL-6 family of cytokines and levels are increased in Th2-mediated diseases including allergic rhinitis (Kang *et al.*, 2005) and asthma (Simpson *et al.*, 2009). Stimulation of nasal epithelial air liquid interface cultures with OSM resulted in compromised tight junction structures, increased dextran flux and decreased transepithelial electrical resistance indicating increased permeability (Pothoven *et al.*, 2015). This impaired barrier function was proposed to enable the infiltration of stimuli such as allergens and pathogens into the nasal mucosa resulting in a chronic inflammatory response (Figure 1.3). Furthermore, stimulation of nasal epithelial cells from AFRS patients directly with Th2 cytokines (IL-4 and IL-13) similarly resulted in decreased transepithelial electrical resistance and reduced tight junction protein expression (Wise *et al.*, 2014) and suggests that the strong Th2 signature in CRSwNP may also play a direct role in disruption of the epithelial barrier function. Moreover, activation of the epithelium by pathogens such as bacteria or virus may also lead to apoptosis of epithelial cells resulting in a compromised immune barrier (White, 2011).

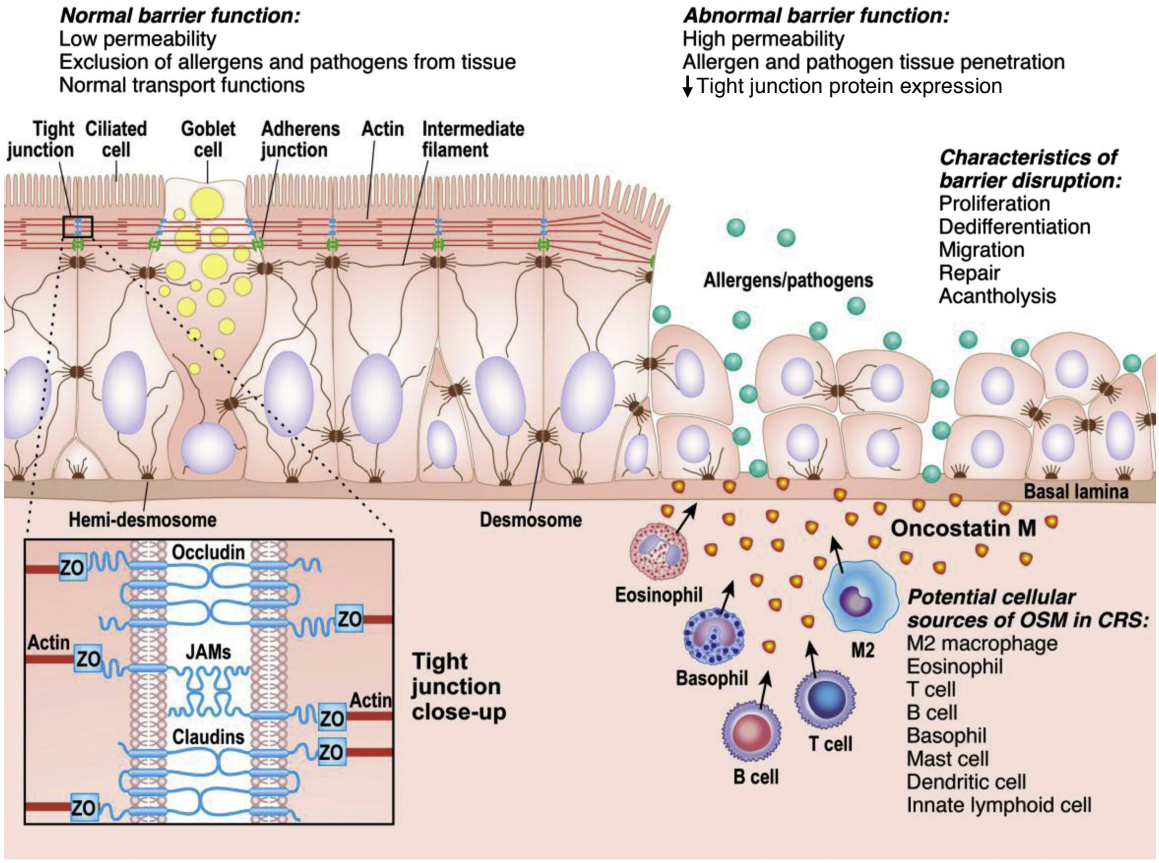


Figure 1.3 Impaired barrier function in CRSwNP

The epithelium in CRSwNP is associated with decreased tight junction protein expression and increased permeability to exogenous stimuli, partly mediated by oncostatin M (OSM). Potential sources of OSM are listed. Figure modified from Pothoven *et al.* (2015).

The epithelium functions in a defensive role in the nasal cavity by acting as a barrier to invading pathogens but also with the release of antimicrobial peptides (AMPs). This is however, compromised in CRSwNP with decreased expression of the calcium-sensing S100 family proteins; S100A7 (psoriasin) and S100A8 (calgranulin A) reported compared to healthy controls (Richer *et al.*, 2008, Tieu *et al.*, 2010). These have all been extensively associated with antimicrobial activity (Boniface *et al.*, 2005, Wolk *et al.*, 2006, Eyerich *et al.*, 2009) and are involved in regulating a wide range of processes including proliferation, inflammation and apoptosis (Donato *et al.*, 2013). For example, S100A7 has been shown to kill *Escherichia coli* in human skin in a zinc-dependent

manner (Glaser *et al.*, 2005). Recalcitrant polyps were associated with reduced expression of the IL-22 receptor (IL-22R) with only 55% of epithelial cells from CRSwNP patients expressing IL-22R compared to 100% of CRSsNP and control samples (Ramanathan *et al.*, 2007b). IL-22 has been shown to induce the secretion of S100A7 and S100A8 by epithelial cells and thus, a deficient or lack of IL22R expression on nasal epithelial cells could be a possible reason for the diminished levels of the S100 family of AMPs in CRSwNP.

Diminished levels of other AMPs are also observed in CRSwNP. The PLUNC family protein – short, palate, lung, and nasal epithelial clone 1 (SPLUNC1) – is expressed and secreted by the nasal epithelium and was demonstrated to be involved in the innate immune response against *Pseudomonas aeruginosa* (Sayeed *et al.*, 2013) and *Mycoplasma pneumonia* (Gally *et al.*, 2011). In line with a defective immune barrier, reduced expression of SPLUNC1 was reported in CRSwNP polyps compared to uncinate tissue from CRSsNP and healthy controls (Seshadri *et al.*, 2012, Wei *et al.*, 2014). In addition to AMPs, Richer *et al.* (2008) have also shown that expression of the serine protease inhibitor kazal type 5 (SPINK5) is reduced in CRSwNP versus healthy control. Although the exact mechanism of action is unknown, SPINK5 has been hypothesised to protect gap junctions from proteases secreted by pathogens to maintain epithelial barrier integrity (Tieu *et al.*, 2009).

In summary, these studies suggest that a defective epithelial immune and mechanical barrier exists in CRSwNP. This inherent defect may lead to the increased penetration by pathogens such as *S. aureus* into nasal tissues resulting in chronic inflammation. Indeed, stimulation of pattern recognition receptors (PRR) by pathogen-associated molecular patters (PAMPS) and danger signals have been extensively linked to an inflammatory response (O'Neill *et al.*, 1998, Akira *et al.*, 2006, Mogensen, 2009). Although the role of toll like receptors (TLRS) in polyposis remains unclear with conflicting studies

regarding their expression - especially for TLR9 - in the literature (Ramanathan *et al.*, 2007a, Zhao *et al.*, 2011, Zhang *et al.*, 2013), polymorphisms in taste receptors expressed by epithelial cells are associated with increased susceptibility to upper respiratory tract infections (Hulse *et al.*, 2015). In a study by Lee *et al.* (2012), the authors showed that the bitter taste receptor T2R38 induces nitric oxide production in response to quorum-sensing molecules secreted by *Pseudomonas aeruginosa*. Individuals able to detect the bitter compound phenylthiocarbamide were associated with the homozygous PAV/PAV 'taster' polymorphism in the T2R38 gene and were able to respond and clear Gram-negative bacteria more effectively compared to PAV/AVI heterozygous or homozygous AVI/AVI 'non-tasters'. A further study has identified that CRSwNP patients displaying the 'taster' polymorphism were less likely to require surgical intervention for nasal polyps compared to heterozygous or 'non-taster' patients (Adappa *et al.*, 2013). Thus, these studies suggest that PRRs may be linked to a defective epithelial immune barrier in the pathogenesis of CRSwNP.

1.2.3 Treatment of CRSwNP

CRSwNP is considered a difficult to treat disease due to its unknown aetiology and heterogeneity of the disease. There is an unmet clinical need for effective treatments in CRSwNP. Current therapy options are limited and mainly involve the management of symptoms along with control of the underlying inflammation as discussed below.

1.2.3.1 Corticosteroids

Intranasal corticosteroids are considered the 'cornerstone of maintenance treatment' (Bachert *et al.*, 2014) by limiting chronic inflammation. A meta-analysis study has also reported that intranasal corticosteroids are effective and safe for post-operative use and was associated with reduced symptom scores and polyp reoccurrence in the first year following sinus surgery (Fandiño *et al.*, 2013). In addition, topical and oral systemic

corticosteroids have both been shown to be effective at improving CRSwNP outcome measure scores (Rudmik *et al.*, 2012, Howard *et al.*, 2013).

1.2.3.2 Biologics

Various biologics aimed at reducing the Th2 response have been examined for use in the context of CRSwNP therapy. Anti-IL-5 monoclonal antibodies represent one such class of biologics that has received considerable interest in recent years. In a double-blind, placebo-controlled, randomised trial with a single intravenous infusion of the humanised anti-IL-5 monoclonal antibody reslizumab, Gevaert *et al.* (2006) reported that serum eosinophil numbers and levels of ECP were reduced in patients administered reslizumab for up to 8 weeks following treatment. Reduction in nasal polyp size was only observed in 50% of patients who received reslizumab at 4 weeks post treatment. Interestingly, nasal IL-5 levels were determined to be a predictive marker of patients that would respond to anti-IL-5 treatment. In another double blind, placebo-controlled, randomised trial with mepolizumab, active treatment resulted in significant improvements in symptom scores at 8 weeks and was associated with significant reduction in nasal polyp size at 4 weeks post treatment (Gevaert *et al.*, 2011). These studies demonstrate that anti-IL-5 biologics may have some potential for treating CRSwNP.

High levels of IgE are detected in CRSwNP and as such, the anti-IgE monoclonal antibody omalizumab has been postulated to be a potential therapeutic. A small double blind, placebo-controlled trial failed to demonstrate any significant improvement in all clinical outcomes measured (Pinto *et al.*, 2010). However, a more recent study has demonstrated clinical efficacy of omalizumab with significant reduction in nasal polyp scores at 16 weeks following treatment accompanied by reduced airway symptoms such as nasal congestion and rhinorrhea (Gevaert *et al.*, 2013a). Together, these small-scale

studies indicate that anti-IL-5 and anti-IgE antibodies are attractive and promising therapeutic strategies for CRSwNP disease. However, longer-term studies with a larger number of subjects will be required to fully determine the clinical effectiveness of these biologics. Dupilumab, a humanised monoclonal antibody against the alpha receptor of the IL-4 receptor, targets both IL-4 and IL-13 and was shown to reduce the number of exacerbations in a subset of patients with persistent asthma (Wenzel *et al.*, 2013). It is now postulated as another potential therapeutic in CRSwNP (De Schryver *et al.*, 2015).

1.2.3.3 Other therapies

Nasal saline sprays and saline rinsing are routinely performed in CRSwNP and numerous studies have shown that saline use is beneficial at relieving symptoms (Cain *et al.*, 2013, Bachert *et al.*, 2014). It is more commonly used as an adjunctive therapy but with no evidence that it is more effective than other pharmacotherapy such as intranasal corticosteroids (Harvey *et al.*, 2007). In addition, the use of anti-leukotrienes, such as the 5-lipoxygenase inhibitor zileuton and particularly the cysteinyl leukotriene receptor 1 (CysLT1) antagonist montelukast, has been explored for CRSwNP therapy. In a systemic review and meta-analysis of clinical trials evaluating the use of anti-leukotrienes in CRSwNP, Wentzel *et al.* (2013) reported that specific symptoms such as rhinorrhea and hyposmia were improved with the use of montelukast compared to intranasal corticosteroids. However, adjunctive therapy of anti-leukotrienes with intranasal steroids showed only slight improvements in symptom scores compared with either treatment alone. Indeed, in the European Position Paper on Rhinosinusitis and Nasal Polyps (EPOS) 2012, they are not recommended for use in the treatment of CRSwNP (Fokkens *et al.*, 2012).

1.2.3.4 Surgical intervention

In cases of nasal polyposis uncontrolled by pharmacotherapy, surgical excision of nasal polyps (polypectomy) by endoscopic sinus surgery (ESS) is the standard treatment strategy. Polyps frequently reoccur following surgery with one study reporting that 60% of patients with severe disease who underwent ESS developed recurrent nasal polyps within a median time frame of 40 months (Wynn *et al.*, 2004). Furthermore, the recurrence rate was reported to be higher in patients with concomitant asthma and AERD (Wynn *et al.*, 2004, Mendelsohn *et al.*, 2011). Patients often require revision surgery thus highlighting the unmet therapeutic need in CRSwNP disease.

1.3 The developing subsets of helper T cells

The role of the Th2 response in allergic disease, and to a lesser extent nasal polyposis, has been extensively investigated since Mosmann *et al.* (1986) first proposed the Th1/Th2 paradigm. More recently, human Th17 (Cua *et al.*, 2003), Th9 (Veldhoen *et al.*, 2008b) and Th22 subsets (Eyerich *et al.*, 2009) which produce their namesake interleukins have been described (Figure 1.4), although the role and significance of these phenotypes in upper respiratory diseases is less clear.

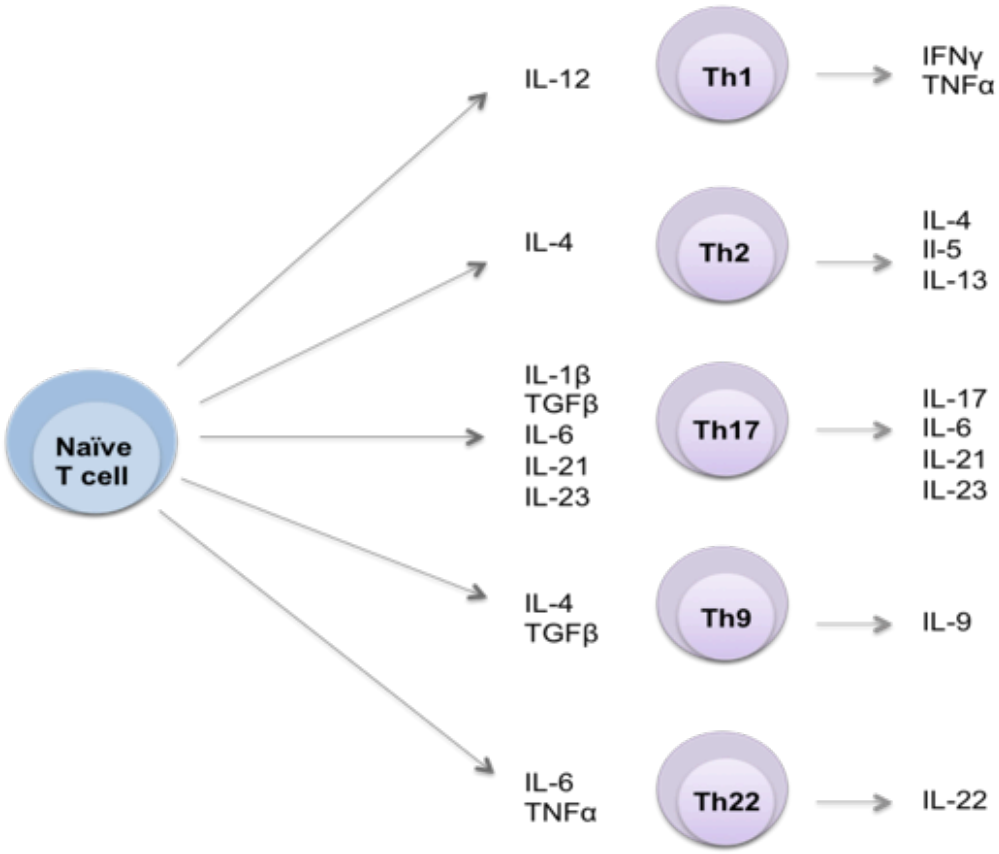


Figure 1.4 T helper cell differentiation

Upon exposure to various cytokines, naïve T cells are polarised to differentiate into one of the known T helper cell subsets: Th1, Th2, Th9, Th17 and Th22. The cytokine combinations inducing polarisation and the prototypical cytokines produced by each T cell subset are shown.

1.3.1 Th1 and Th2 cells

Th1 cells are defined by their expression of the chemokine receptor CXCR3 and are characterised by production of the prototypical cytokine IFNγ as well as IL-12, tumour necrosis factor (TNF)α and TNFβ (Mosmann *et al.*, 1986, Sallusto *et al.*, 1998). Th2 cells on the other hand express CCR4 and chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) and are characterised by the production of IL-4, IL-5 and IL-13 (Cosmi *et al.*, 2000, Romagnani, 2000). Although the chemokine receptors CCR5 and CCR3 have also been reported to be markers of Th1 and Th2 cells respectively (Sallusto *et al.*, 1997, Loetscher *et al.*, 1998), Yamamoto *et al.* (2000) demonstrated that CXCR3 and CCR4 were more efficient at identifying circulating effector Th1 and Th2 cells in the periphery compared to CCR5 and CCR3. Tbet was reported to be the master transcription factor responsible for Th1 lineage commitment (Szabo *et al.*, 2000) with expression of GATA3 associated with lineage commitment to a Th2 cell population (Zhu *et al.*, 2006). Interestingly, reciprocal regulation of Th1/Th2 differentiation has been observed with IL-12, required for Th1 differentiation, able to inhibit Th2 development and the reverse observed with the Th2 polarising cytokine IL-4 (Figure 1.4) (Maggi *et al.*, 1992, O'Garra, 1998).

Th1 cells are primarily involved in the immune response against invading intracellular pathogens by activating macrophages and neutrophils as well as inducing B cell-mediated production of opsonizing and complement-binding antibodies (Romagnani, 2000). In contrast, Th2 cells are mainly involved in responses to extracellular pathogens by inducing IgE antibody production from B cells and activation of eosinophils to clear pathogens including helminths (Sallusto *et al.*, 1999). In addition to their protective roles, both Th1 and Th2 subsets are involved in human disease. The Th1 population was widely believed to be associated with autoimmune pathologies such as multiple sclerosis and rheumatoid arthritis. However, with the discovery of the Th17 cell subset,

this view has now shifted with the relative roles of Th1 and Th17 cells the subject of continued debate (Dardalhon *et al.*, 2008b). Th2 cells have been extensively implicated in mediating allergic diseases and Robinson *et al.* (1992) first reported the involvement of a Th2 cell population in atopic asthma with the increased expression of IL-4 and IL-5 mRNA in bronchoalveolar lavage fluid (BAL) T cells from atopic asthmatics compared to healthy controls.

1.3.2 Th17 cells

Th17 cells express the chemokine receptor CCR6 and are characterised by the production of IL-17A, IL-17F, IL-21 and IL-23 but can also co-express IFNγ and IL-22. Expression of the transcription factor retinoic acid-related orphan nuclear hormone receptor C (RORC) is associated with lineage commitment of human naïve $CD4^+$ cells to Th17 cells (Korn *et al.*, 2009). A number of cytokines are purported to be required for differentiation including TGFβ, IL-21, IL-23, IL-1β and IL-6 (Burgler *et al.*, 2009, de Wit *et al.*, 2011). However, the necessity for each cytokine and their combination is uncertain with contradictory findings reported in the literature, especially regarding the requirement for TGFβ, although this is probably necessary for induction of RORC expression (Acosta-Rodriguez *et al.*, 2007, Manel *et al.*, 2008).

The expression of IL-17A and IL-22 by $CCR6^+$ memory T cells is potently induced by cytokines that share the common-γ (γc) chain such as IL-2, IL-7 and IL-15 (Wan *et al.*, 2011). Culture of *ex vivo* isolated $CCR6^+IL\text{-}17^+$, $CCR6^+IL\text{-}17^-$, and $CCR6^-IL\text{-}17^-$ cells from peripheral blood with IL-2 for 7 days resulted in the detection of a 20-40% $IL\text{-}17^+$ population compared to 5-10% in *ex vivo* $CCR6^+IL\text{-}17^+$ cells. This induction of IL-17 production was observed in cells that were previously $CCR6^+IL\text{-}17^-$, demonstrating that CCR6 expression is a stable marker of IL-17 producing cells. Furthermore, the authors

suggest that this ability to express IL-17 following γc-chain stimulation results in an underrepresentation of the frequency of Th17 cells analysed from tissues *ex vivo*.

Th17 cells may be able to promote airway inflammation as co-culture of *in vitro* differentiated Th17 cells with normal human bronchoepithelial cells (HBEC) resulted in a significant increase in epithelial production of the pro-inflammatory cytokine IL-6 (Burgler *et al.*, 2009). Furthermore, a Th17 cell association with autoimmune inflammatory diseases has been reported with a significantly higher number of Th17 cells detected in the synovial fluid of rheumatoid arthritis patients compared to peripheral blood (Church *et al.*, 2010). A study by Annunziato *et al.* (2007) also reported that in T cells expanded from bowel specimens of Crohn's disease patients, a significant proportion were IL-17^{+}CD4^{+} and had the ability to co-express IL-17 and IFNγ. This Th17/Th1 population was associated with a loss of RORC expression and an increase in Tbet^{+}IFNγ^{+} cells upon culture with IL-12, a finding corroborated by Bending *et al.* (2009) in a non-obese diabetic/severe combined immunodeficient (NOD/SCID) mouse model. This suggests that Th17/Th1 plasticity is probable but the effect of this in the context of human disease remains to be fully determined.

1.3.3 Th22 cells

Th22 cells are the most recently described T cell subset and are present locally in skin inflammation associated with psoriasis (Boniface *et al.*, 2007, Zheng *et al.*, 2007). Th22 cells produce IL-22 in the absence of IL-17 and IFNγ and express CCR6 as well as the skin-homing receptors CCR4 and CCR10 (Duhen *et al.*, 2009). In addition, transcriptome analysis has shown that Th22 cells produce more IL-22 than Th17 cells and do not express CCL20 or the IL-23 receptor (Eyerich *et al.*, 2009). Th22 cells differentiate from naïve cells under IL-6 and TNFα culture conditions (Figure 1.4) and aryl hydrocarbon receptor ligands such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)

have been shown to decrease Th17 polarisation and favour an IL-22 producing subset, suggesting aryl hydrocarbon receptor may be a lineage commitment transcription factor for Th22 differentiation (Ramirez *et al.*, 2010). TCDD increased the population of $CD4^+$ cells that were IL-22^+ but not IFNγ^+ or IL-17^+ and reduced Th17 populations (IL-17^+IL-22^+, IL17^+IL-22^-) by decreasing RORC expression. Furthermore, the authors showed that IL-23 neutralization and IL-1 blockade did not affect IL-22 production induced by TCDD and these IL-22^+ cells were also negative for CD161, a marker of Th17 cells, demonstrating that these cells are a distinct population.

The role of IL-22 in human disease is not fully characterised. An expansion of Th22 cells was observed in the peripheral blood of patients with ankylosing spondylitis and rheumatoid arthritis, together with a positive correlation with Th17 cells (Zhang *et al.*, 2012). Furthermore, circulating Th22 cells are increased in psoriasis patients and correlate with disease severity (Kagami *et al.*, 2010). Culture of keratinocytes with supernatants of activated Th22 cultures resulted in upregulation of pro-inflammatory innate immune genes, which was potentiated in the presence of TNFα (Eyerich *et al.*, 2009) suggesting Th22 cells promote inflammation by amplifying TNFα signals. IL-22 also augmented wound repair in a functional keratinocyte *in vitro* injury model with the addition of TNFα having no effect. IL-22 therefore appears to be pathogenic under inflammatory conditions but may also be tissue protective and involved in regeneration under non-inflammatory conditions. This was also supported by Sonnenberg *et al.* (2010) with IL-22 administration able to protect murine airway epithelial cells from bleomycin-induced apoptosis. However, addition of IL-17 with IL-22 resulted in the loss of the tissue protective ability of IL-22.

1.3.4 Th9 cells

IL-9 was initially considered to be a Th2 cytokine. The subsequent identification of an

IL-9-only producing Th cell population (Dardalhon *et al.*, 2008a, Veldhoen *et al.*, 2008b) and the identification of PU.1 as a lineage commitment transcription factor has led to recognition of Th9 cells as a distinct lineage of Th cells (Chang *et al.*, 2010). Th9 cells in humans are characterised by the release of IL-9 in the absence of Th2 cytokines or IL-10 (Figure 1.4). Differentiation from naïve cells occurs in the presence of IL-4 and TGFβ, with IL-25 able to augment IL-9 production through the IL-25 receptor, IL-17RB (Angkasekwinai *et al.*, 2007). In line with the idea that Th2 cells produce IL-9 however, it has been demonstrated that plasticity between Th9 and Th2 cells exists with TGFβ able to redirect Th2 cells to an IL-9 producing subset (Veldhoen *et al.*, 2008b). This shifting of Th2 cells to the Th9 phenotype with TGFβ has been suggested to be due to PU.1 induction with similarities between Th9 and Th2 cells, such as IL-4-induced STAT6 and interferon regulatory factor-4 (IRF4) expression, allowing for this plasticity to occur (Staudt *et al.*, 2010, Goswami *et al.*, 2012). Furthermore, Perumal *et al.* (2011) have suggested that the epigenetic changes produced by PU.1 induction are able to shift Th9 specific genes from a poised state i.e. expressing both active and repressive histone modification marks, to an active state to allow rapid transcribing and the switch of the T helper cell phenotype from Th2 to Th9.

Studies examining the involvement of Th9 cells in allergy have been confounded by additional cell types that can also produce IL-9 such as innate lymphoid cells (Wilhelm *et al.*, 2011, Stassen *et al.*, 2012). The precise source of IL-9 cannot be conclusively shown to be Th9 cells although the expression of IL-9 in allergy has been extensively documented. IL-9 was selectively produced in an allergen specific manner by peripheral blood mononuclear cells isolated from house dust mite (HDM) allergic patients (Devos *et al.*, 2006). Furthermore, anti-IL-9 antibody treatment in an HDM-induced murine asthma model was able to prevent airway remodelling and mast cell infiltration into the lung (Kearley *et al.*, 2011). Moreover, IL-9 mRNA levels were found to be upregulated

in nasal biopsy specimens from allergic rhinitis patients, correlating with eosinophil recruitment which was inhibited by immunotherapy (Nouri-Aria *et al.*, 2005). These studies suggest that IL-9 is involved in allergic diseases but the exact role of Th9 cells in this process remains to be defined.

Collectively, these studies illustrate the potential complexity of the allergic response within the context of evolving knowledge of effector T cell phenotypes. Further work is still required to characterise the roles played by these recently described T-helper cell subsets in respiratory mucosal allergic airway disease and also to understand their interplay and co-regulation with each other.

1.4 Innate lymphoid cells

The description of an innate non-B/non-T cell population capable of producing a Th2 response was first reported in a study involving intranasal IL-25 administration in mice infected with *Nippostrongylus brasiliensis* (Hurst *et al.*, 2002). These cells were identified to belong to a non–B/non–T, c-kit^{+}, FcεR1^{-} population by Fallon *et al.* (2006) and produced IL-13 at an early stage of infection. Subsequent studies from separate groups using IL-4/IL-13 reporter mice showed that effective clearance of *N. brasiliensis* required the presence of this innate cell population, initially termed 'nuocytes' (Neill *et al.*, 2010) and 'innate type 2 helper cells' (Price *et al.*, 2010), and that expansion in response to IL-25 and IL-33 was required for effective worm clearance. The first demonstration of a type 2 innate cell in human disease was reported by Mjosberg *et al.* (2011) with a population of CRTH2^{+}CD161^{+}IL-7Rα^{+} cells that were present in lung, gut and nasal tissues. These cells responded to IL-25 and IL-33 with enhanced IL-13 production and culture of foetal gut CRTH2^{+} cells showed that they were a stable cell lineage. Furthermore, the authors found that these cells expressed the IL-25 receptor, also known as IL-17RB in addition to the receptor for IL-33, ST2. These innate populations are now collectively termed type 2 innate lymphoid cells (ILC2s).

ILC2s are required for the induction of the adaptive Th2 response. In a papain-induced model of allergic lung inflammation, Halim *et al.* (2014) showed that early infiltration of eosinophils and neutrophils into the BAL and lung tissue and low level Th2 cytokine production were dependent on the presence of ILC2s but not T or B cells as RAG$^{-/-}$ mice showed similar responses. However, this was not observed in *Rora*$^{sg/sg}$ bone marrow transplanted (BMT) ILC2-deficient mice. RORα transcription factor is required for ILC2 differentiation (Halim *et al.*, 2012, Wong *et al.*, 2012) and *Rora*$^{sg/sg}$ BMT mice were generated from the transplantation of bone marrow from ILC2-deficient *Rora*$^{sg/sg}$

mice into irradiated wild-type mice. Furthermore, the authors also demonstrated that at day 20 following papain challenge, the adaptive Th2 cell response and lung inflammation was drastically reduced in *Rora*$^{sg/sg}$ BMT mice compared to wild-type BMT mice. This was shown to be due to the lack of ILC2-mediated IL-13 production required for lung dendritic cell (DC) activation and the subsequent promotion of Th2 cell differentiation from naïve T cells (Figure 1.5).

In accordance with their role in mediating Th2 responses, ILC2s have been implicated in a number of allergic diseases. Numerous studies have shown that increased numbers of ILC2s are observed in nasal polyps from CRSwNP patients compared to healthy nasal tissue (Mjosberg *et al.*, 2011, Licona-Limon *et al.*, 2013, Shaw *et al.*, 2013, Miljkovic *et al.*, 2014). In addition, ILC2s have also been implicated in atopic dermatitis (Kim *et al.*, 2013, Salimi *et al.*, 2013), allergic rhinitis (Doherty *et al.*, 2014) and in animal models of allergic lung inflammation (Klein Wolterink *et al.*, 2012, Li *et al.*, 2013). Furthermore, the number of ILC2s present in the periphery during the pollen season is decreased by subcutaneous grass pollen immunotherapy (Lao-Araya *et al.*, 2014).

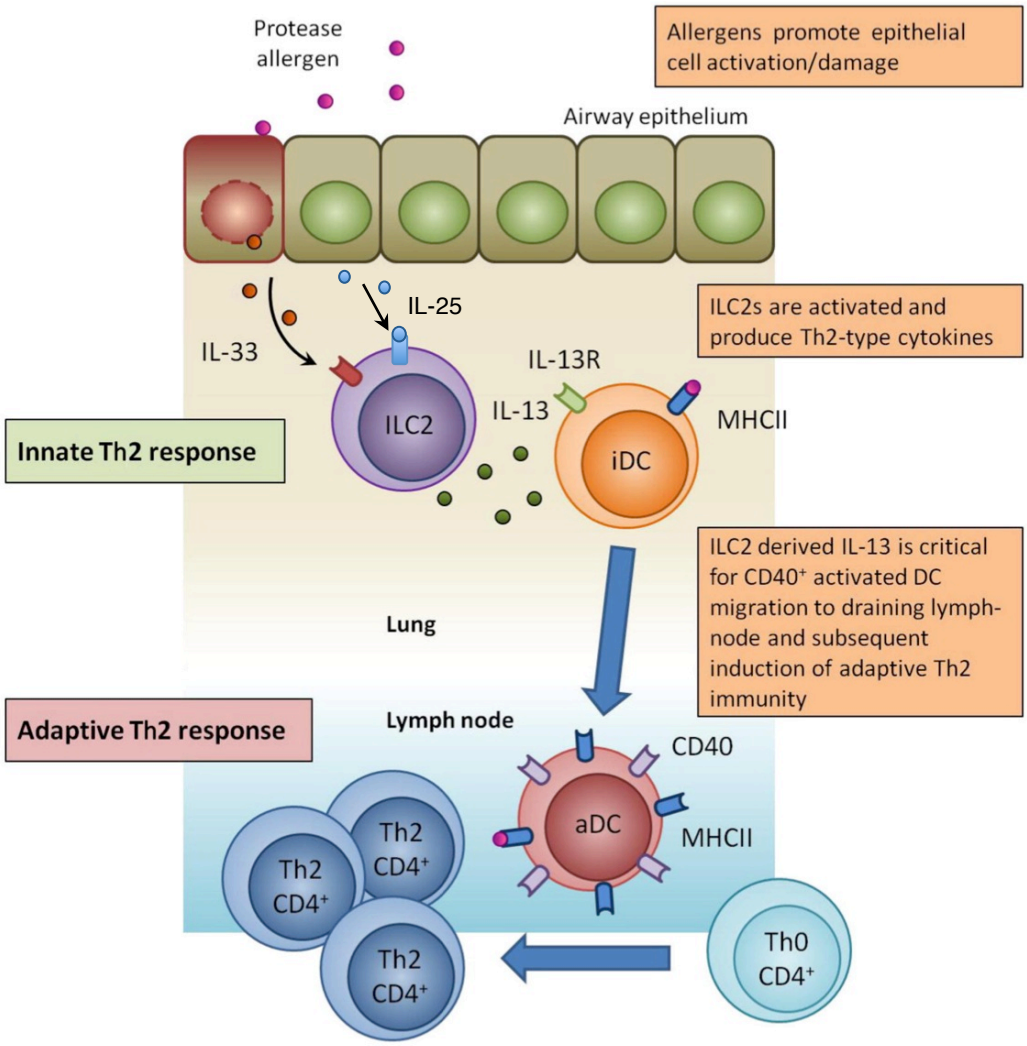


Figure 1.5 ILC2s are capable of inducing the adaptive Th2 response

ILC2s respond to IL-25 and IL-33 produced by epithelial cells in response to allergens and other danger signals leading to the production of IL-13. ILC2-derived IL-13, via lung DCs, induces the differentiation of Th2 cells from naïve T cells. Figure modified from Halim *et al.* (2014).

1.5 Epithelial cell derived cytokines

Recently, the three novel epithelial cell-derived cytokines IL-25, IL-33 and TSLP have received considerable interest. As reviewed by Divekar *et al.* (2015), all three cytokines are involved in promoting type 2 responses and are recognised to play important roles in allergic diseases.

1.5.1 Interleukin-25

IL-25, also known as IL-17E, is the newest member of the IL-17 family of cytokines sharing 16-20% homology with IL-17A, IL-17B and IL-17C (Lee *et al.*, 2001). However, IL-25 plays a distinct role and is involved in promoting type 2 responses and the subsequent pathology of allergic diseases (Fort *et al.*, 2001, Hurst *et al.*, 2002, Tamachi *et al.*, 2006, Ballantyne *et al.*, 2007, Gregory *et al.*, 2012). In contrast, IL-17A and IL-17F are involved in promoting type 1 responses against bacteria and other infections (Ishigame *et al.*, 2009, Reynolds *et al.*, 2010).

IL-25 is produced by a wide variety of immune and structural cells. Ikeda *et al.* (2003) reported that mast cells expressed IL-25 mRNA upon activation of FcεR1 receptors by IgE crosslinking. $CD15^{+}CD16^{-}CD45^{+}$ eosinophils were found to be responsible for the release of IL-25 in peripheral blood samples from Churg-Strauss syndrome patients, a Th2-dominated disease associated with eosinophilia, vasculitis and asthma (Terrier *et al.*, 2010). Furthermore, IL-5 was able to induce eosinophils from the peripheral blood of both healthy and atopic patients to secrete IL-25 protein (Wang *et al.*, 2007). The authors also reported that IgE crosslinking on basophils resulted in IL-25 release with levels in atopic patients twice that observed in healthy individuals. In addition, numerous studies have demonstrated the production of IL-25 by epithelial cells (Angkasekwinai *et al.*, 2007, Takahashi *et al.*, 2011). Corrigan *et al.* (2011a) have shown that IL-25 can be detected in the epithelium of bronchial mucosa from asthmatic

patients and epithelial cell-derived IL-25 is required for the development of murine asthma (Suzukawa *et al.*, 2012). Higher levels of IL-25 mRNA were found in sinus mucosal samples from CRSwNP patients compared to CRSsNP or healthy controls (Lam *et al.*, 2013) and was reported to be predominantly produced by epithelial cells in an Asian cohort of CRSwNP patients (Shin *et al.*, 2015).

The mechanism for the induction of IL-25 expression from epithelial cells is unclear but may involve allergens and respiratory viruses. Primary mouse epithelial cells release IL-25 when stimulated with airborne allergens such as *Aspergillus* (Angkasekwinai *et al.*, 2007) and normal human bronchoepithelial cells were reported by Kouzaki *et al.* (2013) to respond in a similar manner upon house dust mite stimulation, although this was probably due to the presence of proteases (Asokananthan *et al.*, 2002). Rhinovirus infection also increases the capacity for IL-25 release from cultured primary bronchoepithelial cells of asthmatic patients (Beale *et al.*, 2014).

1.5.1.1 IL-25 receptor

IL-25 forms a homodimer to bind to its heterodimeric receptor IL-25R (also known as IL-17RB). This receptor is formed from IL-17RA and IL-17RB and both subunits are required for IL-25 function (Rickel *et al.*, 2008). IL-17B is also a ligand for IL-17RB however, IL-17RB binds IL-25 with a much higher affinity. Furthermore, IL-17B has not been demonstrated to signal via the IL-25R (Chang *et al.*, 2011, Iwakura *et al.*, 2011). Expression of IL-25R has been observed on a number of immune cells including Th2 cells, Th9 cells, basophils, macrophages, invariant natural killer T (iNKT) cells, ILC2s, DCs, airway smooth muscle cells (ASMC), fibroblasts and endothelial cells (Pappu *et al.*, 2012). The cell surface expression of IL-25R is upregulated upon T cell activation and Wong *et al.* (2007) have demonstrated that IL-25R signals via the intracellular JNK, p38 MAPK and NFκB pathways. Furthermore, memory Th2 cell

expression of IL-25R is strongly upregulated by TSLP-activated dendritic cells and this upregulation was greater compared to anti-CD3/CD28 stimulation (Wang *et al*, 2006). Moreover, the authors also demonstrated that only activated Th2 cells could respond to IL-25. No IL-25-mediated enhancement of memory Th2 cell proliferation or induction of effector memory differentiation was observed in cultures stimulated with medium only compared to TSLP-DC stimulated cultures.

The pathological role of IL-25R in a number of allergic diseases has been demonstrated. IL-25R is constitutively expressed by lung fibroblasts and IL-25R mRNA is upregulated in response to TNFα stimulation (Letuve *et al.*, 2006). The authors showed that TNFα and IL-25 are able to act synergistically to induce granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-8 production from fibroblasts, leading to the initiation and maintenance of eosinophilic infiltration in allergic inflammation. IL-25R expression is also upregulated in primary human ASMC by TNFα, with IL-25 shown to increase expression of extracellular matrix components such as procollagen-αI suggestive of a role in airway remodelling (Lajoie-Kadoch *et al.*, 2006). This role was further supported by studies on human endothelial cells, which constitutively express IL-25R and respond to IL-25 to promote expression of the angiogenic and remodelling cytokine basic fibroblast growth factor (Wang *et al.*, 2012) and expression of vascular endothelial growth factor (VEGF) and its receptor VEGFR to promote angiogenesis in asthma (Corrigan *et al.*, 2011b, Keglowich *et al.*, 2015). Moreover, IL-25 and IL-25R mRNA transcripts are increased in asthma and atopic dermatitis (Wang *et al.*, 2007) and IL-25R expression by eosinophils is increased, along with plasma IL-25 levels, in allergic asthma patients compared to healthy controls (Tang *et al.*, 2014).

1.5.2 IL-33

IL-33 is a member of the IL-1 family of cytokines consisting of IL-1, IL-18 and IL-36. Similar to IL-25 however, it plays a distinct role compared to other members of its family. IL-18 and IL-1β have been implicated in mediating pro-inflammatory and autoimmune diseases such as rheumatoid arthritis, psoriasis and inflammatory bowel disease (Arend *et al.*, 2008) and although the function of IL-36 is still relatively uncharacterised, it has been suggested to play a role in the pathology of psoriasis (Towne *et al.*, 2012). This is in contrast to the function of IL-33, which has been demonstrated to promote type 2 responses, display chemotactic activity for Th2 cells (Komai-Koma *et al.*, 2007) and is involved in the pathology of allergic diseases including atopic dermatitis and asthma (Kurowska-Stolarska *et al.*, 2008, Kearley *et al.*, 2009, Salimi *et al.*, 2013, Divekar *et al.*, 2015). Moreover, IL-33 activity was reported to be independent of the requirement for caspase-1 processing which is essential for IL-18 and IL-1β activity (Talabot-Ayer *et al.*, 2009) further highlighting its distinct role in the family.

IL-33 is constitutively expressed in the nucleus of fibroblasts, endothelial cells and epithelial cells (Moussion *et al.*, 2008) and several studies have shown that it is released in response to danger and stress signals as an 'alarmin' (Carriere *et al.*, 2007). Mechanical stretching of primary human fibroblasts and stimulation of human monocytes with the acute phase protein, serum amyloid A, result in increased concentrations of IL-33 being released (Kakkar *et al.*, 2012, Sun *et al.*, 2014). In an asthma exacerbation murine model, rapid release of IL-33 was observed upon *Alternaria* challenge resulting in eosinophil and ILC2 infiltration and greater pulmonary inflammation compared to controls (Snelgrove *et al.*, 2014). This was dependent on *Alternaria* serine protease activity, as this effect was not observed with heat-killed *Alternaria*. Kouzaki *et al.* (2011) have corroborated this finding *in vitro* with HBECs. In

addition, the authors showed that this process involved another danger signal, adenosine triphosphate (ATP), as blockade of the P2Y2 ATP receptor was able to inhibit IL-33 release. Furthermore, stimulation of polyp epithelial cells with ATP and high-mobility group box-1 (HMGB-1), both recognised to be damage-associated molecular patterns (DAMPS), induced significantly upregulated expression of IL-33 mRNA and protein compared to control nasal epithelial cells (Paris *et al.*, 2014).

IL-33 has been implicated in a number of diseases including psoriasis (Kay *et al.*, 2015), asthma (Kurowska-Stolarska *et al.*, 2009, Prefontaine *et al.*, 2010), rheumatoid arthritis and Crohn's disease (Carriere *et al.*, 2007). An IL-33 responsive ILC2 population was first described in nasal polyps by Mjosberg *et al.* (2011) and expression of IL-33 mRNA was reported to be threefold higher in epithelial cell cultures derived from recalcitrant CRSwNP compared to treatment-responsive CRSwNP patients (Reh *et al.*, 2010). Furthermore, rhinovirus infection was shown to induce IL-33 release from HBECs leading to type 2 inflammation during rhinovirus-induced asthma exacerbations (Jackson *et al.*, 2014). Moreover, a study by Kurowska-Stolarska *et al.* (2009) has suggested that IL-33 may also play a role in the polarisation of macrophages to the alternatively activated M2 phenotype and contribute to airway inflammation.

1.5.2.1 IL-33 receptor

ST2 was established as the receptor for IL-33 and is composed of the ST2 subunit and the signalling chain, IL-1RAcP (Schmitz *et al.*, 2005, Molofsky *et al.*, 2015). Different isoforms exist with initial discovery of the soluble isoform, sST2, followed by identification of the full length transmembrane (TM) ST2 (also known as ST2L) (Yanagisawa *et al.*, 1993, Kakkar *et al.*, 2008). sST2 is purported to act as a decoy receptor for IL-33 as it binds directly to IL-33 to prevent interaction with TM ST2 and sST2 pre-treatment in an ovalbumin (OVA)-induced murine model of allergic

inflammation resulted in reduced Th2 cytokine production by splenocytes (Hayakawa *et al.*, 2007). Interestingly, sST2 levels are reportedly higher in the sera of asthmatic and allergic rhinitis patients compared to healthy controls with sST2 expression correlating with the severity of asthma exacerbations (Oshikawa *et al.*, 2001, Baumann *et al.*, 2013).

Many cell types express ST2 including ILC2s, macrophages, mast cells, eosinophils, basophils and natural killer (NK) cells (Mjosberg *et al.*, 2011, Shaw *et al.*, 2013, Cayrol *et al.*, 2014, Molofsky *et al.*, 2015). Th2 cells were shown to produce sST2 with low-level expression of TM ST2 at the cell surface only upon activation (Lecart *et al.*, 2002). Recently, expression of ST2 has been reported on murine colonic Tregs which respond to IL-33 upon tissue damage with increased proliferation and upregulated expression of FOXP3 to limit intestinal inflammation (Schiering *et al.*, 2014). A population of human $ST2^+$ Tregs has also very recently been demonstrated in visceral adipose tissue with a postulated role in limiting obesity-associated inflammation (Vasanthakumar *et al.*, 2015).

Elevated expression of ST2 is observed in a number of diseases. An increase in the number of $ST2^+$ eosinophils is observed in the subepithelial layer of nasal tissue from eosinophilic CRSwNP compared to noneosinophilic CRSwNP patients (Baba *et al.*, 2014). Furthermore, mRNA levels of both IL-33 and ST2 are significantly higher in the nasal mucosa of allergic rhinitis patients compared to healthy volunteers and were shown to mediate production of the pro-inflammatory cytokines GM-CSF and IL-8 by human nasal epithelial cells (Haenuki *et al.*, 2012, Kamekura *et al.*, 2012). ST2 is involved in the persistence of airway hyperresponsiveness (AHR) in an OVA-induced allergic inflammation model with blockade of ST2 able to abrogate AHR to levels observed in control mice (Kearley *et al.*, 2009). Furthermore, its role in asthma pathogenesis has been supported by the identification of both *IL33* and *ILRL1* genes

(the gene for ST2) as major asthma susceptibility loci in genome-wide association studies (Moffatt *et al.*, 2010, Bonnelykke *et al.*, 2014).

1.5.3 TSLP

Thymic stromal lymphopoietin (TSLP) was originally discovered in a mouse thymic stromal cell line and identified to be a growth factor for B cells (Friend *et al.*, 1994). The receptor for TSLP is heterodimeric and consists of the IL-7Rα and TSLPR chains (Ziegler *et al.*, 2013). TSLP is primarily produced by epithelial cells and keratinocytes although recent studies have suggested that fibroblasts, ASMCs, DCs as well as mast cells are able to produce TSLP (Takai, 2012, Cianferoni *et al.*, 2014). Similar to IL-25 and IL-33, epithelial cells are induced to release TSLP in response to various danger or stress signals. Allakhverdi *et al.* (2007) demonstrated that stimulation of human airway epithelial cells with the pro-inflammatory cytokines IL-1β and TNFα was able to induce TSLP production. Bacterial peptidoglycan and poly I:C stimulation were also able to elicit the same effect, indicative of TSLP production in response to bacterial or viral infections. Furthermore, proteases such as that derived from *Alternaria* have also been reported to induce TSLP production by HBECs (Kouzaki *et al.*, 2009).

The role of TSLP in mediating Th2 responses is exemplified by its ability to polarise DCs to drive Th2 cell differentiation (Ito *et al.*, 2005). The authors showed that TSLP induced the expression of OX40 ligand (OX40L) on DCs, which in turn binds to OX40 expressed on naïve T cells for the promotion of Th2 differentiation. Furthermore, TSLP can also drive Th2 cell differentiation directly in the absence of antigen presenting cells. Omori *et al.* (2007) reported a 4-fold increase in the number of IL-4^{+} T cells detected in anti-CD3 antibody stimulated murine CD4^{+}CD62L^{high} naïve splenic T cell cultures, in the presence of TSLP compared to in the absence of TSLP. This was not observed in *Tslpr*$^{-/-}$ mice, indicative of a direct response to TSLP.

TSLP is implicated in the pathology of a number of allergic diseases and animal models of allergic inflammation. Keratinocyte expression of TSLP is higher in acute and chronic atopic dermatitis lesions compared to healthy skin (Soumelis *et al.*, 2002) and mRNA expression is also increased in the nasal mucosa of allergic rhinitis patients compared to non-allergic controls (Zhu *et al.*, 2009). He *et al.* (2008) showed that $TSLPR^{-/-}$ mice sensitised to OVA in an allergic skin inflammation model did not develop eosinophilia and showed reduced IL-4 and IL-13 expression compared to wild-type mice. Furthermore, TSLP gene expression has been shown to directly correlate with expression of Th2-related genes and eosinophil numbers in asthmatic bronchial biopsies (Shikotra *et al.*, 2012). TSLP is implicated in CRSwNP disease with increased TSLP mRNA expression detected in nasal polyps compared to the atopic nasal mucosa (Kimura *et al.*, 2011). This finding has been corroborated in a study by Nagarkar *et al.* (2013) however, the authors reported a decrease in TSLP protein expression in nasal polyps compared to control uncinate tissue. Protease-mediated cleavage of TSLP was observed in nasal polyps but interestingly, the cleaved form of TSLP was able to significantly enhance IL-1β induced IL-5 production from human mast cells in a TSLP bioactivity assay, suggestive of an enhanced ability of TSLP to mediate Th2 inflammation in nasal polyps.

1.6 Hypotheses

The body of work in this thesis addressed the hypotheses that:

1. Nasal mucosal T cells display distinct tissue-specific phenotypes compared to T cells from peripheral blood.
2. Nasal mucosal T cells derived from nasal polyps display disease-specific pro-inflammatory phenotypes that may represent therapeutic targets in the treatment of CRSwNP disease.

1.7 Aims

The overall aim of this thesis was to re-examine the nasal mucosal T cell phenotype in health and inflammatory disease, using chronic rhinosinusitis with nasal polyps (CRSwNP) as the model. This programme of work aimed to:

1. Identify phenotypic differences in human nasal mucosal vs. peripheral T cells.
2. Identify local nasal mucosal T cell subsets restricted to CRSwNP disease.
3. Examine the phenotype of T cells in the healthy nasal mucosa.
4. Determine the function of the T cell phenotypes present in the nasal mucosa.

Chapter 2 Materials and Methods

2.1 Peripheral blood and tissue samples

Peripheral blood samples (50ml) and nasal polyp tissue were collected from CRSwNP patients during nasal polypectomy surgery. Normal nasal mucosal biopsies and peripheral blood were collected from healthy volunteers. Nasal inferior turbinate tissue was collected from patients undergoing turbinectomy surgery. Demographic data for CRSwNP patients and healthy volunteers are shown in Table 3.1 and Table 3.2 respectively. Skin punch biopsies were obtained from the forearm of participants in the PollenLITE low dose intradermal grass pollen immunotherapy trial (Slovick *et al.*, 2013). All subjects provided written, informed consent and all studies were performed with Research Ethics Committee approval. Collected tissue was treated in accordance with the Human Tissue Act 2004.

2.1.1 Cell isolation from peripheral blood

Peripheral blood was diluted 1 in 2 with Hank's Balanced Salt Solution (HBSS) (Life Technologies, Paisley, UK) and layered on top of Lymphoprep™ solution (Axis-Shield, Dundee, UK). Samples were then centrifuged for 20 minutes at 800g and 22°C. The resultant peripheral blood mononuclear cell (PBMC) layer was drawn off into a 50ml Falcon tube and topped up with HBSS. Samples were centrifuged (250g, 10 minutes, 4°C) and supernatant subsequently discarded. Pellets were resuspended in 50ml phosphate buffered saline (PBS) containing 2% foetal calf serum (FCS) before washing (200g, 10 minutes, 4°C). Pellets were then resuspended in 50ml of 2% FCS/PBS for viable cell counting with 0.4% Trypan Blue solution (Sigma-Aldrich, Poole, UK) and a haemocytometer.

2.1.1.1 PBMC cultures

PBMCs were washed before resuspension to $1x10^6$ cells/ml with Roswell Park Memorial Institute media-1640 (RPMI) (Life Technologies) containing 10% FCS,

penicillin (100 U/ml), streptomycin (100 μg/ml) and L-glutamine (2mM), hereafter referred to as complete medium. PBMCs were plated at $1x10^6$ cells/ml, supplemented with recombinant IL-2 (50 U/ml; Novartis, Camberley, UK) and cultured at 37°C in 5% CO_2 for 2-3 days. Cells were then stimulated by seeding onto tissue culture plates pre-coated with anti-CD3 antibody (1 mg/ml; OKT3, ECACC) and anti-CD28 antibody (1 mg/ml; 15E8; Sanquin, Amsterdam, Netherlands). Cells were removed from stimulation after 3-4 days and cultured for a total of 7 days before analysis. Fresh complete medium and IL-2 were added every 2-3 days or when required. In some experiments, recombinant human (rh) IL-25 (125 ng/ml; R&D Systems, Abingdon, UK) and rhIL-33 (50 ng/ml; R&D Systems) were added to samples on day 0 of culture or on day 7 post-stimulation.

2.1.1.2 $CD4^+$ cell cultures

Following viable cell counting with Trypan Blue, PBMC samples were washed and resuspended in 5ml 2% FCS/PBS. $CD4^+$ cells were then isolated with the use of the Dynal® CD4 positive isolation kit (Life Technologies). An aliquot of CD4 Dynabeads® to allow a concentration of 4 beads per $CD4^+$ cell was then washed on a DynaMag™ magnet (Life Technologies) and subsequently added to the PBMC sample for 20 minutes at 4°C. Cells were then placed on the magnet for 5 minutes and supernatant containing the $CD4^-$ cells removed. Cells were washed three times with 2% FCS/PBS on the magnet to remove residual $CD4^-$ cells. Bound $CD4^+$ cells were resuspended in 1ml of 2% FCS/PBS and 200μl of DETACHaBEAD® CD4 was added and incubated for 45 minutes at 4°C. Following incubation, cells were placed on the magnet as before and supernatant containing the $CD4^+$ cells harvested. Beads were then washed three times to ensure the maximal recovery of $CD4^+$ cells. Live cell numbers were determined with Trypan Blue solution before washing with 2% FCS/PBS. $CD4^+$ cells were subsequently resuspended to $1x10^6$ cells/ml in complete medium supplemented with IL-

2. Cells were cultured in plates pre-coated with anti-CD3 and anti-CD28 antibodies at 37°C and 5% CO_2 for 2-3 days before removal from stimulation. Cells were cultured for a total of 7 days before analysis.

2.1.1.3 Th17 *in vitro* differentiation cultures

To purify naïve $CD4^+$ T cells, 10µl of purified anti-CD45RO antibody (UCHL1; eBioscience, Hatfield, UK) was added per $10x10^6$ $CD4^+$ cells. Cells were then incubated on ice for 20 minutes and washed twice before resuspension in 1ml of 2% FCS/PBS. Pan-mouse IgG Dynabeads (300µl; Life Technologies) were washed and added to $CD4^+$ cells to bind to $CD45RO^+$ cells. Cells were incubated at 4°C for 20 minutes before being placed on the magnet and supernatant containing $CD45RO^-$ cells collected. $CD45RO^-$ cells were then washed twice and resuspended in 1ml of complete medium for viable cell counting with Trypan Blue solution. After cell counting, $CD45RO^-$ cells were resuspended to $1x10^6$ cells/ml and seeded onto plates pre-coated with anti-CD3/CD28 antibodies. For Th17 differentiation, culture medium (RPMI, Iscove's Modified Dulbecco's Medium (IMDM), X-vivo 20 medium and AIM-V medium) was supplemented with rhIL-1β (10 ng/ml; eBioscience), rhIL-23 (40 ng/ml; eBioscience), rhIL-6 (50 ng/ml; R&D), rhTGFβ (50 ng/ml; R&D), anti-IFNγ antibody (5 µg/ml; 4S.B3; eBioscience) and anti-IL-4 antibody (5 µg/ml; 7A3-3, ECACC). Cells were restimulated every 7 days with fresh complete medium and differentiating cytokines/antibodies added every 2-3 days, up to a total of 28 days.

2.1.2 Nasal mucosal tissue

2.1.2.1 Explant cultures

Nasal polyp tissue, nasal turbinate tissue and normal nasal mucosal tissue were dissected and resuspended in complete medium. Tissues were cultured at 37°C in 5% CO_2 in the presence of IL-2 (50 U/ml). After 2-3 days, cells were passed through a

0.2μm cell strainer to obtain a single cell suspension and restimulated by culturing on anti-CD3/CD28 antibody coated plates. Cells were cultured for a further 7 days before analysis with removal from stimulation after 3-4 days. Blood and parallel tissue samples were analysed on the same day. In some experiments, rhIL-25 (125 ng/ml) and rhIL-33 (50 ng/ml) were added to cultures on day 0 or on day 7 post anti-CD3/CD28 restimulation.

2.1.2.2 Collagenase digested cultures

Nasal polyp tissue was placed in a petri dish containing 3mM $CaCl_2$/HBSS for mechanical dissociation. Tissue was subsequently transferred to a 24 well plate and 100μl Liberase (thermolysin low) collagenase (2.5 mg/ml; Roche, Burgess Hill, UK) was added to wells to a final volume of 2 ml/well. The plate was wrapped in foil and placed on a rocking platform overnight at 4°C. The next day, dissociated cells were passed through a 100μM cell strainer and washed before resuspension in complete medium. Cells were then either counted and analysed by flow cytometry or placed in culture for 7 days with supplementary IL-2 and fresh complete medium added every 2-3 days. Cells were then restimulated with plate-bound anti-CD3/CD28 antibodies on day 7 and cultured for a further 7 days before analysis.

2.1.3 Nasal turbinate tissue

2.1.3.1 $CD326^+$ epithelial cell isolation

Inferior turbinate tissue was dissected off the turbinate bone in a sterile petri dish containing 2% FBS/HBSS. Tissue was resuspended in 9ml 2% FBS/HBSS and 3ml transferred to each well of a 6 well plate. Liberase collagenase (2.5 mg/ml) was added (100μl per well) and the plate was incubated at 37°C for 1 hour. The plate was then centrifuged at 200g for 10 minutes and supernatant was discarded. Digested tissue was resuspended in 3ml fresh 2% FBS/HBSS per well before a second wash. Bronchial

epithelial cell growth medium (BEGM) was prepared by supplementing 500ml of bronchial epithelial basal medium (BEBM) (Lonza, Slough, UK) with the contents of the BEGM SingleQuots™ Kit (Lonza) containing: 2ml bovine pituitary extract, 0.5ml insulin, 0.5ml hydrocortisone, 0.5ml gentamycin and amphotericin, 0.5ml retinoic acid, 0.5ml transferrin, 0.5ml triiodothyronine, 0.5ml epinephrine, and 0.5ml human epidermal growth factor. Tissue was resuspended in a 50:50 mix of complete RPMI medium and BEGM and incubated overnight at 37°C.

The following day, digested turbinate tissue was mechanically dissociated with a pipette before cells were passed through a 0.2µm cell strainer to obtain a single cell suspension. Cells were centrifuged (200g for 10 minutes) and the cell pellet resuspended in 300µl of magnetic-activated cell sorting (MACS) buffer (50ml PBS, 200µl ethylenediaminetetraacetic acid (EDTA), 1ml FBS) in a screw cap eppendorf. FcR blocking reagent (50µl; Miltenyi Biotec) and CD326 MicroBeads (100µl; Miltenyi Biotec) were added to cells. The eppendorf was then placed on a roller and incubated for 30 minutes at 4°C. Following incubation, cells were resuspended in a total volume of 10ml MACS buffer in a V-bottomed universal before centrifugation (300g for 10 minutes). Cells were subsequently resuspended in 500µl of MACS buffer.

MACS LS columns (Miltenyi Biotec) were placed in MidiMACS Separator magnets (Miltenyi Biotec) attached to a MACS MultiStand (Miltenyi Biotec). Columns were primed by adding 3ml MACS buffer to columns and allowing the buffer to drip out. Cells in 500µl of MACS buffer were then added to LS columns and flow-through (containing $CD326^-$ cells) discarded. Columns were washed 3 times with 3ml MACS buffer before being taken off the magnet. MACS buffer (5ml) was added to columns and the plunger inserted to wash out $CD326^+$ cells. $CD326^+$ cells were then washed three times with 10ml 2% FCS/HBSS (300g, 10 minutes). After the final wash, $CD326^+$

cells were resuspended in 8ml BEGM media and transferred to a Nunc™ cell culture treated T25 flask with filter caps (Thermo Scientific) and cultured at 37°C in 5% CO_2.

2.1.3.2 Culture and passage of epithelial cells

Cells were fed with fresh BEGM medium day 1 post $CD326^+$ isolation and every 3 days until 90% confluent. Cells were then passaged with trypsin/EDTA. Briefly, the culture supernatant was discarded and 5ml HEPES ((4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) was added to wash the flask. Trypsin/EDTA (2ml; Lonza) was then added to flasks and incubated for 30 seconds at 37°C. Flasks were observed under the microscope to determine if >90% of adherent cells had detached. If less than 90% of cells had detached, the flask was incubated for a further 30 seconds at 37°C. Trypsin neutralising buffer (5ml; Lonza) was then added to flasks and contents were transferred to a V-bottomed universal. Cells were centrifuged at 250g for 7 minutes before the cell pellet was resuspended in 12ml fresh BEGM medium and transferred to a T75 flask. Cells, now at passage 1, were cultured as previously with fresh BEGM added to flasks the day after passaging. $CD326^+$ cells were then cultured until 90% confluent at passage 2 before cryopreservation.

2.1.3.3 Cryopreservation of epithelial cells

Epithelial cells were detached from flasks with trypsin/EDTA as previously performed. Following centrifugation at 250g for 7 minutes, cells were resuspended in 1ml of BEGM medium and 500µl aliquoted per cryotube (Starlab, Milton Keynes, UK). Freezing medium (40% FCS, 10% dimethyl sulfoxide (DMSO), 50% BEGM) was added drop wise to a total volume of 1ml. Cryotubes were then placed in a Mr Frosty freezing container (Sigma-Aldrich) and stored at -80°C overnight. Cryotubes were then transferred to liquid nitrogen for long-term storage.

2.1.3.4 Epithelial cell/ nasal explant supernatant co-cultures

Cryotubes containing epithelial cells were removed from liquid nitrogen storage and thawed for 1 minute in a water bath at 37°C. Fresh BEGM medium (6ml) was then added for a total volume of 7ml. Epithelial cells were aliquoted at 250µl/well in a 48 well plate and cultured overnight at 37°C. The next day, supernatant was aspirated and cells were fed with fresh BEGM medium and cultured until 75% confluent. Upon reaching 75% confluence, epithelial cells were co-cultured with supernatants from nasal turbinate explant cultures stimulated with anti-CD3/CD28 antibodies for 24 hours. Wells containing epithelial cells and no explant culture supernatant (0% RPMI) were also cultured as a negative control. Cells were incubated at 37°C for 24 hours before culture supernatants were harvested and stored at -20°C ready for ELISA/CBA analysis.

2.1.4 Skin biopsy tissue

Skin punch biopsies were obtained from the forearms of participants in the PollenLITE trial (Slovick *et al.*, 2013). Briefly, participants were challenged with diluent and allergen (grass pollen), one on each arm and skin punch biopsies were taken at the same time. Biopsies were processed and cultured in the tissue explant model as for nasal polyp tissue.

2.2 Cell restimulation

2.2.1 Stimulation with anti-CD3/anti-CD28 antibodies

Nunclon™Δ surface multiwell plates (Thermo Fisher Scientific, Loughborough, UK) were coated with anti-CD3 and anti-CD28 antibodies. Plates were coated by incubating wells with anti-CD3 antibody (1 mg/ml; OKT3, ECACC) and anti-CD28 antibody (1 mg/ml; 15E8; Sanquin, Amsterdam, The Netherlands), diluted in HBSS, for at least 1 hour at 37°C. Cells in culture were then washed and resuspended to $1x10^6$ cells/ml and seeded onto pre-coated wells.

For experiments comparing restimulation methods, cells were resuspended to $2x10^6$ cells/ml and 500µl of cells added per pre-coated well. Fresh complete medium (500µl) was also added per well for a total volume of 1ml/well. All cells were supplemented with IL-2 and cultured for a further 7 days. Cells were split once confluent and fresh complete medium and IL-2 added every 2-3 days.

2.2.2 Stimulation with irradiated PBMCs and PHA

Prior to culture of cells with phytohaemagglutinin (PHA), a dose response experiment to PHA was performed utilising a carboxyfluorescein diacetate, succinimidyl ester (CFSE) cell proliferation assay to determine the optimal working concentration.

2.2.2.1 CFSE proliferation assay

Cells were labelled with CFSE with the use of the CellTrace™ CFSE cell proliferation kit (Life Technologies). Freshly isolated PBMCs were resuspended to $2x10^6$ cells/ml in PBS containing 5% FCS. CFSE was prepared from the stock solution according to the manufacturer's instructions and 2 µl/ml added to PBS. Equal volumes of prepared CFSE solution and cells were then mixed together and immediately vortexed. Final staining concentrations of CFSE and cells were 5µM and $1x10^6$ cells/ml respectively.

Samples were incubated in the dark for 10 minutes at 37°C before staining was quenched by the addition of 5 times the volume of ice-cold 5% FCS/PBS. Labelled cells were left on ice for 5 minutes and washed three times (300g, 5 minutes, 4°C). Cells were then resuspended in fresh complete medium at $2x10^6$ cells/ml and cultured at 1 ml/well with IL-2 (50 U/ml) added to all wells. PHA was also added as required for final concentrations of 0-100 μg/ml. Cells were cultured at 37°C in 5% CO_2 and fresh complete medium and IL-2 added every 2-3 days. Proliferation of $CD4^+$ and $CD8^+$ cells was analysed on days 4 and 7.

2.2.2.2 Determination of PHA working concentration

The CFSE analysis of proliferating $CD4^+$ and $CD8^+$ cells on days 4 and day 7 of culture is shown in Figure 2.1a. PHA at concentrations ≥ 0.1 μg/ml was required for at least 80% proliferation by day 4 with no substantial difference observed between the $CD4^+$ and $CD8^+$ populations. By day 7, the percentage of divided cells from the same cultures ranged from 86.5% to 100% (Figure 2.1b). However, $CD8^+$ cells with a PHA concentration of 0 or 0.01 μg/ml showed only around half the comparable amount of divided cells observed at higher PHA concentrations even at day 7. This suggests that a minimum concentration of 0.1 μg/ml of PHA is required for effective cell proliferation in both $CD4^+$ and $CD8^+$ cells.

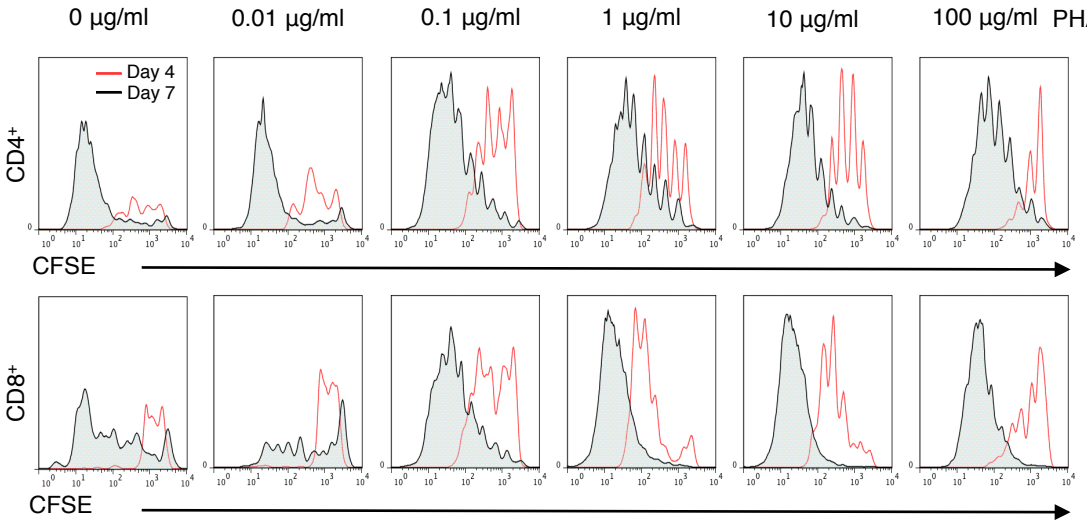
Examination of the number of cells that were in each cell division cycle showed that on day 4, cultures with PHA at concentrations of 1 and 10 μg/ml resulted in the highest amount of cells at division cycle 4 or above (Figure 2.2a). By day 7, most cells were at division cycle 6 or above when a PHA concentration ≥ 0.1 μg/ml was utilised in cultures. This shows that cells cultured with a PHA concentration ≥ 0.1 μg/ml were undergoing numerous cell divisions and proliferating readily, further supporting the use of the higher PHA concentrations to stimulate cells.

To determine that the cells in culture were healthy, cells were stained with the viability dye eFluor®780. Figure 2.2b shows that cells cultured with a PHA concentration of 1 μg/ml had the highest percentage of live cells compared to 100 μg/ml of PHA on both day 4 (76.8% vs. 39.5%) and day 7 (84.3% vs. 72.9%). PHA concentration of 1 μg/ml was therefore selected for use in further experiments as this concentration resulted in the highest percentage of viable proliferating cells.

2.2.2.3 PHA/irradiated PBMC stimulation

Freshly isolated PBMCs were resuspended to $2x10^6$ cells/ml in complete medium and irradiated at a dose of 3000 rads with a Nordion GC-1000S v2.09 cell irradiator (Nordion, Ottawa, Canada). Isolated PBMCs and $CD4^+$ cells on day 4 of culture were washed and resuspended to $2x10^6$ cells/ml. Irradiated PBMCs (500μl) were then added per well with 500μl of cultured cells for a total volume of 1ml. Cultures were supplemented with IL-2 (50 U/ml) as before with the addition of 1 μg/ml of PHA. Cells were cultured for a further 7 days and split once confluent with fresh complete medium and IL-2 added every 2-3 days.

a



b

	CD4		CD8	
PHA (µg/ml)	**Day 4**	**Day 7**	**Day 4**	**Day 7**
0	17.1%	100.0%	3.6%	54.1%
0.01	11.3%	97.3%	5.6%	56.7%
0.1	88.8%	86.5%	89.0%	97.7%
1	89.7%	100.0%	83.1%	99.9%
10	89.8%	90.1%	79.9%	98.9%
100	85.9%	97.3%	93.3%	98.5%

Figure 2.1 Dose-dependent cell proliferation in response to PHA.

(a) PBMCs ($1x10^6$ cells/ml) were labelled with CFSE (5µM) and cultured in the presence of PHA (0-100 µg/ml). Surface staining for $CD4^+$ and $CD8^+$ cells was carried out on day 4 (red open histogram) and day 7 (grey solid histogram). Data is representative of two independent experiments. (b) Percentages of proliferating $CD4^+$ and $CD8^+$ cells at each concentration of PHA are shown for days 4 and 7.

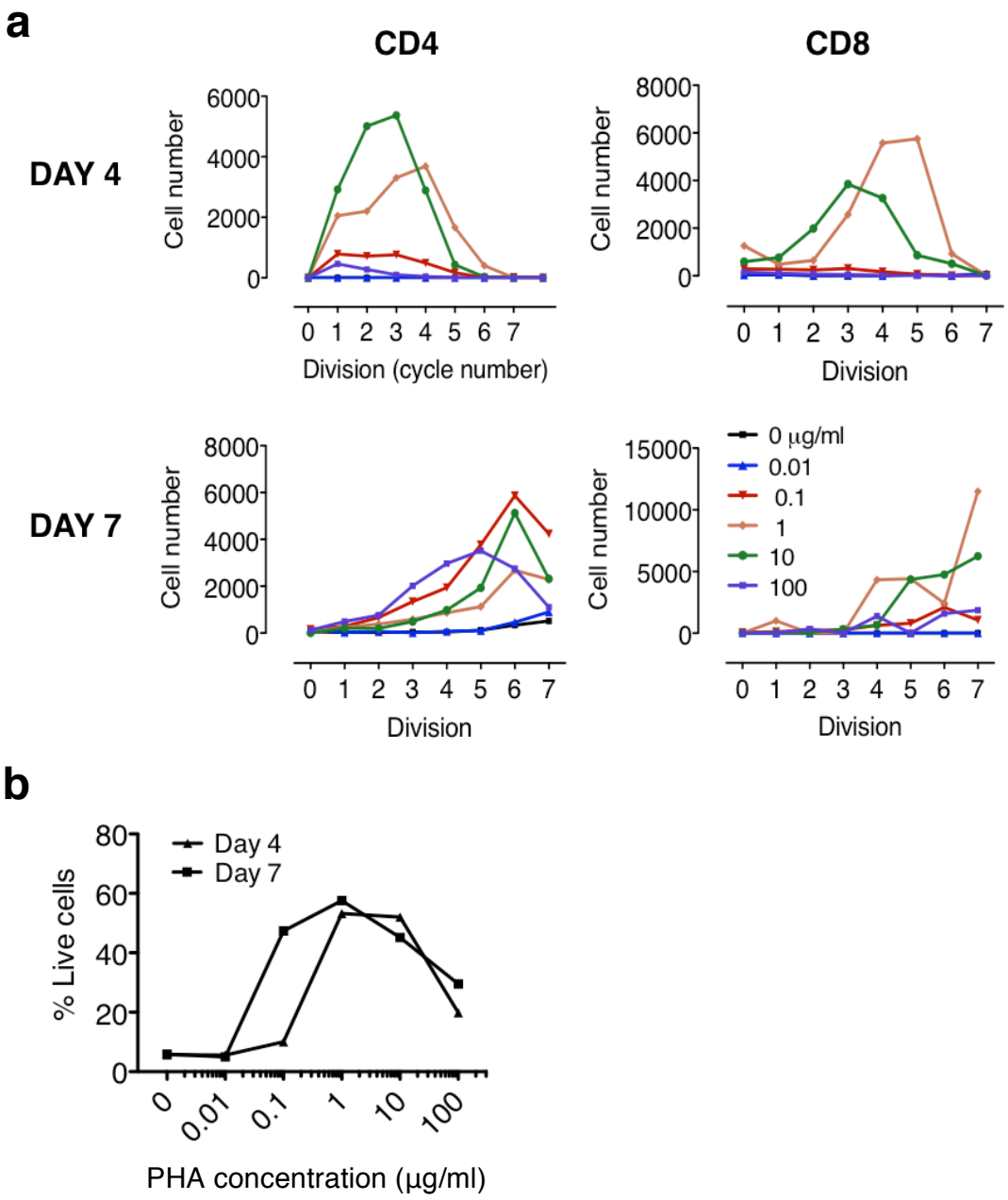


Figure 2.2 Irradiated PBMCs/PHA and anti-CD3/CD28 antibodies are comparable cell stimulation routes.

(a) Cell numbers for each division cycle are shown for $CD4^+$ and $CD8^+$ cells responding to varying concentrations of PHA. (b) Percentage of live cells over varying PHA concentrations was measured with eFluor780 viability dye.

2.3 Flow cytometry

2.3.1 Antibodies

Anti-human antibodies utilised for flow cytometry are listed in Table 2.1.

2.3.2 Surface staining

Cells were prepared for surface staining by washing with 2ml of cold FACSflow™ sheath fluid (BD Biosciences). Cells were then resuspended in FACSFlow and stained with the fixable viability dye eFluor®780 (eBioscience) on ice for 30 minutes in the dark to exclude dead cells. Cells were then washed twice with 1ml FACSFlow and supernatant discarded. Fc receptor (FcR) blocking reagent (10µl; Miltenyi Biotec, Surrey, UK) was added to the residual volume in the tube and samples incubated on ice for a further 10 minutes. Single colour compensation tubes were prepared by washing the appropriate AbC™ capture and negative compensation beads (Life Technologies) for reactivity against the antibody host species or by using the cells of interest. Primary labelled antibodies were then added to samples and appropriate control/compensation tubes and incubated on ice (except CRTH2 staining, performed at room temperature) for 30 minutes in the dark. Samples were subjected to two washes before final resuspension in FACSFlow and analysis on a FACSCanto II (BD Biosciences) flow cytometer. Data were acquired with the use of FACS DIVA™ software (BD Biosciences) and analysed using FlowJo™ v7.6 software (Tree Star, Inc., Oregon, USA).

2.3.3 Intracellular cytokine staining

Cells for intracellular cytokine staining were activated by incubation with ionomycin (500 ng/ml) and phorbol 12-myristate 13-acetate (PMA) (5 ng/ml) at 37°C for 4 hours. Cells with no PMA/ionomycin added were used as resting controls. For the final 2 hours of incubation, monensin was diluted 1:1 with 2mM sodium hydroxide and 2 µl/ml

added to wells. Cells were then washed (200g, 5 minutes, 4°C) with 1ml FACSFlow and supernatant discarded. The wash was repeated and cells were stained with the viability dye eFluor®780 for 30 minutes on ice. Cells were washed twice with FACSFlow before addition of 500µl of cytofix/cytoperm™ solution (BD Biosciences) to tubes. Cells were incubated on ice for 20 minutes before two more washes. Perm/wash™ solution (500µl; BD Biosciences) was then added to cells and centrifuged before supernatant was discarded. FcR blocking reagent (10µl) was added to each tube and incubated on ice for 10 minutes. Subsequent steps proceeded as before for surface staining except Perm/wash™ solution was utilised for the first wash of cells after primary antibody incubation.

2.3.4 Fluorescence-activated cell sorting

Cells for sorting were stained with monoclonal antibodies as above, with the exception that 2% FCS/PBS was used in all wash steps. Cells were resuspended in a final volume of 1ml in 2% FCS/PBS and sorted with the FACSAria II (BD Biosciences) flow cytometer in the National Institute for Health Research (NIHR) Biomedical Research Centre (BRC) Flow Core Facility at Guy's Hospital. Purity of sorted cells was determined to be >95%.

Table 2.1 Anti-human antibodies used in flow cytometry experiments

Molecule	Colour	Clone	Company
CCR6	PE/Cy7	G034E3	BD Biosciences
CCR6	FITC	G034E3	Biolegend
CD3	PE	UCHT1	BD Biosciences
CD3	eFluor450	OKT3	eBioscience
CD4	APC	OKT4	eBioscience
CD4	FITC	RPA-T4	eBioscience
CD4	PerCP/Cy5.5	OKT4	Biolegend
CD4	PE/Cy7	RPA-T4	eBioscience
CD4	Brilliant Violet (BV) 510	OKT4	eBioscience
CD45	APC	H130	eBioscience
CD45	PerCP/Cy5.5	H130	Biolegend
CD45RA	FITC	HI100	eBioscience
CD45RO	PE	UCHL1	eBioscience
CD49a	Alexa Fluor (AF) 647	TS2/7	Biolegend
CD62L	APC	DREG-56	Biolegend
CD8	PE	RPA-T8	eBioscience
CD8	Pacific Blue	RPA-T8	Biolegend
CD8	BV510	RPA-T8	BD Biosciences
CD8	PerCP/Cy5.5	RPA-T8	Biolegend
CLA	FITC	HECA-452	Biolegend
CRTH2	PE	BM-16	Biolegend
CXCR3	APC	IC6/CXCR3	BD Biosciences
CXCR3	BV421	G025H7	Biolegend
IFNγ	Labelled with AF488 labelling kit (Life Tech)	7R2/A4	ECACC
IFNγ	PE	4s.B3	eBioscience
IL-10	AF647	JES3-9D7	eBioscience
IL-13	PE	JES10-SA2	Biolegend
IL-13	PerCP/Cy5.5	JES10-SA2	Biolegend
IL-17A	AF647	BL168	Biolegend
IL-17A	BV421	N49-653	BD Biosciences
IL-17F	eFluor710	SHLR17	eBioscience
IL-22	PE	142928	R&D

IL-25R (IL-17BR)	Labelled with AF647 labelling kit (Life Tech)	D9.2	Dr. A. McKenzie (University of Cambridge)
IL-5	PE	TRFK5	BD Biosciences
IL-9	AF647	MH9A4	eBioscience
IL-9	PE	MH9D1	eBioscience
iNKT	PE	6B11	Biolegend
Mouse IgG	FITC	MOPC-21	BD Biosciences
Mouse IgG	AF647	MOPC-21	Biolegend
Mouse IgG	PerCP/Cy5.5	MOPC-21	Biolegend
Mouse IgG	BV421	MOPC-21	Biolegend
Mouse IgG	PE	MOPC-21	BD Biosciences
Mouse IgG	APC	MOPC-21	BD Biosciences
Mouse IgG	PE/Cy7	M1-14D12	eBioscience
Mouse IgG2a	PE	eBM2a	eBioscience
Mouse IgG2b	PE/Cy7	MPC-11	Biolegend
Mouse IgG2b	PerCP/Cy5.5	MPC-11	Biolegend
Mouse IgG2b	FITC	eBMG2b	eBioscience
PSGL1	PE	KPL-1	Biolegend
Rat IgG	efluor710	eBRG1	eBioscience
Rat IgG2a	PE	KLH/G2a	Beckman Coulter
Rat IgG2a	PE/Cy7	R35-95	BD Biosciences
Rat IgM	FITC	RTK2118	Biolegend
T cell receptor (TCR) αβ	PE/Cy7	IP26	Biolegend
TCR γδ	APC	B1	Biolegend
TNFα	FITC	MAb11	eBioscience

2.4 Enzyme-linked immunosorbent assay (ELISA)

ELISA assays were performed on cell culture supernatant samples following 24 hour stimulation with immobilised anti-CD3/CD28 antibodies. IL-22 was measured using the Ready-SET-Go!® ELISA kit from eBioscience according to the manufacturer's instructions. Human beta-defensin (hBD) 1 and hBD2 levels were measured using the mini ELISA development kits from Peprotech (London, UK) according to the manufacturer's instructions. Matched antibody pairs and standards from BD Biosciences (Oxford, UK) were used for the following cytokines: IL-4, IL-5, IL-13, IL-10 and IFNγ. Diluents, buffers and substrate details for ELISAs from each company are shown in Table 2.2.

2.4.1 Protocol for ELISA with BD Biosciences antibodies

Buffers used in this protocol are shown in Table 2.3. Capture antibody was diluted to 1 μg/ml and 50μl per well was used to coat a Nunc-Immuno™ 96 MicroWell™ plate (Thermo Fisher Scientific). Plates were incubated at 4°C overnight and washed four times with PBS the next day. Standards were serially diluted with complete medium. Standards and samples were added in duplicate (50μl per well) to the appropriate wells of the plate before overnight incubation at 4°C. The next day, plates were washed four times. The detection antibody was diluted to 1 μg/ml and 50μl added per well. Plates were incubated at room temperature for 2 hours before being subjected to four further washes. ExtrAvidin®-alkaline phosphatase (50μl volume of 1:5000 dilution; Sigma-Aldrich) was added per well following the last wash and plates incubated for 30 minutes at room temperature. Substrate solution was prepared by dissolving one 4-nitrophenyl phosphate disodium salt hexahydrate substrate tablet (Sigma-Aldrich) per 20ml of 1x diethanolamine buffer and left to warm to room temperature. Plates were then washed six times and 100μl of substrate solution was added to all wells. Plates were incubated

for a final 30 minutes at room temperature and reactions were stopped by addition of 3M NaOH (50 μl/well). Plates were read on a μQuantTM microplate spectrophotometer (Bio-Tek Instruments, Winooski, USA) at a wavelength of 450nm.

Table 2.2 Solutions used in ELISA protocol from each company

	BD Biosciences	eBioscience	Peprotech
Coating buffer	Sodium carbonate/ bicarbonate coating buffer	PBS	PBS
Detection range	10 – 50,000 pg/ml	8 – 1000 pg/ml	4 – 1000 pg/ml (hBD1) 16 – 2000 pg/ml (hBD2)
Diluent	Complete RPMI medium	Assay diluent	0.05% Tween-20/ 0.1% BSA/ PBS
Detection antibody diluent	0.5% Tween-20/ 0.5% mouse serum/ PBS	Assay diluent	0.05% Tween-20/ 0.1% BSA/ PBS
Wash buffer	PBS	0.05% Tween-20/ PBS	0.05% Tween-20/ PBS
Substrate	Alkaline phosphatase (ALP)	Horseradish peroxidase (HRP)	Horseradish peroxidase (HRP)
Substrate Solution	4-Nitrophenyl phosphate disodium salt hexahydrate	3,3',5,5' Tetramethylbenzidine (TMB)	2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)

Table 2.3 Buffer recipes for ELISAs using BD Biosciences matched antibody pairs

Buffer	Recipe
Sodium carbonate/ bicarbonate coating buffer (pH 9.7)	4.24g Na_2CO_3 5.04g $NaHCO_3$ Make up to 1 litre with distilled water
Diethanolamine buffer (pH 9.8)	101mg $MgCl_2$ $6H_2O$ 97ml diethanolamine Adjust pH to 9.8 with HCl and make up to 1 litre with distilled water

2.5 Cytometric bead array (CBA) immunoassay

CBA assays were performed on cell culture supernatants using CBA Flex Sets (BD Biosciences) for the following cytokines: IL-4, IL-5, IL-6, IL-13, IL-17A, IL-17F, IL-9, IL-10, IFNγ, GM-CSF and TNFα. Standard curves (1.5-50,000 pg/ml) were prepared for each cytokine by serial dilution of the top standards with in-house buffer (FACSFlow, 1% FCS, 0.05% Tween-20, 2mM EDTA). The top standards for each cytokine were pooled before serial dilution to create one standard curve 'mix' for all cytokines assayed. Each standard and sample (50µl) was then added in duplicate to a U bottomed 96-well plate (Thermo Fisher Scientific) before addition of CBA capture beads (50µl volume diluted 1:250 with in-house buffer) to each well. To ensure beads were evenly mixed, plates were placed on a shaker for 5 minutes before incubation at room temperature for 3 hours. Plates were then centrifuged (5 minutes, 200g) and supernatant decanted. Plates were subsequently washed twice with 200µl of FACSFlow and 50µl of CBA detection beads (diluted 1:250 with in-house buffer) was added to each well. Plates were again shaken for 5 minutes and incubated for a further 2 hours with the detection beads. Centrifugation of plates was followed by two washes with FACSFlow and final resuspension of beads in each well in 150µl of FACSFlow. Plates were analysed on a LSR Fortessa flow cytometer (BD Biosciences) with a high throughput sampler plate reader platform. Data was analysed utilising FlowJo™ v7.6 software and GraphPad Prism® 5 software.

2.6 Histology

2.6.1 Tissue fixation

Fixation of nasal polyp and healthy normal nasal mucosal tissue used for IL-25 and IL-33 staining was performed by Dr. Mikila Jacobson and Dr. Nara Orban at Imperial College London. Nasal polyp tissue used for IL-17A and CCR6 staining was immersed in 4% paraformaldehyde (PFA) and fixed at 4°C. Following fixation, tissue samples were washed twice in 15% sucrose solution for one hour at 4°C. Samples were left overnight at 4°C in fresh 15% sucrose solution before being embedded in OCT compound (VWR International, Lutterworth, UK) the next day. Embedded tissues were stored at -80°C until required for cryosectioning.

2.6.2 Cryosectioning

OCT embedded tissue was placed in the chamber of a Bright OFT5000 cryostat (Bright Instruments, Huntingdon, UK) for 30 minutes before cryosectioning. Sections were cut to a thickness of 7μm and transferred to Polysine™ slides (Thermo Fisher Scientific) before air-drying overnight at room temperature. Slides were stored at -80°C pending immunostaining.

2.6.3 Immunohistochemistry

Details of dilutions and reagents used can be found in Table 2.4. Slides were inserted into staining racks and submerged in wash buffer. Slides were then placed on a rocking platform and washed twice (15 minutes per wash). Endogenous biotin and avidin binding sites were blocked with the avidin/biotin blocking kit (Vector Labs, Peterborough, UK) and serum blocking buffer (60μl) was applied to each section. Slides were then incubated for 1 hour at room temperature in a humidified chamber. Primary

antibody or isotype control (60µl) was applied to each section and incubated overnight at 4°C in a humidified chamber.

The following day, slides were washed three times and secondary antibody (60µl) applied to each section. Slides were then incubated for 1 hour at room temperature before being washed three times. For IL-17A and CCR6 staining, 60µl of alkaline phosphatase anti-alkaline phosphatase (APAAP) enzyme immune complex was applied to each section and incubated for 1 hour at room temperature in a humidified chamber. This method was used as it provides additional sensitivity compared to the indirect two-step staining method due to the larger number of alkaline phosphatase molecules that are attached per target site. For IL-25 and IL-33 staining, streptavidin-biotin complex labelled with alkaline phosphatase (VECTORSTAIN® ABC-AP mouse IgG reagent; Vector Labs) was added to slides for 30 minutes. This technique was again used to enhance sensitivity, as the large numbers of streptavidin molecules in the streptavidin-biotin complex are able to bind to the biotinylated secondary antibody with high affinity.

Slides were washed three times and fast red substrate solution prepared using SIGMAFAST™ Fast Red TR/Naphthol AS-MX tablets (Sigma-Aldrich) with addition of levamisole to reduce endogenous alkaline phosphatase activity. Substrate solution (60µl) was applied to each section. After development for up to 20 minutes, slides were washed under running tap water for 5 minutes. Sections were then counterstained with Harris's haematoxylin (Sigma-Aldrich) for approximately 30-60 seconds and subsequently washed under running tap water for 5 minutes. Slide covers were mounted with glycergel or faramount mounting medium (see Table 2.4). Immunostaining was visually inspected with an Olympus BX40 light microscope (Olympus-Europe, Hamburg, Germany) and images captured with a JVC KY-F55B camera (London, UK) using Zeiss KS300 software (Cambridge, UK). Alternatively, whole slide imaging was

performed with a Hamamatsu NanoZoomer (Hamamatsu Photonics, Welwyn Garden City, UK) in the Research Oncology Department at Guy's Hospital and viewed with the Hamamatsu NDP.view2 software.

Table 2.4 Dilutions and reagents used in immunohistochemistry

	IL-17A	CCR6	IL-25	IL-33
Primary antibody	Mouse anti-human IL-17A (eBio64DEC17; eBioscience)	Mouse anti-human CCR6 (53103; R&D)	Mouse anti-human IL-25 (182203, R&D)	Mouse anti-human IL-33 (Nessy-1; Enzo, Exeter, UK)
Dilution	1:20	1:200	1:200	1:100
Primary antibody diluent	PBS	PBS	5% human serum/ TBS	5% human serum/ TBS
Isotype antibody	Mouse IgG1 isotype (MOPC 21; Sigma-Aldrich)	Mouse IgG2b isotype (73009; R&D)	Mouse IgG isotype control (DAK-GO1, Dako)	Mouse IgG isotype control (DAK-GO1, Dako)
Secondary antibody	Rabbit anti-mouse polyclonal (Dako, Ely, UK)	Rabbit anti-mouse polyclonal (Dako)	Biotinylated horse anti-mouse IgG (BA-2000, Dako)	Biotinylated horse anti-mouse IgG (BA-2000, Dako)
Dilution	1:50	1:50	1:100	1:100
Secondary antibody diluent	5% rabbit serum/ PBS	5% rabbit serum/ PBS	10% human serum/ TBS	10% human serum/ TBS
Detection antibody	APAAP mouse monoclonal (AP7/6/7; Dako)	APAAP mouse monoclonal (AP7/6/7; Dako)	VECTORSTAIN® ABC-AP mouse IgG reagent (Vector Labs, Peterborough, UK)	VECTORSTAIN® ABC-AP mouse IgG reagent (Vector Labs)
Dilution	1:30	1:30	-	-
Diluent	5% rabbit serum/ PBS	5% rabbit serum/ PBS	-	-
Wash buffer	PBS	PBS	0.1% Tween-20/ TBS (TBST)	0.1% Tween-20/ TBS
Serum block	10% rabbit serum	10% rabbit serum	3% horse serum in TBST	3% horse serum in TBST
Mounting medium	Glycergel (Dako)	Glycergel (Dako)	Faramount (Dako)	Faramount (Dako)

2.7 Gene expression studies

2.7.1 RNA Isolation

RNA was isolated from cell pellets with the Qiagen miRNeasy mini kit (Manchester, UK). Samples were resuspended in 700µl of QIAzol™ lysis reagent (Qiagen) to lyse and denature proteins and rested for 5 minutes. Chloroform (140µl) was then added and samples vortexed, before being rested for a further 2 minutes. Tubes were subsequently spun at 12,000g in a benchtop centrifuge for 15 minutes. Following centrifugation, the top aqueous phase layer for each sample (containing RNA) was transferred to fresh individual 1.5ml collection tubes. The remaining interphase and organic phase layers (containing DNA) were stored at 4°C for DNA isolation. A volume of 100% ethanol, equal to 1.5 times the volume of the aqueous phase, was added per tube. Samples were then mixed by pipetting and 700µl of each sample was transferred to individual spin columns (contain a silica membrane to bind RNA). Columns were centrifuged at 8,000g for 15 seconds. Flow-through was then discarded before centrifugation was repeated with remaining sample. RPE buffer (500µl) was subsequently added and centrifuged for 15 seconds to wash spin columns. The wash step was repeated with columns centrifuged for 2 minutes to remove residual liquid. To ensure that the silica membranes in the columns were completely dry, spin columns were transferred to new 2ml collection tubes and spun at full speed for 1 minute. Spin columns were then transferred to new 1.5ml collection tubes and 50µl of RNase-free water added. Finally, tubes were centrifuged at 8,000g for 1 minute for the elution of RNA.

2.7.2 Genomic DNA removal

RNA samples were subjected to DNase I digestion to remove genomic DNA contamination. Master mix with the reagents listed in Table 2.5 was added to RNA samples for a final reaction volume of 100µl. Samples were incubated for 1 hour in a water bath at 37°C before proceeding to RNA purification.

Table 2.5 DNase I digest reagents

	1 x Master Mix (per reaction)
10µl	10X Turbo DNase enzyme (2 U/µl) (Life Technologies)
10µl	10X Turbo DNase buffer
30µl	RNase-free water

2.7.3 RNA clean up

Following DNase I digestion, RNA samples were purified with the RNeasy MinElute cleanup kit (Qiagen). RLT buffer (350µl) and 100% ethanol (525µl) was added to each sample. Samples were mixed by pipetting and 700µl was transferred to mini elute columns and centrifuged for 15 seconds at 8,000g. Flow-through was discarded and centrifugation repeated with remaining samples. RPE buffer (500µl) was subsequently added and spun for 15 seconds to wash the columns. This was followed by a further wash and centrifugation step, except columns were spun for 2 minutes to dry the membrane. Columns were then transferred to new 2ml collection tubes and spun for 5 minutes at maximum speed with the lids open to ensure that the membranes were completely dry. Columns were then placed in fresh 1.5ml collection tubes and 20µl of RNase-free water was added. Tubes were centrifuged for 1 minute (maximum speed) for the elution of RNA and stored at -20°C until required.

2.7.4 Bioanalyser

To check the quality and quantity of RNA isolated from samples, total RNA was analysed by on-chip electrophoresis using the Agilent 2100 Bioanalyser platform. Agilent RNA 6000 Pico chips (Agilent Technologies, Stockport, UK) were set up according to the manufacturer's instructions. Integrity of RNA was expressed as a RNA integrity number (RIN); RIN greater than 7, together with the presence of ribosomal peaks corresponding to 18S and 28S in the output electropherogram, was considered to indicate good quality, non-degraded RNA (Figure 2.3). Distinct bands in the gel-like image also indicated intact RNA. Quantity of RNA was expressed as pg/µl. RNA (5ng) was aliquoted and either diluted with RNase-free water or vacuum concentrated in an Eppendorf vacufuge vacuum concentrator (Stevenage, UK) to a final volume of 5µl in preparation for complementary DNA (cDNA) synthesis and amplification.

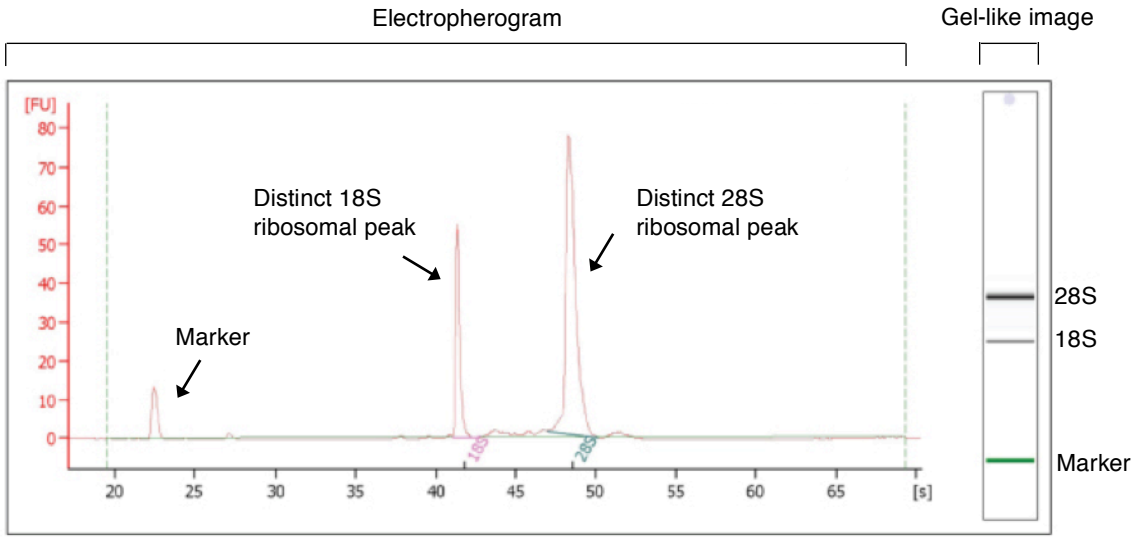


Figure 2.3 Example electropherogram trace and gel-like image

Example electropherogram and corresponding gel-like image of intact RNA with RIN score of 10 (Agilent Technologies, 2013).

2.7.5 cDNA synthesis and amplification

NuGEN Ovation PicoSL WTA system V2 kit (NuGEN Technologies, Leek, The Netherlands) involving Ribo-Single Primer Isotheral Amplification (Ribo-SPIA) technology was used for the synthesis and subsequent amplification of cDNA according to the manufacturer's instructions (Figure 2.4). Briefly, the process involved generation of the first strand of cDNA by reverse transcription, with a DNA/RNA chimeric 'SPIA tag' at the 5' end of the cDNA strand. Heat-induced degradation of mRNA produced mRNA fragments that served as primers for second strand synthesis. Addition of ribonuclease H (RNase H) resulted in cleavage of the RNA portion of the SPIA tag, allowing binding of the SPIA DNA/RNA amplification primer. Primer extension and strand displacement by DNA polymerase, followed by subsequent cleavage of the RNA portion of the SPIA primer resulted in continuous rounds of cDNA amplification with a typical yield of 2-5 µg of cDNA from 500pg of RNA. Clean up of cDNA was carried out using the MinElute reaction cleanup kit (Qiagen) according to the manufacturer's instructions.

Purity of amplified cDNA was measured using an Agilent RNA 6000 Nano chip on the 2100 Bioanalyser platform. Yield of cDNA was determined with the NanoDrop 2000 spectrophotometer (Thermo Scientific) on the single-stranded DNA setting.

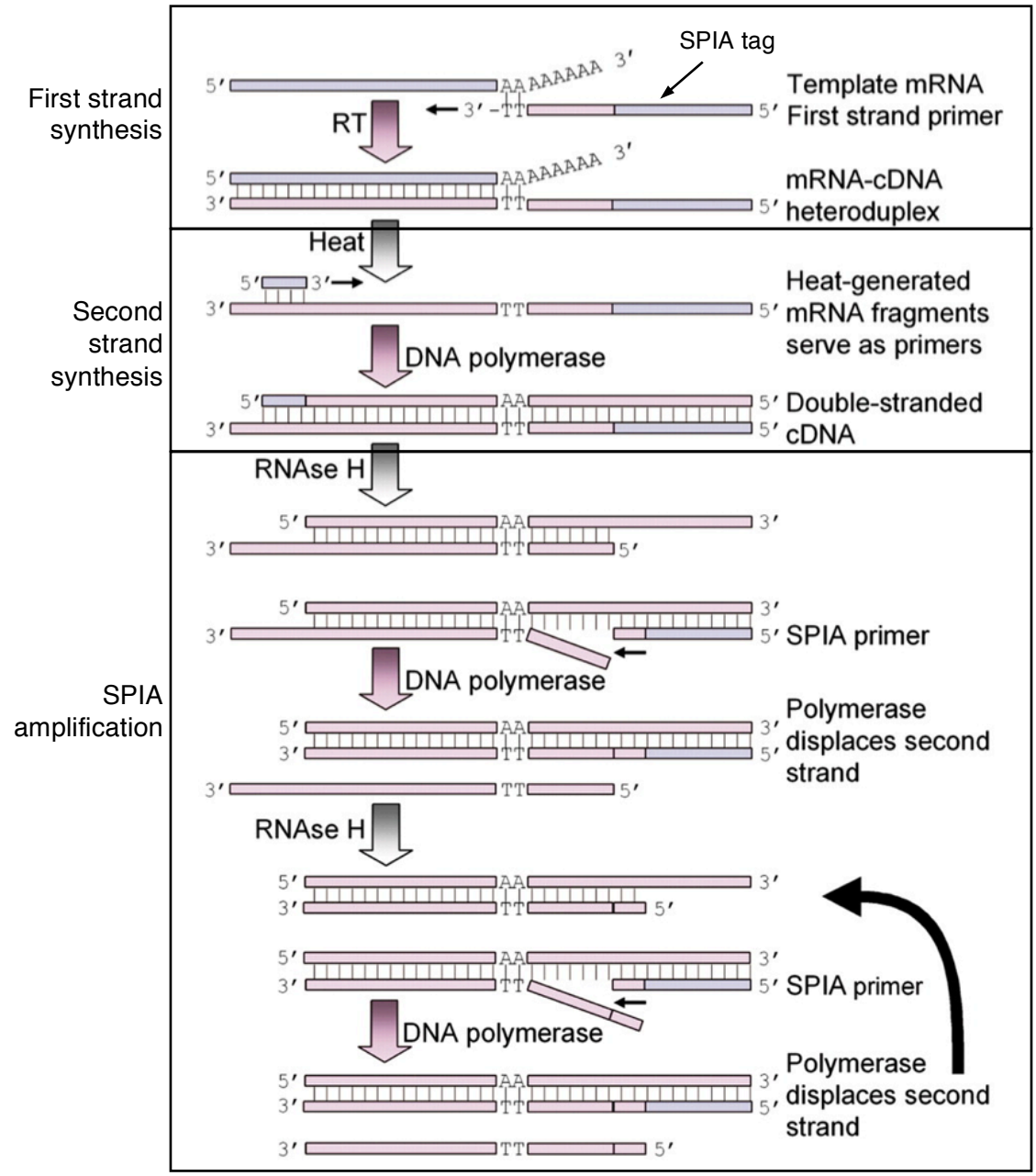


Figure 2.4 Ribo-SPIA technology

Illustration of Ribo-SPIA technology from NuGEN used in Ovation PicoSL WTA system V2 kit. Blue, RNA; pink, DNA. Modified from Singh *et al.* (2005).

2.8 Quantitative real-time polymerase chain reaction (qRT-PCR)

TaqMan primer/probe sets labelled with FAM™ (Life Technologies) used in relative quantification qRT-PCR reactions are shown in Table 2.6. Eukaryotic 18s ribosomal RNA (rRNA) (VIC label) (Applied Biosystems, Paisley, UK) was used as the reference gene. cDNA was diluted to 1 ng/µl and master mix for each probe set was made according to amounts shown in Table 2.7.

Master mix (8.5µl) was pipetted into each well of a 384-well plate for multiplex assays and 1.5µl of cDNA (1 ng/µl) added to appropriate wells in triplicate. Plates were spun at 1000g for 2 minutes to remove air bubbles prior to reading on the ViiA™ 7 Real Time PCR System qPCR machine (Applied Biosystems) utilising the ViiA™ 7 software (Applied Biosystems).

Data values acquired were averaged before further analysis. Thresholds for cycle threshold (Ct) values were set to the linear, lower third of the amplification plot to ensure quantification of cDNA in the exponential amplification phase. Data was normalised to 18s rRNA expression and analysed utilising the comparative $2^{-\Delta\Delta Ct}$ method: [delta][delta]Ct = [delta]Ct,sample - [delta]Ct,reference (Livak *et al.*, 2001).

Table 2.6 TaqMan probe sets used in qRT-PCR reactions

Target (FAM label)	Probe ID
AIM2	Hs00915710_m1
CCL20	Hs01011368_m1
GATA3	Hs00231122_m1
IFNG	Hs00174143_m1
IL-5	Hs00174143_m1
IL13	Hs00174379_m1
IL17A	Hs00174383_m1
IL17BR (IL25R)	Hs00218889_m1
IL17F	Hs00369400_m1
IL1RL1 (ST2, transmembrane)	Hs00249389_m1
IL1RL1 (ST2, soluble)	Hs01073300_m1
IL4	Hs00929862_m1
KLRB1 (CD161)	Hs00174469_m1
LTA	Hs00236874_m1
PMCH	Hs00173595_m1
PTGS2 (COX2)	Hs00153133_m1
RORC	Hs01076122_m1

Table 2.7 qRT-PCR master mix components per reaction

	1 x Master Mix (per reaction)
TaqMan universal master mix II, no uracil-DNA glycosylase (UNG) (Applied Biosystems)	5µl
Target gene primer (FAM)	0.33µl
18s (VIC)	0.17µl
RNase-free water	3µl
Total volume	8.5µl

2.2 Gene expression microarray

Amplified cDNA was biotin labelled with the NuGEN Encore BiotinIL module according to the manufacturer's instructions. Briefly, the process involves the cleavage of uracil bases incorporated during SPIA amplification by uracil-DNA glycosylase (UNG) treatment. This was followed by biotin labelling at the cleavage sites. Purification of biotin-labelled cDNA was performed using the MinElute reaction cleanup kit (Qiagen) and eluted cDNA (10µl volume) quantified with the NanoDrop 2000 spectrophotometer. Labelled cDNA (750ng) was then aliquoted and either diluted with RNase-free water or vacuum concentrated in an Eppendorf vacufuge vacuum concentrator (Stevenage, UK) to a final volume of 5µl.

Direct hybridisation to the Illumina HumanHT-12 v4 Expression BeadChip (Illumina United Kingdom, Essex UK), with subsequent washing, blocking and streptavidin-Cy3 staining steps were performed by the NIHR BRC Genomics Facility at Guy's Hospital. Illumina HumanHT-12 v4 Expression BeadChip allows the expression analysis of more than 47,000 genes. The BeadChip was scanned with the iScan system (Illumina) using GenomeStudio software (Illumina). Data was analysed with the Partek Genomics Suite™ software (Partek Incorporated, Missouri, USA) using the 3 way-ANOVA model. Genes were considered significantly differentially expressed at $p<0.05$, together with a >2-fold difference in expression.

2.9 T cell receptor (TCR) repertoire analysis

2.9.1 DNA isolation

Interphase phase and organic phase layers stored from samples during RNA isolation were spun down in tubes for 10 minutes at maximum speed. Any remaining aqueous phase overlaying the interphase was removed and 700µl of 100% ethanol added to precipitate DNA. Tubes were then vortexed for 15 seconds, left to rest for 2-3 minutes and subsequently centrifuged at 12,000g for 5 minutes. Supernatant (containing proteins) was discarded with DNA pellet remaining in the bottom of tube.

DNA pellets were washed with 700µl of 0.1M sodium citrate/10% ethanol, gently vortexed and left to incubate for 30 minutes at room temperature with occasional mixing by inversion. Tubes were subsequently centrifuged at 12,000g for 5 minutes and supernatant removed and discarded. The wash step was repeated before 1ml of 75% ethanol was added per tube. This was followed by a 10-20 minute incubation step with occasional mixing by inversion. Tubes were centrifuged (12,000g for 5 minutes) and supernatant discarded. DNA pellets were air-dried before resuspension in 8mM sodium hydroxide. DNA purification was performed utilising the MinElute reaction cleanup kit (Qiagen) and samples eluted in 10µl volume. DNA was stored at -20°C until required.

2.9.2 TCR Vβ sequencing

DNA samples were shipped on dry ice to Adaptive Biotechnologies (Seattle, USA) for TCR Vβ sequencing utilising the immunoSEQ assay. The proprietary technology involves a combination of high throughput sequencing and a multiplex PCR strategy to amplify the complementarity-determining region 3 (CDR3) region of the T cell receptor (TCR). This spans the variable region formed by the junction of the V, D and J segments and their associated non-templated insertions.

Data was analysed with the immunoSEQ analyser software (Adaptive Biotechnologies). Nucleotide sequences of the CDR3 region up to 60 nucleotides long were obtained and clonality of the TCR Vβ repertoire established with the use of the proprietary software.

2.10 AIM2 functional experiment

Poly(dA:dT) is a synthetic ligand of the AIM2 inflammasome and consists of a (poly(deoxyadenylic-deoxythymidylic) acid sodium salt and a repetitive double-stranded DNA sequence of poly(dA-dT)•poly(dT-dA).

Polyp explant T cells were harvested for functional experiments 7 days post anti-CD3/CD28 antibody stimulation and incubated with poly(dA:dT) (InvivoGen, Toulouse, France). For transfection of poly(dA:dT) into cells, poly(dA:dT) (1μg) was mixed with 100μl of LyoVec™, a cationic lipid transfection reagent (InvivoGen). The mixture was incubated at room temperature for 15 minutes to allow the formation of a poly(dA:dT):LyoVec complex.

Polyp explant-derived cells were resuspended in complete RPMI media and plated onto a 48 well plate at 350 μl/well. Varying concentrations of the poly(dA:dT)/LyoVec complex were added (1-2 μg/ml). Wells with LyoVec only and complete media were used as vehicle and negative controls. Cells were incubated for 36 hours at 37°C before cells were harvested for intracellular cytokine staining.

2.11 Statistical analysis

All statistical analysis was performed using GraphPad Prism® 5 software. Results were considered statistically significant if $p<0.05$. Significance was determined as $p<0.05$*, $p<0.01$** and $p<0.001$***.

Chapter 3 Characterisation of nasal tissue-specific T cell phenotypes in health and polyposis

3.1 Introduction

Since the pivotal roles for Th2 cells and their cytokines in allergic asthma were established (Robinson *et al.*, 1992, Wills-Karp *et al.*, 1998), the function and importance of the Th2 response in allergic asthma and other human allergic airway diseases such as allergic rhinitis has been extensively reviewed (Greiner *et al.*, 2011, Sin *et al.*, 2011, Ingram *et al.*, 2012, Cohn *et al.*, 2014). However, few studies have examined the immunobiology of T cells in the normal nasal mucosa.

One such study by Till *et al.* (2001) has shown compartmentalisation of T cells in the normal nasal mucosa and peripheral blood. A predominance of the Th1 subset was found in the normal nasal mucosa with reduced expression of the Th2 cytokines IL-4 and IL-5 compared to the periphery. Furthermore, a parallel decrease in expression of CCR3, the chemokine receptor first described by Sallusto *et al.* (1997) to be Th2-associated, was observed in the nasal mucosa. Additionally, compartmentalisation of CCR6 expression has been reported with elevated expression by nasal mucosal T cells compared to peripheral blood T cells (Francis *et al.*, 2008). This finding was similarly observed in both bronchial mucosal and skin biopsy samples suggesting a general tissue-homing role for CCR6. Overall, these studies highlight the importance of studying the local T cell response as opposed to exclusively examining the peripheral response. Thus, in this thesis, analysis of both local and peripheral T cells was performed in order to characterise phenotypic differences between the two sites.

Since the publication of these studies, CCR6 has been reported to be a marker of IL-17-producing Th17 cells (Maggi *et al.*, 2010). Additional novel T helper cell subsets such as Th9 and Th22 cells have also been identified (Veldhoen *et al.*, 2008b, Duhen *et al.*, 2009). Levels of mRNA for the Th9-associated cytokine IL-9 were upregulated in nasal biopsy specimens from allergic rhinitis patients (Nouri-Aria *et al.*, 2005) and polyp

tissue homogenates from CRSwNP patients (Lin *et al.*, 2014). Limited literature exists on the role of IL-22-producing Th22 cells in the respiratory tract. In the few IL-22 studies published in animal models of allergen-induced inflammation, expression of this cytokine was associated with a decrease in allergic inflammation (Schnyder *et al.*, 2010, Fang *et al.*, 2014). However, studies of these subsets in local T cells direct from the human upper respiratory tract are lacking. This illustrates that there is a clear need to re-examine the T cell phenotypes present in the nasal mucosa.

In light of these studies, this project set out to reinvestigate the immunobiology of local nasal mucosal T cells in health and in nasal polyposis. CRSwNP was selected for study in this thesis for a number of reasons. Nasal polyps represent a source of chronically inflamed nasal mucosal tissue, typically abundant in Th2 cells and are associated with eosinophilic inflammation (Hamilos, 2000, Zhang *et al.*, 2008). Symptoms of CRSwNP are often severe however, no effective treatments are currently available. This is partly due to the unknown aetiology and the absence of an identified antigen involved in disease pathogenesis, making CRSwNP an intriguing disease to study. In addition, polyp tissue is relatively accessible as a by-product of routine polypectomy surgery.

Specifically, aims of the work presented in this chapter were to investigate:

1. The phenotype of the local nasal mucosal T cell vs. the peripheral response.
2. These phenotypes in health and CRSwNP.
3. The phenotype of T cells in the nasal mucosa vs. the skin.

3.1 Results

3.1.1 Optimisation of T cell stimulation in short-term expansion cultures *in vitro*

T cell activation and expansion *in vitro* requires periodic stimulation through the CD3/TCR complex and non-cognate engagement of co-stimulatory molecules such as CD28 and CD80/86. There are a number of different approaches for *in vitro* stimulation of cells with two of the most common methods being the use of 1) anti-CD3 and anti-CD28 antibodies and 2) irradiated feeder cells in conjunction with a mitogen such as phytohaemagglutinin (PHA). Both methods have been previously used in the Cousins group (Cousins *et al.*, 2002) and Till group (Till *et al.*, 2001). Preliminary experiments were therefore carried out utilising peripheral blood to establish if either of the two methods was optimal for this study.

3.1.1.1 Irradiated PBMC/PHA and anti-CD3/CD28 stimulation result in comparable cell numbers and T cell subsets

PBMC and $CD4^+$ cells were isolated from peripheral blood and restimulated with irradiated PBMC/PHA or anti-CD3/CD28 antibodies on day 0. On day 7 post-restimulation, cell numbers and the percentage of $CD4^+$ and $CD8^+$ cells were determined. Figure 3.1 shows that although the average number of cells per ml of culture volume was slightly higher for cultures stimulated with irradiated PBMC/PHA, this was not substantially different from the anti-CD3/CD28 stimulated cultures. Furthermore, no sizeable difference in cell numbers was observed when starting culture populations of pure $CD4^+$ T cells and PBMC were compared.

For PBMC cultures, the percentage of $CD4^+$ vs. $CD8^+$ cells in expanded cultures is shown in Figure 3.1b. A larger percentage of $CD4^+$ cells was observed compared to $CD8^+$ cells for both restimulation methods. Cultures restimulated with anti-CD3/CD28 showed a trend for higher CD4:CD8 ratio compared to stimulation with irradiated PBMC/PHA (80.9% ± 3.6 vs. 62.5% ± 8) although this was not statistically significant. Taken together, the data above shows that both restimulation methods are comparable in terms of cell numbers and subsets produced at the end of the 7 day short-term culture. Anti-CD3/CD28 was therefore selected for use in further experiments as this removed the need for an on-going supply of PBMCs and facilitated standardisation of culture conditions.

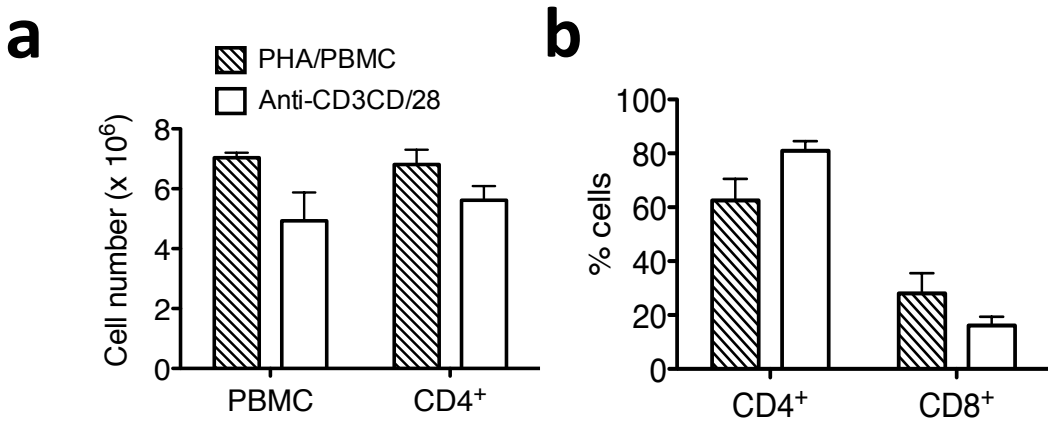


Figure 3.1 Irradiated PBMC/PHA and anti-CD3/CD28 antibodies are comparable cell stimulation methods.

(a) Live cell numbers on day 7 following stimulation with irradiated PBMC/PHA or anti-CD3/CD28 was calculated by staining with propidium iodide in the PBMC and $CD4^+$ populations. Data is presented as mean ± SEM (n=6). (b) Percentages of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells in PBMC cultures on day 7 following stimulation are shown (n=6).

3.1.2 Characterisation of the T cell surface phenotype in the polyp nasal mucosa vs. in the periphery

Polyp tissue was obtained from CRSwNP patients undergoing routine polypectomy surgery. Demographic and clinical data are shown in Table 3.1. Tissue was processed on day of receipt as previously described (Chapter 2, page 60). To characterise the T cell phenotype of polyp explants and blood-derived T cells, extensive flow cytometry for T cell surface markers was performed following short-term explant culture. Cells were harvested for analysis on day 7 post stimulation with anti-CD3/CD28 antibodies. Isotype controls were used in all experiments as control for non-specific staining.

Table 3.1 CRSwNP patient demographic and clinical data.

Code	Gender	Ethnicity	Age	Aspirin sensitive	Asthma	Prednisolone	Inhalers	Tablets	Nasal	Atopic	Smoker	Other information
004HKP	F	Caucasian	52	+	+		Symbicort			-	N	
005HKP	M	Caucasian	39	+	+		Symbicort	Singulair	Flixonase	+	Y	
006HKP	F	Caucasian	32	+	+		Symbicort		Flixonase	-	N	
007HKP	M	Caucasian	35	+	+	5 weeks before surgery	Symbicort			+	N	
008HKP	M	Caucasian	50	+	+	6 weeks before surgery	Symbicort		Flixonase	-	N	
012HKP	M	Caucasian	48	-	+		Seretide		Flixonase	+	N	
013HKP	M	Caucasian	47		+	2 weeks before surgery	Seretide		Flixonase	-	N	
015HKP	M	Caucasian	46	+	+	3 weeks before surgery	Symbicort	Singulair	Flixonase	-	N	
016HKP	M	Asian		+	+		Seretide		Flixonase	N.K	N	
018HKP	M	African-Caribbean	58	+	+		Symbicort		Flixonase	-	N	
020HKP	F	Caucasian		+	+	15mg dose daily	Symbicort		Flixonase	+	N	
021HKP	M	Indian	38		+	8 weeks before surgery	Symbicort		Flixonase	-	N	
022HKP	M	Caucasian	49	+	+	4 weeks before surgery	Becotide	Singulair	Flixonase	+	N	
023HKP	F	African-Caribbean	43	+	+		Seretide		Flixonase	-	N	
025HKP	F	Caucasian	42	-	+				Flixonase	+	N	
026HKP	M	Caucasian	65	-	-					-	N	
027HKP	M	Caucasian	34	+	+		Seretide		Flixonase	-	N	
031HKP	F	Caucasian	54	-	+		Symbicort	Singulair	Flixonase	N.K *	N	pANCA+
032HKP	F	Caucasian	65		+		Seretide			+	N	
033HKP	F	Caucasian	37		+		Becotide	Singulair	Flixonase	+	N	
034HKP	F	Caucasian	36	+	+		Symbicort		Flixonase	+	N	Mepolizumab 6 months before
035HKP	F	Caucasian	45	+	+		Seretide	Singulair	Avamys	-	N	
036HKP	F	Caucasian	42	-	+	5mg dose daily	Becotide	Cetirizine	Flixonase	+	N	Citalopram
039HKP	M	Caucasian	59	+	+	10 days before surgery	Symbicort			-	Y	Co-amoxiclav
040HKP	F	Caucasian	49	+	+		Seretide	Cetirizine	Betnesol	+	N	

N.K, not known. pANCA, perinuclear anti-neutrophil cytoplasmic antibodies * Patient was dermographic.

3.1.2.1 Effector memory phenotype of peripheral blood and polyp-derived T cells

$CD4^+$ T cells (Figure 3.2a) and $CD8^+$ T cells (Figure 3.2b) were analysed for expression of TCRαβ, CD45RO, CD62L and CCR7. The overwhelming majority of T cells were determined to be $TCR\alpha\beta^+$ (Figure 3.3a). Both blood and polyp-derived populations expressed high levels of CD45RO consistent with a memory phenotype after restimulation, although expression by $CD4^+$ cells from polyp explants was higher than $CD4^+$ cells from blood cultures (polyp, 98.9% ± 0.8 vs. blood, 93.2% ± 2.8; $p<0.01$) (Figure 3.3b). Furthermore, CD62L and CCR7, expression of which defines central memory T cells (T_{CM}), were significantly lower in polyp-derived $CD45RO^+$ cells compared to blood-derived cells (polyp $CD4^+$, 5.8% ± 2.1 vs. blood $CD4^+$, 13.1% ± 3.4; $p<0.001$) (Figure 3.3c). The converse was true for $CD62L^-CCR7^-CD45RO^+$ effector memory T cells (T_{EM}), with significantly higher percentages of T_{EM} cells detected in polyp-derived cultures compared to blood-derived cultures (polyp $CD4^+$, 39.1% ± 3.5 vs. blood $CD4^+$, 23.5% ± 6.2; $p<0.05$) (Figure 3.3d). Expression of CD69 by polyp-derived $CD4^+$ cells was also significantly elevated compared to blood-derived cells (polyp $CD4^+$, 39.4% ± 7.5 vs. blood $CD4^+$, 21.8% ± 3.5; $p<0.05$) (Figure 3.3e). These data suggest that in polyp and blood cultures, T_{EM} cells constitute the major cell population although numbers were significantly higher in the tissue.

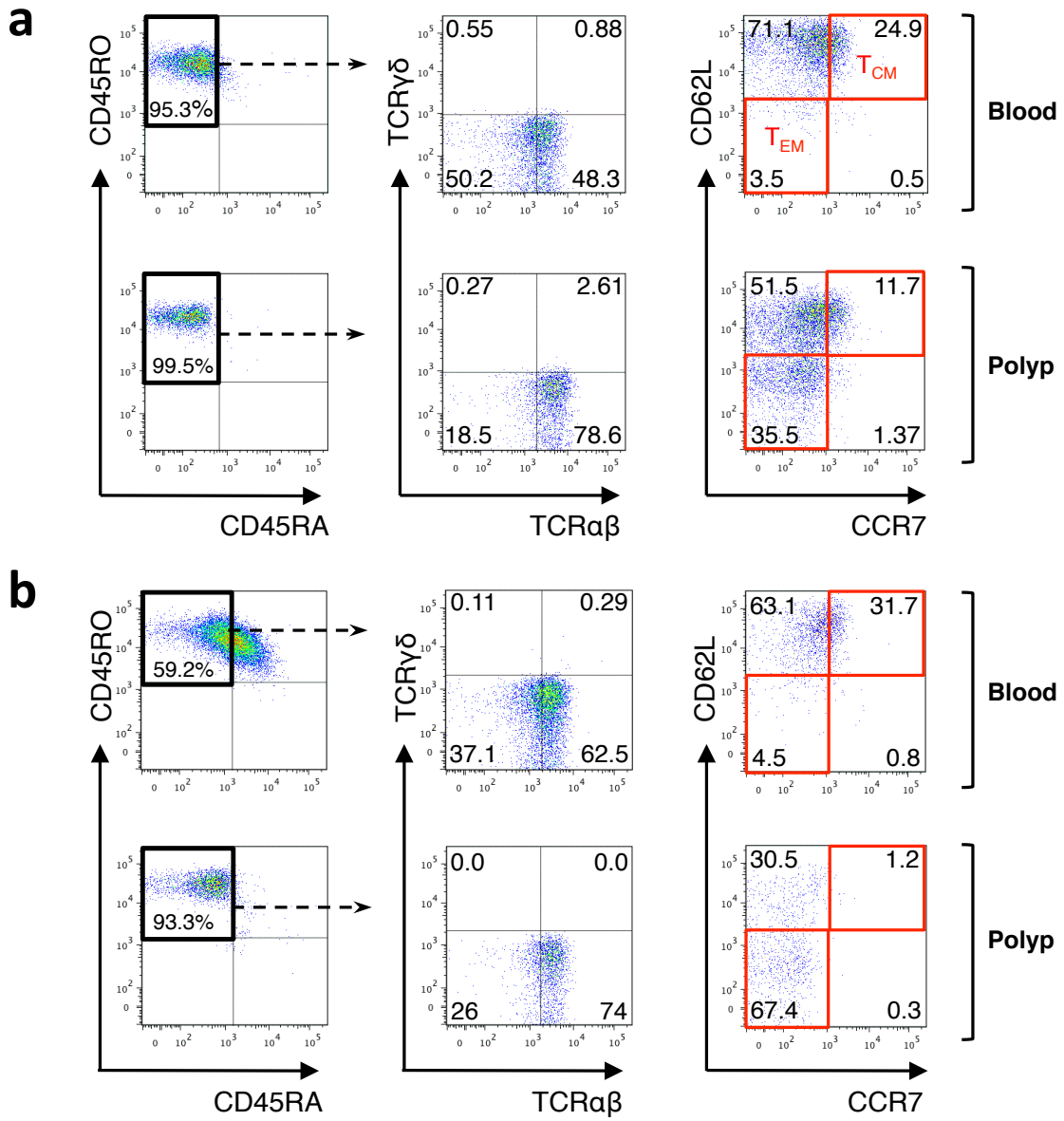


Figure 3.2 Representative flow cytometry staining for TCRαβ and memory markers from nasal polyp explant and blood cultures.

Representative flow cytometry staining for (a) $CD4^+$ cells and (b) $CD8^+$ cells derived from blood and polyp tissue. The black highlighted quadrant denotes the gate for memory $CD45RO^+$ T cell population used for subsequent analysis of TCRαβ, TCRγδ, CD62L and CCR7 expression. Red highlighted quadrants represent the T_{CM} subset ($CD62L^+CCR7^+$) and T_{EM} subset ($CD62L^-CCR7^-$).

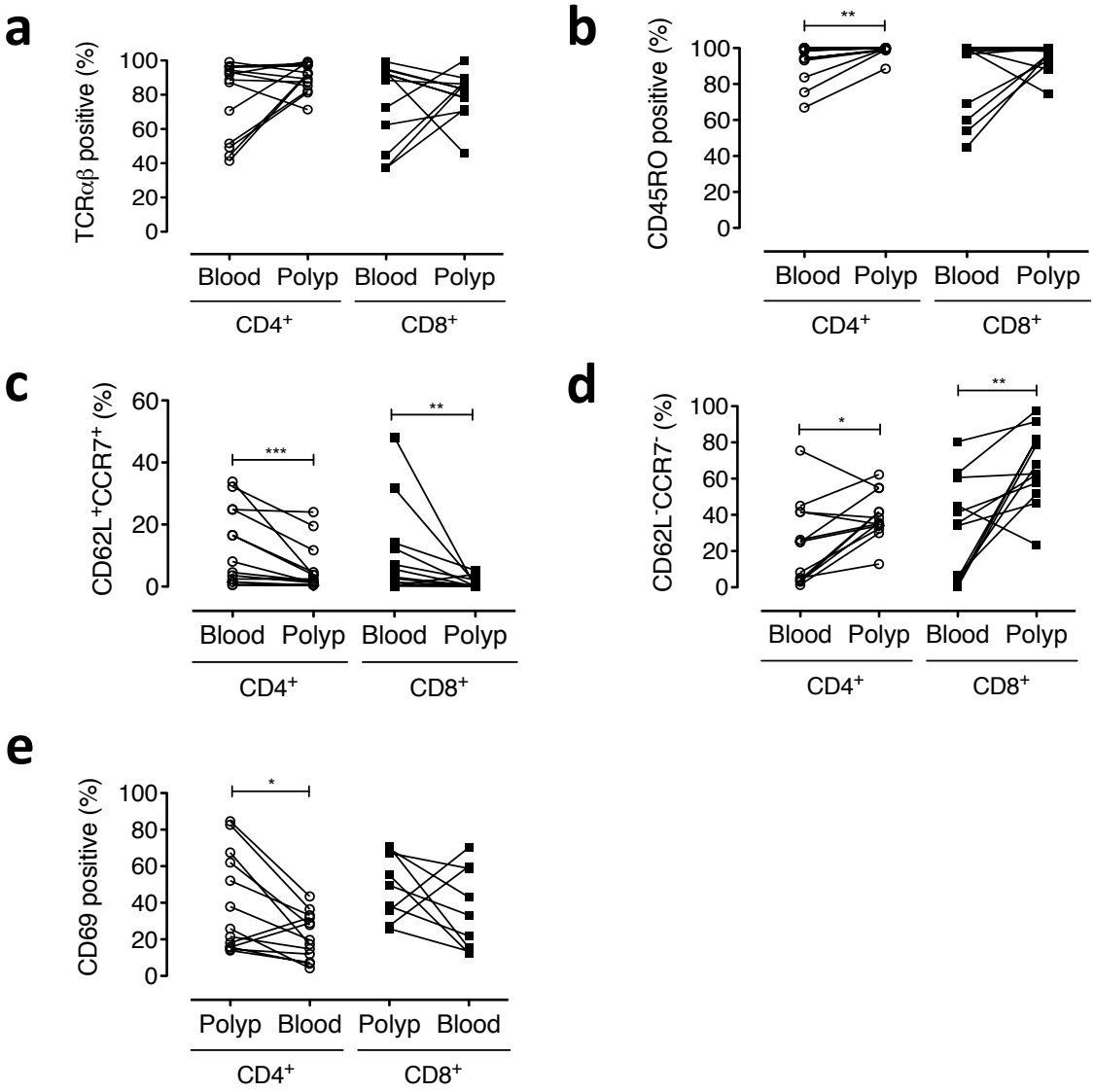


Figure 3.3 Nasal polyp explants contain a higher percentage of effector memory TCRαβ T cells compared to peripheral blood.

Percentage of $CD4^+$ cells (n=14) and $CD8^+$ cells (n=12) expressing (a) CD45RO are shown from blood vs. polyp-derived tissue. The percentage of $CD45RO^+$ cells expressing (b) TCRαβ, (c) $CD62L^+CCR7^+$ (T_{CM}), (d) $CD62L^-CCR7^-$ (T_{EM}) and (e) CD69 are also shown. Wilcoxon matched-pairs signed rank test. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$.

3.1.2.2 Adhesion molecules and skin homing receptor CLA expression by polyp-derived T cells

In a previous study, Purwar *et al.* (2011) evaluated the expression of the adhesion molecules CD49a (VLA-1), p-selectin glycoprotein ligand-1 (PSGL1), the skin homing receptor cutaneous lymphocyte antigen (CLA) and the gut homing receptor $\alpha_4\beta_7$ in lung tissue-resident memory T cells. In this study, the expression of these markers were also examined in $CD4^+$ T cells (Figure 3.4a) and $CD8^+$ T cells (Figure 3.4b) from nasal polyp and blood cultures. Almost all cells (>98%) expressed PSGL1 (Figure 3.4e). CD49a expression was more than 1.5-fold higher in polyp-derived cells compared to blood cells (Figure 3.4c) for $CD4^+$ and $CD8^+$ populations. No detectable expression of the gut homing receptor $\alpha_4\beta_7$ was found (data not shown). Interestingly, CLA expression (Figure 3.4d) was significantly higher (approximately 2-fold) in both polyp $CD4^+$ and $CD8^+$ cells compared to cells from the peripheral blood.

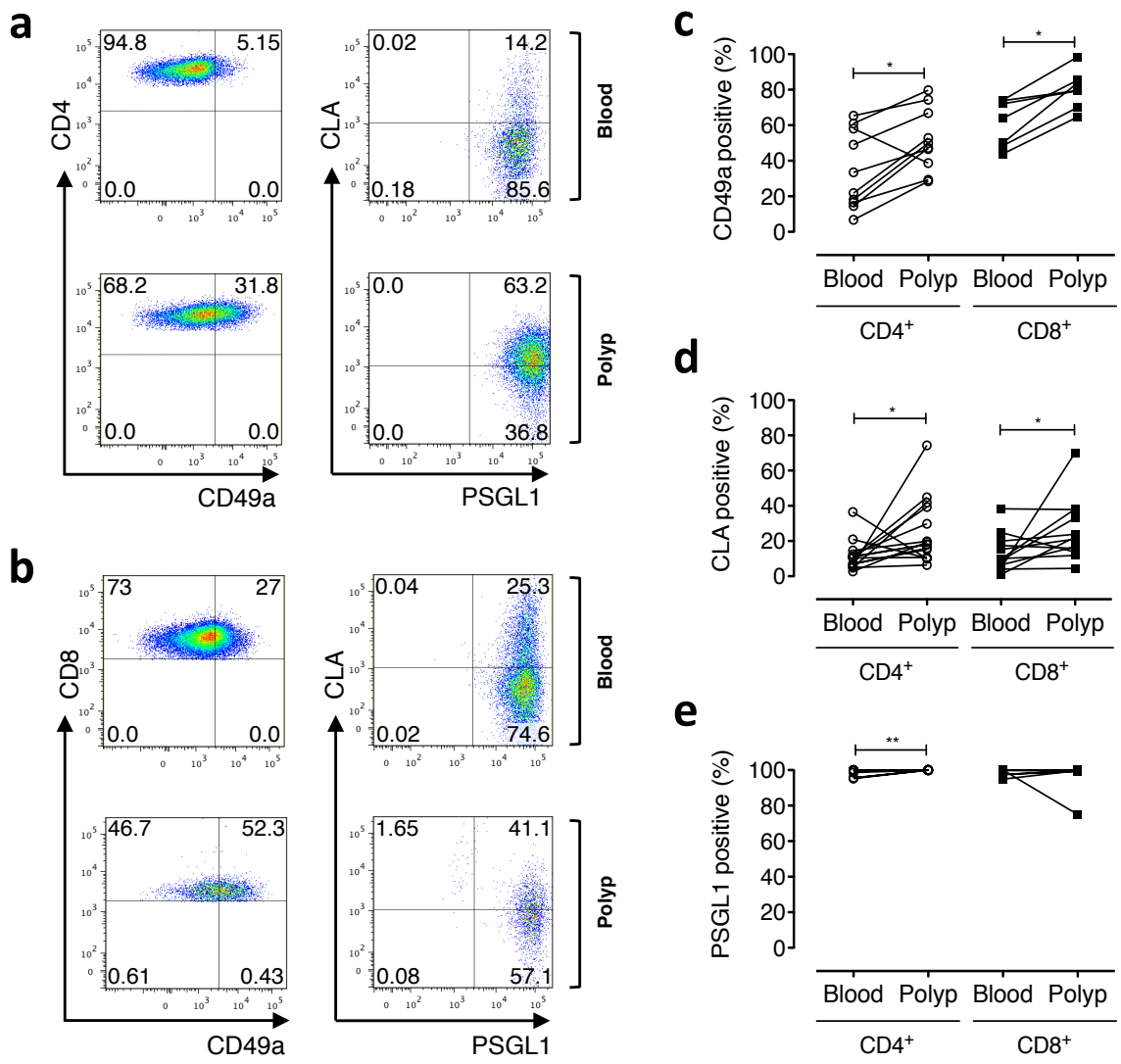


Figure 3.4 Expression of adhesion molecule CD49a and skin homing receptor CLA by nasal polyp explants compared to peripheral blood.

Representative flow cytometry staining for (a) $CD4^+$ cells and (b) $CD8^+$ cells derived from blood and polyp tissue. Expression of (c) CD49a, (d) CLA and (e) PSGL1 by $CD4^+$ cells (n=14, n=10 for CD49a) and $CD8^+$ cells (n=11, n=7 for CD49a) from blood vs. polyp-derived tissue. Wilcoxon matched-pairs signed rank test. *, $p<0.05$, **, $p<0.01$.

3.1.2.3 Nasal polyp-derived cells express significantly higher percentages of CCR6, CRTH2 and IL-25R compared to the periphery

The expression of different T helper cell subset markers was examined. Expression of the Th17 marker CCR6 (Figure 3.5b) and Th2 markers CRTH2 (Figure 3.5d) and IL-25R (Figure 3.5e) were significantly higher in $CD4^+$ polyp cells compared to $CD4^+$ cells derived from peripheral blood (CCR6, polyp 63.5% ± 4.7 vs. blood 9.4% ± 3.7; $p<0.001$; CRTH2, polyp 4.6% ± 0.9 vs. blood 1.6% ± 0.7; $p<0.01$; IL-25R, polyp 15.4% ± 3.4 vs. blood 1.7% ± 0.4; $p<0.001$). Conversely, expression of the Th1 marker CXCR3 was 2-fold lower in $CD4^+$ polyp cells compared to $CD4^+$ blood cells (polyp 26% ± 5.3 vs. blood 51.7% ± 6.8; $p<0.01$) (Figure 3.5c). Although $CD8^+$ cells showed similar expression patterns to $CD4^+$ cells, levels of expression of all phenotypic markers examined were lower in $CD8^+$ cells.

Notably, of the markers that were differentially expressed in the $CD4^+$ population, $CCR6^+$ and IL-$25R^+$ cells represented the two most abundant populations in nasal polyp explants (CCR6, 63.5% ± 3.7; IL-25R, 15.4% ± 3.4). IL-25R showed the greatest relative expression in polyp-derived cells compared to cells from the blood (9-fold higher on average) of all T cell subset markers examined. Figure 3.6 illustrates the difference in IL-25R expression in blood vs. polyp samples from 7 CRSwNP patients.

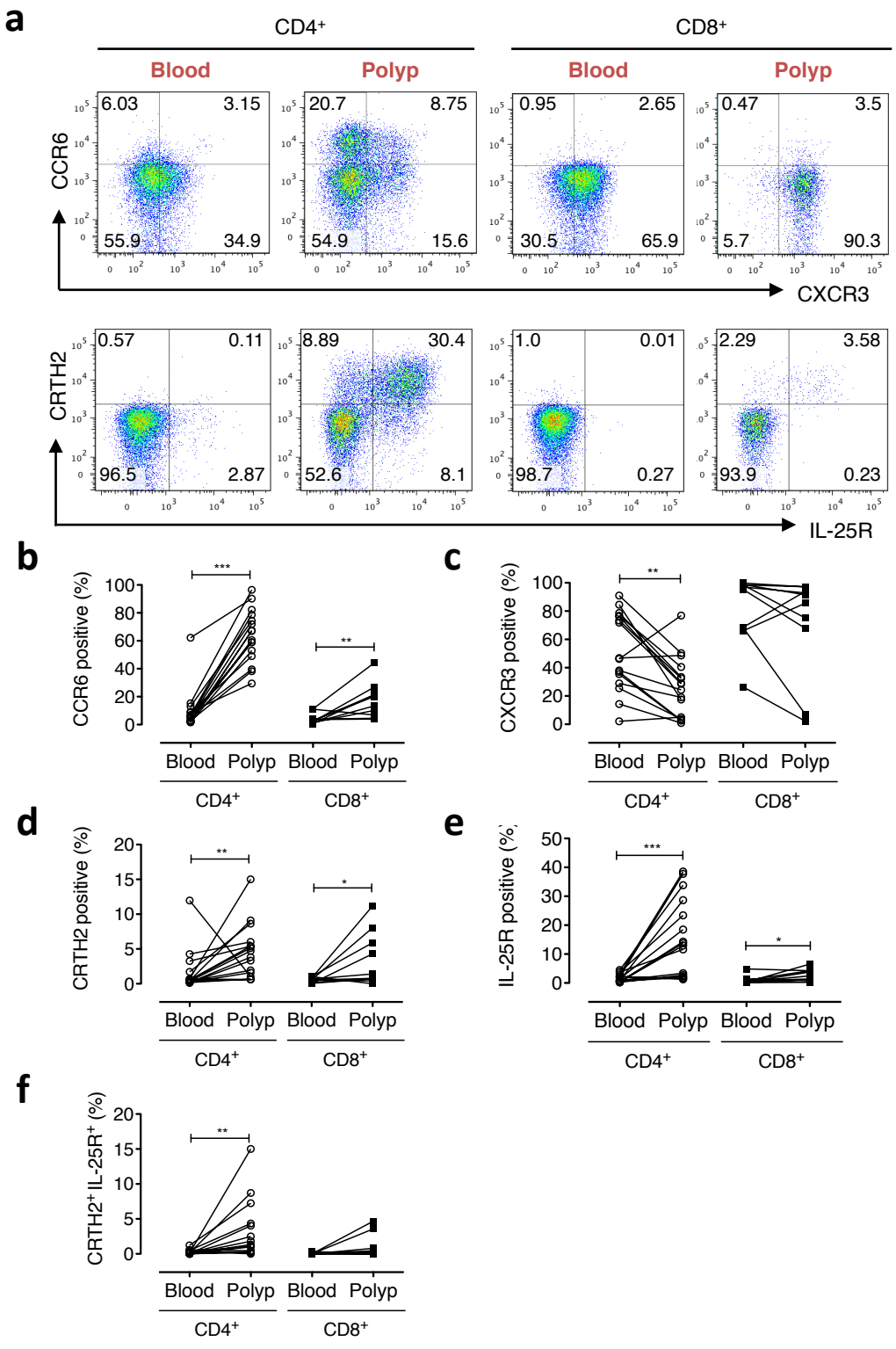


Figure 3.5 Higher percentages of CCR6$^+$, CRTH2$^+$ and IL-25R$^+$ T cells are detected in nasal polyp explants compared to peripheral blood.

Representative flow cytometry staining of T cell subset surface markers for CD4$^+$ cells and CD8$^+$ cells derived from blood and polyp tissue are shown in (a). Expression of (b) CCR6, (c) CXCR3, (d) CRTH2, (e) IL-25R and (f) CRTH2$^+$IL-25R$^+$ by CD4$^+$ cells (n=16) and CD8$^+$ cells (n=10) from blood vs. polyp-derived tissue. Wilcoxon matched-pairs signed rank test. *, p<0.05, **, p<0.01, ***, p<0.001.

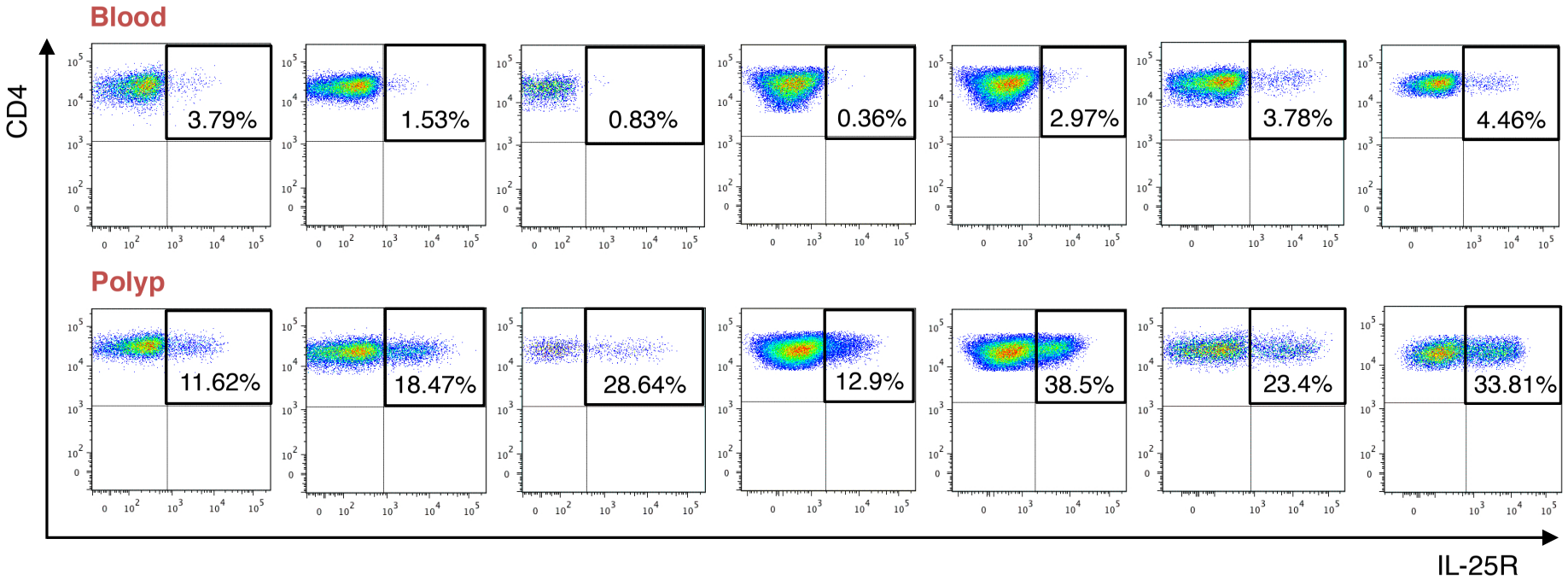


Figure 3.6 IL-25R expression by polyp-derived T cells vs. blood-derived T cells.

Flow cytometry staining for CD4 and IL-25R expression is shown for polyp and blood-derived cells from 7 independent experiments. Each column represents matched polyp and blood samples from the same patient.

3.1.2.4 Explant-derived polyp cells are comparable to cells from collagenase digested polyp tissue

To address the possibility that the tissue microenvironment during culture affected T cell phenotypes, experiments were also performed using T cells expanded from collagenase digested tissue (Chapter 2, page 61). Additionally, comparison of T cell subset populations derived from explant cultures and following collagenase digest was performed to address whether results represented an experimental artefact due to the short-term culture used in the explant model.

Viable cell counts prior to analysis on day 7 post restimulation showed that higher cell numbers were obtained from polyps that were undigested (explant model) compared to polyps that were digested with collagenase (data not shown). This difference was maintained even when cells from digested tissue were subjected to a second round of stimulation with anti-CD3/CD28 and cultured for a further 7 days to boost cell numbers (Figure 3.7e).

T cells expanded from polyp on day 14 of culture revealed similar T cell subset expression profiles to T cells from undigested explant cultures after 7 days expansion (Figure 3.7). Expression of CCR6 (Figure 3.7a), CRTH2 (Figure 3.7c) and IL-25R (Figure 3.7d) was elevated in $CD4^+$ digested polyp cells compared to peripheral blood cells by 7-fold, 2.5-fold and 4-fold respectively. Moreover, CXCR3 expression (Figure 3.7b) in digested polyp T cell cultures was also reduced by around half of that observed in the peripheral blood T cell culture. $CD8^+$ cells also showed the same pattern as $CD4^+$ cells.

Having established that the expression profile of T cell subset markers was similar from collagenase digested and non-digested explant cultures, it was decided to use the non-digested explant method to culture cells from polyp tissue in further experiments. This

was due to the higher cell yields obtained from explant cultures (required for subsequent experiments). Moreover, explant cultures did not require the second round of anti-CD3/CD28 stimulation and the additional 7 day culture period to boost cell numbers, thus minimising the time cells spent in *in vitro* culture.

To examine the effect of T cell expansion, T cell populations were compared when expanded from explants or when analysed direct from polyp digests. Polyp tissue was digested with collagenase overnight. Cells were then washed and stained for flow cytometric analysis (no 7 day short-term culture). Figure 3.7f shows that IL-25R expression was detectable in polyp tissue T cells *ex vivo* and was higher in polyp-derived cells compared to blood-derived cells (polyp, 4.4% ± 1.8 vs. blood, 1.3% ± 0.6). The IL-25R expression profile *ex vivo* was therefore comparable to cultured cells.

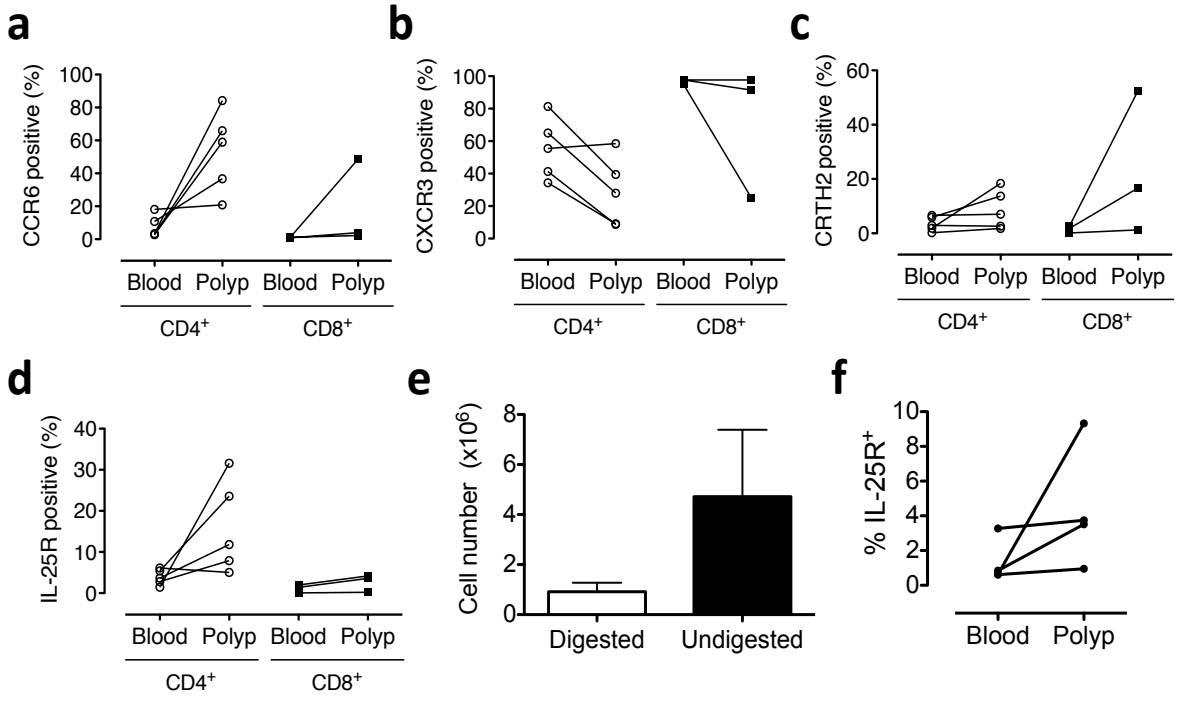


Figure 3.7 Expression of T cell surface markers by T cells expanded from digested and undigested polyp explant cultures.

Polyp tissue was digested with Liberase collagenase (2.5 mg/ml) overnight and dissociated cells restimulated with immobilised anti-CD3/CD28 antibodies. Flow cytometry staining for T cell subset markers (a) CCR6, (b) CXCR3, (c) CRTH2 and (d) IL-25R was performed on day 14 of culture for $CD4^+$ (n=5) and $CD8^+$ cells (n=3). (e) Viable cell numbers at the end of the culture period (14 days, digested; 7 days, undigested) were determined by the Trypan Blue exclusion method (n=3). (f) *Ex vivo* analysis of IL-25R expression by flow cytometry in digested polyp and peripheral blood samples (n=4).

3.1.3 Characterisation of the T cell surface phenotype in the polyp nasal mucosa vs. healthy nasal mucosa

The T cell phenotype in the healthy normal nasal mucosa was examined next to determine whether T cell phenotypes in the nose differ in health and nasal polyposis. Nasal turbinate biopsies and paired blood samples were obtained from healthy volunteers. Demographic and clinical data for the healthy volunteers is shown in Table 3.2. Samples were processed in an identical manner to tissue and blood samples from CRSwNP patients as detailed in Chapter 2. PBMC and biopsy explant cells were restimulated with anti-CD3/CD28 and cultured in the presence of IL-2. Cells were analysed on day 7-post restimulation by flow cytometry.

Table 3.2 Demographic and clinical data of healthy volunteers.

Code	Gender	Ethnicity	Age	Atopic	Smoking status	Total serum IgE (IU/ml)
401	M	Caucasian	48	-	Y	8
402	M	Caucasian	38	-	N	5
403	F	Caucasian	44	-	N	10
404	F	Caucasian	41	-	N	1
405	M	Caucasian	20	-	N	31
406	F	Caucasian	46	-	N	3
407	M	Caucasian	53	-	N	8

No volunteers were receiving medication at time of biopsy.

3.1.3.1 Effector memory cells form the major cell population in the normal nasal mucosa

The naive vs. memory T cell phenotype of $CD4^+$ T cells derived from normal nasal biopsies and blood from healthy volunteers was analysed. The majority of cells were determined to be memory $CD45RO^+$ cells (Figure 3.8a, c). Expression of the central memory T cell marker CD62L was reduced in biopsy-derived cells compared to blood-derived cells (biopsy, 49% ± 3.9 vs. blood, 54.8% ± 6.4) consistent with a higher prevalence of effector memory ($CD62L^-$) $CD4^+$ T cells in the nasal mucosa (Figure 3.8a, b). This was analogous to the expression pattern observed for polyp-derived T cells vs. blood from CRSwNP patients although expression of CD62L was higher overall in CRSwNP samples compared to healthy samples (CRSwNP, blood 80.5% ± 6.6, polyp 63% ± 4.5 vs. healthy, blood 54.8% ± 6.4, biopsy 49% ± 3.9; $p<0.05$ for polyp vs. normal nasal mucosa) (Figure 3.8b). Expression of CLA was also significantly elevated in the normal nasal mucosa compared to the periphery (biopsy, 9.2% ± 3.1 vs. blood, 3.2% ± 0.6; $p<0.05$) (Figure 3.8d) but was significantly lower compared to polyp-derived cells (polyp, 26% ± 4.5; $p<0.05$ for biopsy vs. polyp). Taken as a whole, these data suggest that effector memory T cells also constitute the major cell population in healthy nasal tissue.

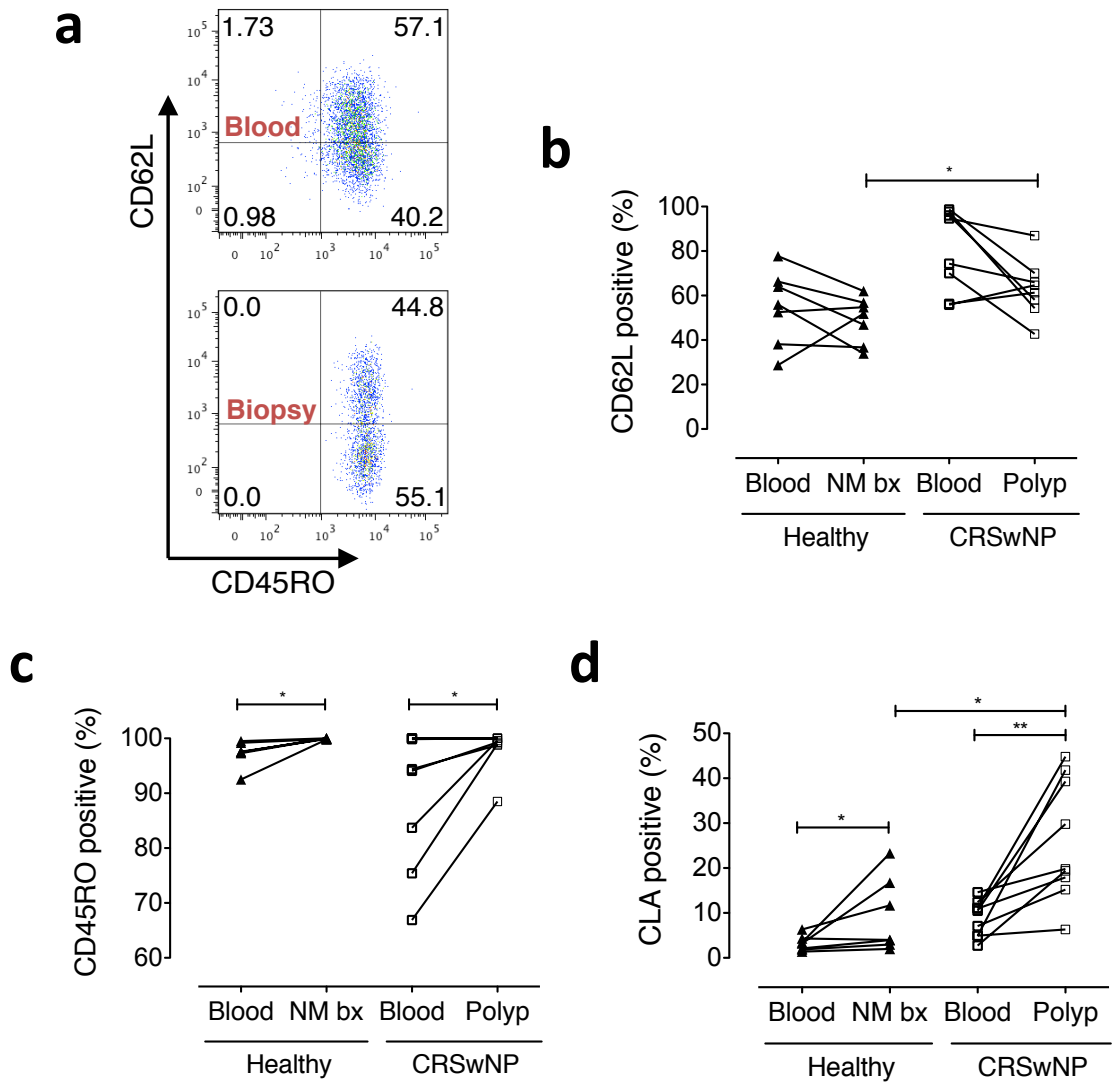


Figure 3.8 Expression of effector memory markers by T cells derived from healthy normal nasal mucosal biopsies and diseased nasal polyp tissue.

Representative flow cytometry staining for $CD4^+$ cells derived from blood and normal mucosa biopsy tissue is shown in (a). Expression of (b) CD62L, (c) CD45RO and (d) CLA by blood and normal mucosa biopsy (NM bx) from healthy volunteers (n=7) vs. blood and polyp tissue from CRSwNP patients (n=9). Blood vs. tissue: Wilcoxon matched-pairs signed rank test. Healthy nasal mucosa vs. polyp: Mann Whitney Test. *, $p<0.05$, **, $p<0.01$.

3.1.3.2 CCR6 is a feature of normal nasal mucosal T cells but IL-25R is expressed only in T cells from the diseased nasal polyp

T cell expression of IL-25R, CRTH2, CCR6 and CXCR3 was determined in normal healthy biopsy explant and blood cultures (Figure 3.9a). Minimal expression of the Th2 markers IL-25R and CRTH2 was observed and this was significantly lower than in T cells from nasal polyps (IL-25R, biopsy, 2.4% ± 1.6 vs. polyp, 21.7% ± 3.6; $p<0.01$, CRTH2, biopsy, 0.6% ± 0.3 vs. polyp, 54% ± 1.3; $p<0.01$) (Figure 3.9b, c).

Expression of the Th17 marker CCR6 was consistently higher in healthy nasal mucosa T cells compared to blood-derived cells (biopsy, 91.8% ± 1.8 vs. blood, 7.9% ± 1.8; $p<0.05$). This pattern was analogous to that observed between diseased nasal polyp and blood from CRSwNP patients (Figure 3.9d). However, the percentage of cells expressing CCR6 was significantly higher in healthy nasal mucosa-derived T cells compared to polyp-derived T cells (biopsy, 91.8% ± 1.8 vs. polyp, 57.9% ± 4.5; $p<0.001$). Furthermore, expression of CXCR3 by biopsy- and blood-derived cells from healthy patients also followed the same pattern of expression as CRSwNP patients (Figure 3.9e) with a lower percentage of $CXCR3^+$ cells in normal biopsies vs. the periphery (biopsy, 32.9% ± 3.3 vs. blood, 62.2% ± 4.1; $p<0.05$).

In summary, these data support the hypothesis that T cell phenotypes differ between healthy nasal tissue and the periphery. $CCR6^+$ Th17 cells are abundant in the nasal mucosa in both health and disease but not in the periphery. In addition, putative Th2 cells characterised by expression of CRTH2 and IL-25R were detectable only from diseased polyp tissue associated with Th2 eosinophilic inflammation. $CCR6^+$ T cells therefore represent a nasal tissue-specific phenotype whilst $IL\text{-}25R^+$ and $CRTH2^+$ T cells are specific to nasal polyposis.

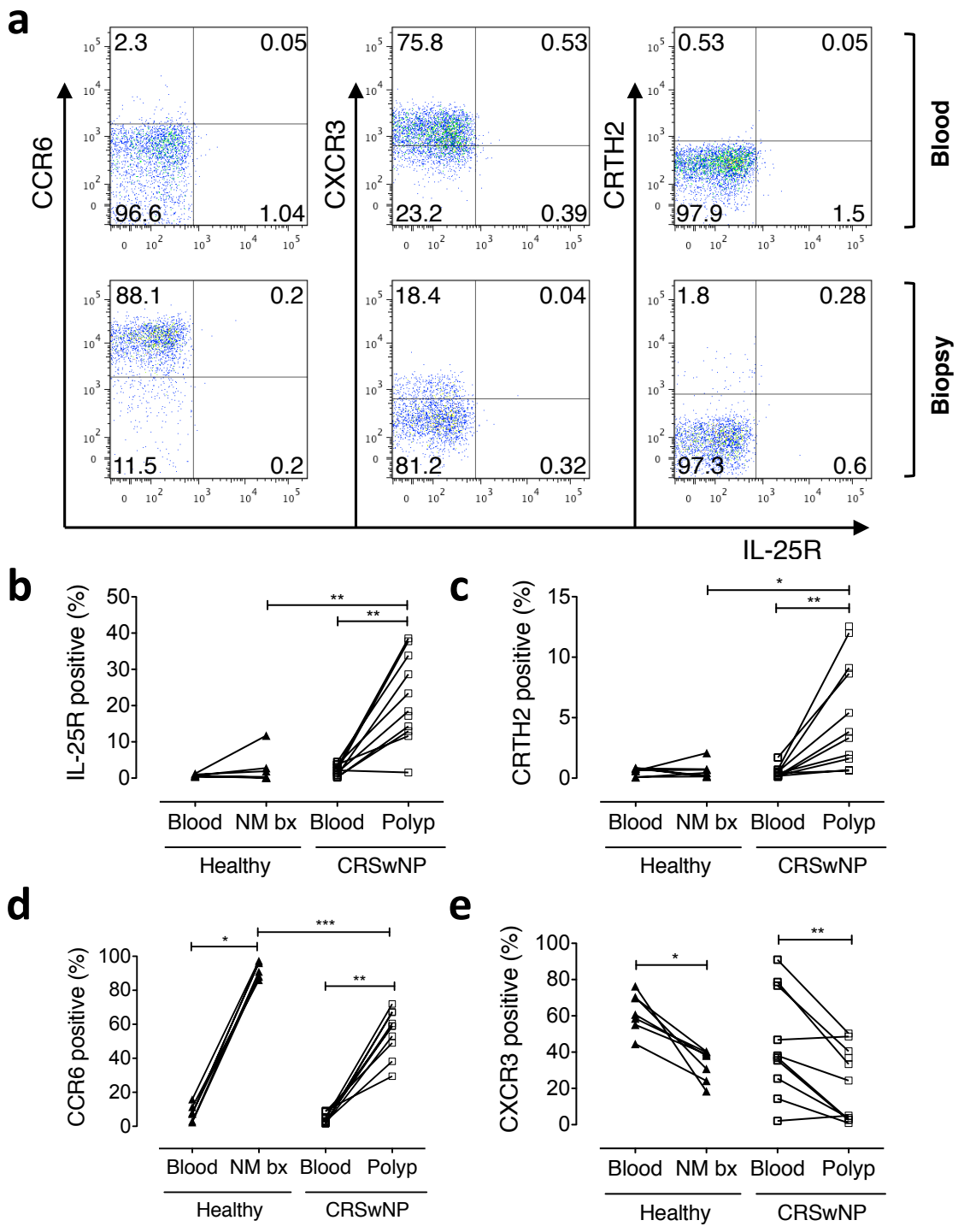


Figure 3.9 IL-25R is expressed only by polyp-derived cells but CCR6 is expressed by both polyp and normal nasal mucosa-derived cells.

Representative flow cytometry staining for $CD4^+$ cells derived from blood and normal mucosa biopsy tissue is shown in (a). Expression of (b) IL-25R, (c) CRTH2, (d) CCR6 and (e) CXCR3 by blood and normal mucosa biopsy (NM bx) from healthy volunteers (n=7) vs. blood and polyp tissue from CRSwNP patients (n=10). Blood vs. tissue: Wilcoxon matched-pairs signed rank test. Healthy nasal mucosa vs. polyp: Mann Whitney Test. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$.

3.1.4 Grass-pollen challenge upregulates IL-25R and CRTH2 expression in the skin

Having observed higher CRTH2 and IL-25R expression in polyp-derived T cells, the next step was to examine whether this observation was specific to eosinophilic polyps or a feature of tissue Th2 responses. In order to address this, the phenotype of T cells from allergen-challenged skin biopsies was examined.

Allergic rhinitis patients participating in the PollenLITE low dose intradermal grass pollen immunotherapy trial were subjected to intradermal diluent and allergen challenge with grass pollen. Punch biopsies were obtained 24 hours post challenge and halved. Half of each punch biopsy was then processed using the same methodology as for polyp explant cultures (Chapter 2). Cells were stimulated with anti-CD3/CD28 and analysed day 7 post restimulation. Following unblinding of the trial, data was decoded and divided for participants who had received placebo treatment or the active vaccine against grass pollen. Data analysed and shown in this thesis are from participants treated with the placebo vaccine. This was to enable the examination of skin T cells under normal (diluent challenged) and allergic inflammatory conditions (allergen challenged) without the confounding effects of the active vaccine.

Diluent challenged samples yielded very few T cells in culture. Hence, flow cytometric analysis was possible for only 3 out of 9 placebo-treated samples received. However, following allergen challenge a larger number of T cells were recovered following culture which enabled analysis by flow cytometry for all 9 samples. Meaningful statistical analysis between diluent and allergen challenged skin was precluded by the small diluent challenged sample number (Figure 3.10). Nonetheless, the data tentatively suggests that CCR6 and CXCR3 expression was decreased following allergen challenge (CCR6, diluent 72.1% ± 11.3 vs. allergen 54.4% ± 5.7; CXCR3, diluent 73.9% ± 8.7 vs.

allergen 56.3% ± 4.2). Interestingly, IL-25R showed increased expression following allergen challenge (diluent 3.3% ± 0.7 vs. allergen 14.9% ± 4.6). However, amongst the 3 diluent challenged samples, there was wide variation in CRTH2 expression and no trend was evident.

Paired peripheral blood specimens from skin biopsy donors were not available. However, the expression of CCR6 in diluent and allergen challenged skin biopsies was significantly higher than in peripheral blood from CRSwNP patients (Figure 3.10a) and expression was comparable to that observed in polyp-derived T cells (diluent challenged skin, 72.1% ± 11.3; allergen challenged skin, 54.4% ± 5.7; blood, 5.9% ± 1.1; polyp, 63.5% ± 4.7). In contrast, CXCR3 expression by T cells in skin biopsies were comparable to blood-derived cells (diluent challenged skin, 73.9% ± 8.7; allergen challenged skin, 56.3% ± 4.2; blood, 51.7% ± 6.8; polyp, 26% ± 5.3). The percentages of T cells positive for CXCR3 in both diluent and allergen challenged skin biopsies were significantly higher than in polyp explants (Figure 3.10b).

A significantly higher percentage of cells positive for CRTH2 was detected in allergen challenged skin biopsies compared to peripheral blood (Figure 3.10c). This pattern of expression was also observed for IL-25R (Figure 3.10d). However, expression of IL-25R was observed in T cells from skin biopsies only following allergen challenge and expression in the 3 diluent challenged skin samples was minimal (diluent challenged skin, 3.3% ± 0.7; allergen challenged skin, 14.9% ± 4.6; blood, 1.7% ± 0.4; polyp, 15.4% ± 3.4).

Taken together, these data indicate that the expression of CCR6, IL-25R and CRTH2 by T cells in the skin, following allergen challenge, is similar to expression in the inflamed polyp nasal mucosa. However, CXCR3 expression appears to be higher on skin-derived T cells than polyp-derived T cells.

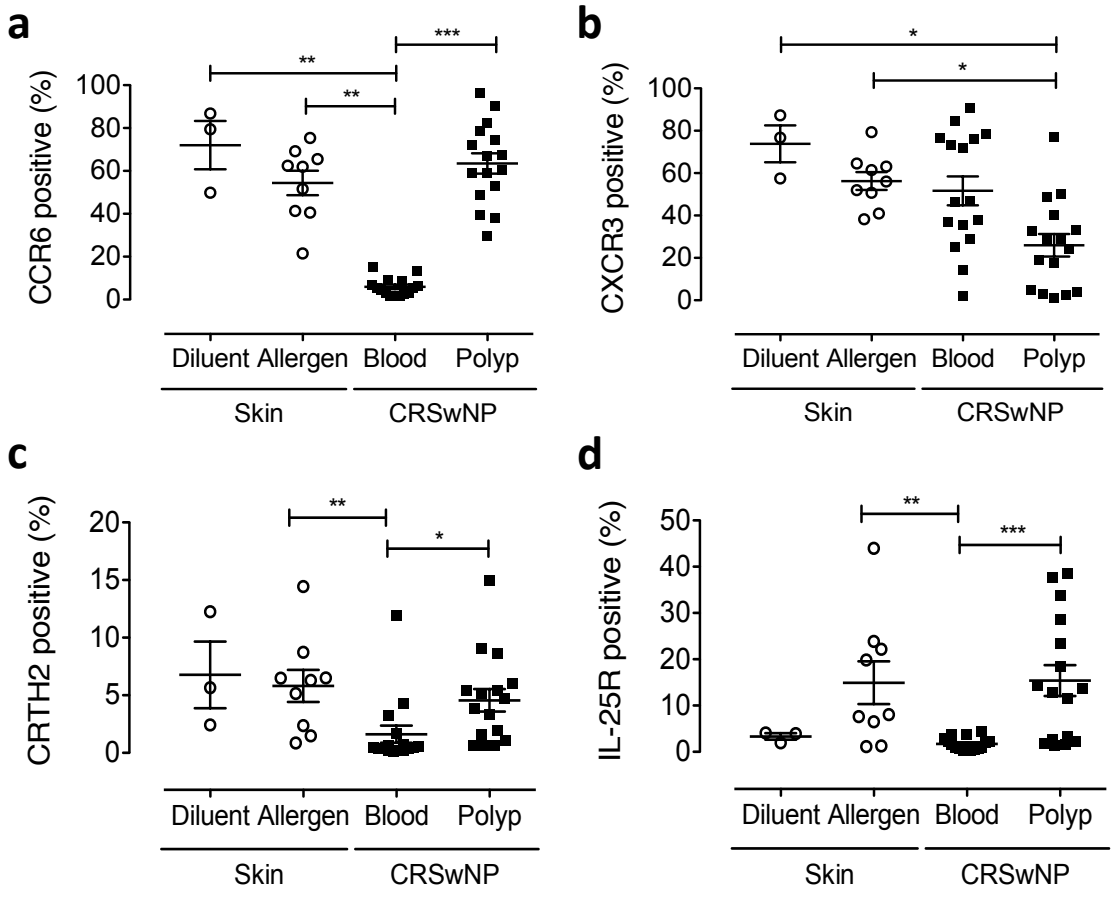


Figure 3.10 Expression of T cell subset markers by skin-derived CD4$^+$ T cells following intradermal diluent and allergen challenge.

Skin punch biopsies were obtained following allergen and diluent challenge from the forearm of participants treated with placebo in the PollenLITE low dose intradermal grass pollen immunotherapy trial. Expression of the T cell subset markers (a) CCR6, (b) CXCR3, (c) IL-25R and (d) CRTH2 was analysed by flow cytometry from allergen (n=9) and diluent (n=3) challenged biopsies on day 7 of culture following restimulation. Expression of T cell surface markers by blood- and polyp-derived cells from CRSwNP patients is shown for comparison (n=16). Kruskal-Wallis test with Dunn's post hoc test. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$.

3.1.5 Analysis of cytokines produced in the polyp nasal mucosa vs. the periphery

To further characterise the T cell phenotype in polyps, the next experiments compared the profile of cytokines produced by T cells derived from polyp explant cultures to those from peripheral blood cultures.

Cells were analysed for intracellular cytokine production on the same day as surface phenotypic analysis by flow cytometry i.e. day 7 post anti-CD3/CD28 restimulation. To elicit measurable cytokine production, T cells were activated with PMA/ionomycin for 4 hours prior to intracellular staining. For the final 2 hours of activation, monensin was added to allow cytokines to accumulate in the Golgi body. Representative intracellular cytokine staining data for $CD4^+$ T cells and $CD8^+$ T cells derived from polyp explant and blood cultures are shown in Figure 3.11 and Figure 3.12 respectively. No cytokine production was detected by resting cells and expression was only observed in activated cells.

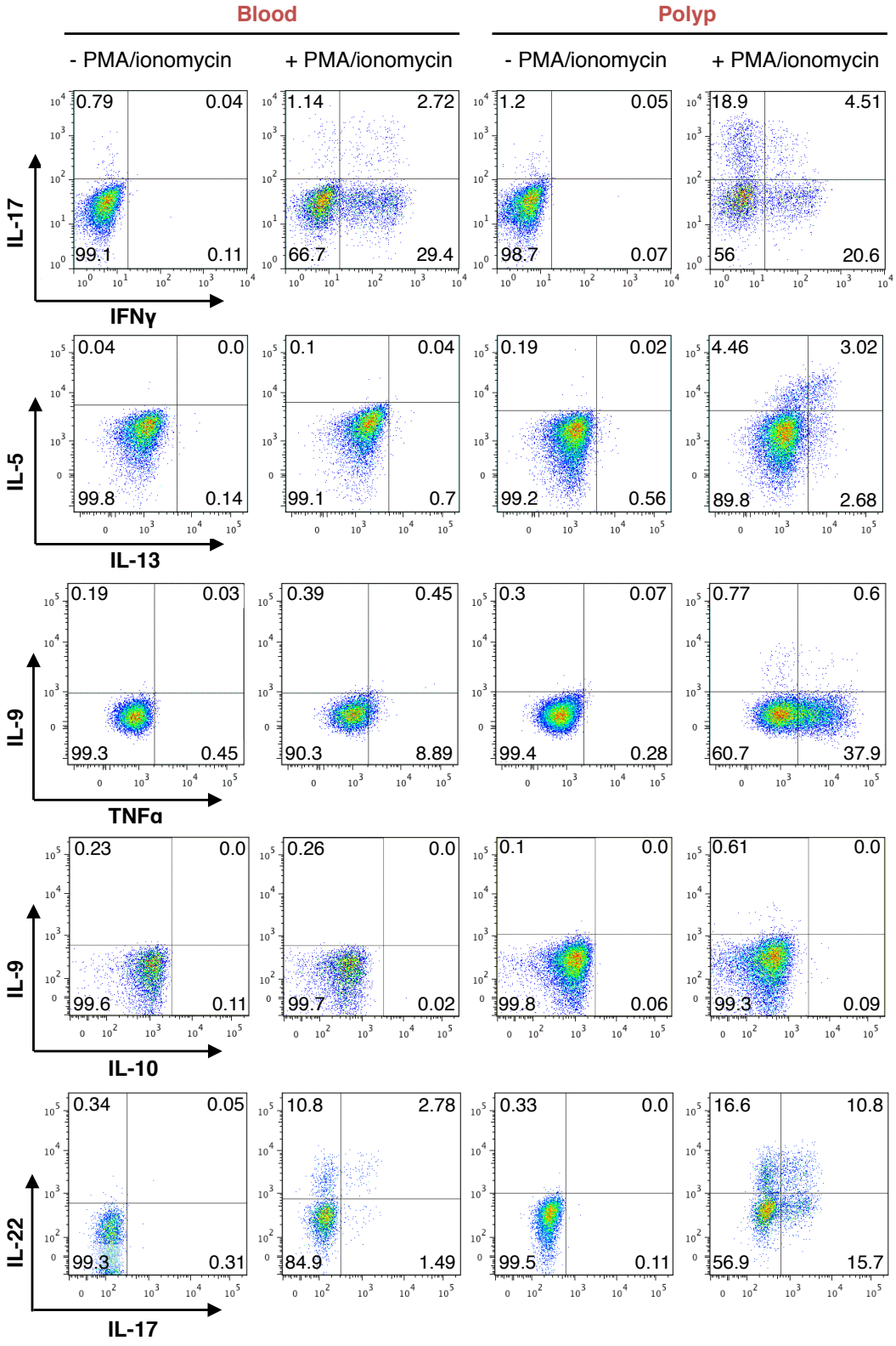


Figure 3.11 Cytokine expression profile by CD4$^+$ T cells derived from peripheral blood and nasal polyp tissue.

Cells were activated with PMA/ionomycin for 4 hours or remained under resting state before intracellular cytokine analysis. Representative flow cytometry staining for blood- and polyp-derived CD4$^+$ T cells under resting or activated state is shown.

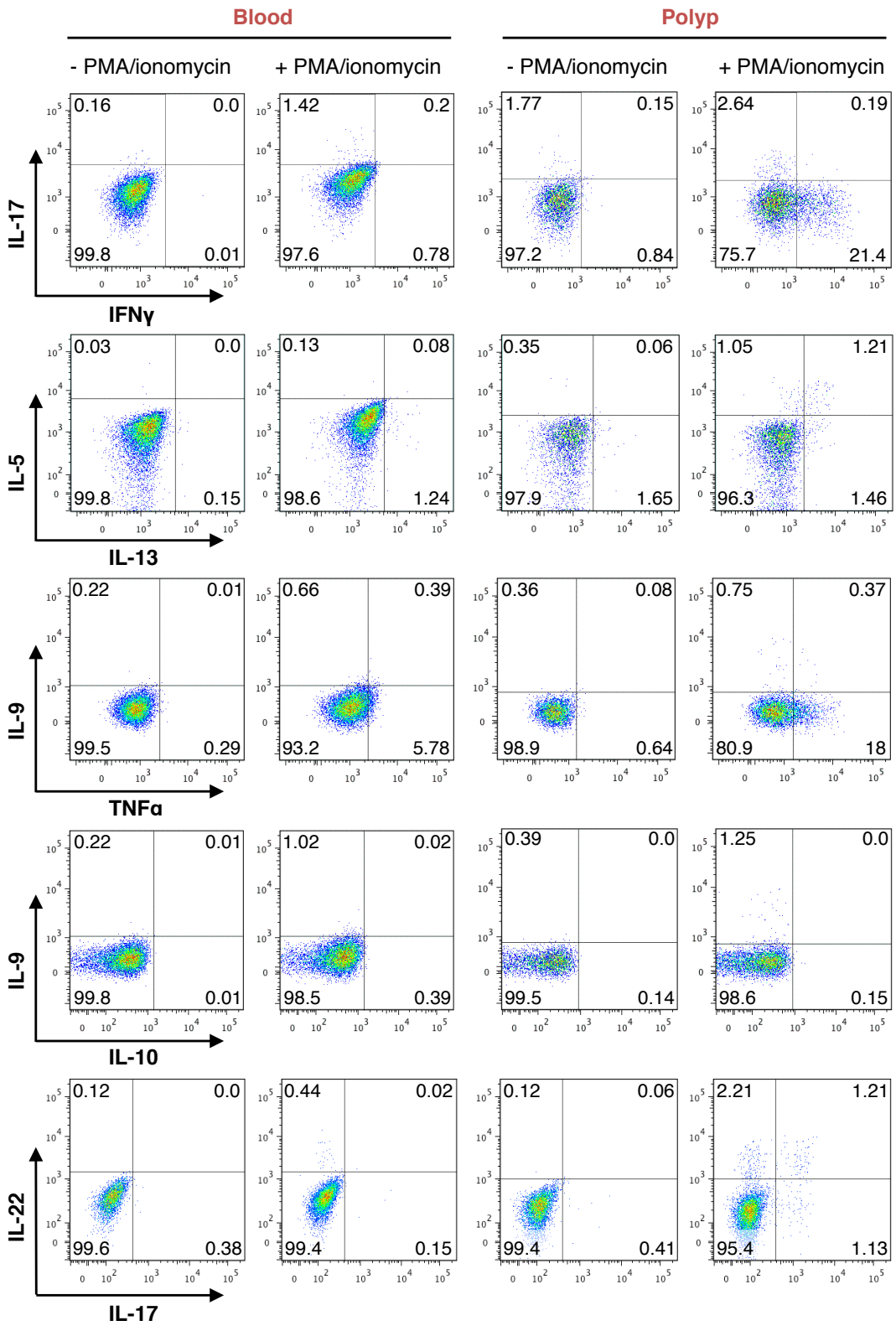


Figure 3.12 Cytokine expression profile by CD8$^+$ T cells derived from peripheral blood and nasal polyp tissue.

Cells were activated with PMA/ionomycin for 4 hours or remained under resting state before intracellular cytokine analysis. Representative flow cytometry staining for blood- and polyp-derived CD8$^+$ T cells under resting or activated state is shown.

3.1.5.1 Polyp-derived cells produce Th2 and Th17 cytokines

Expression of cytokines by $CD4^+$ and $CD8^+$ cells from blood and polyp explants showed similar patterns except for IFNγ, IL-17 and IL-22. The percentage of cells producing IFNγ was significantly higher in polyp-derived $CD8^+$ cells compared to polyp-derived $CD4^+$ cells ($CD8^+$ polyp, 57.6% ± 6.4 vs. $CD4^+$ polyp, 20.2% ± 3.4; $p<0.001$) with the same observed in the periphery ($CD8^+$ blood, 39.1% ± 7.5 vs. $CD4^+$ blood, 21.5% ± 4.2; $p<0.001$) (Figure 3.13b). The percentage of $IFN\gamma^+$ cells was also significantly higher in $CD8^+$ cells derived from polyps compared to $CD8^+$ cells from the blood ($p<0.05$) although no difference in expression was detected for $CD4^+$ cells.

Minimal numbers of Th17 cytokine (IL-17 and IL-22) producing cells were detected in both blood and polyp-derived $CD8^+$ cells (Figure 3.13a, h). $IL\text{-}17^+$ cells constituted a significantly lower percentage of $CD8^+$ cells compared to $CD4^+$ cells derived from polyp explant cultures ($CD8^+$ polyp, 1.2% ± 0.3 vs. $CD4^+$ polyp, 19.8% ± 2.9; $p<0.001$) and blood cultures ($CD8^+$ blood, 0.7% ± 0.3 vs. $CD4^+$ blood, 3.3% ± 1.2; $p<0.05$). IL-22 also showed the same pattern of expression although statistical significance was not reached due to the lower number of cases. Interestingly, although typically less than 2% of $CD8^+$ cells produced IL-17, the number of $IL\text{-}17^+$ cells was still significantly higher in polyp-derived $CD8^+$ T cells than blood $CD8^+$ cells ($p<0.05$). Striking differences in IL-17 and IL-22 production were observed in the $CD4^+$ T cell populations. The percentages of $IL\text{-}17^+$ and $IL\text{-}22^+$ cells were both significantly higher in polyp-derived $CD4^+$ cells compared to blood-derived $CD4^+$ cells (IL-17: $p<0.0001$, IL-22: $p<0.05$), correlating with CCR6 expression (Figure 3.5b).

In addition to the differential expression of IL-17, IL-22 and IFNγ by $CD8^+$ cells, the percentages of $CD8^+$ Th2 cytokine producing cells (IL-5 and IL-13) derived from polyp explants showed a trend decrease in expression compared to $CD4^+$ cells (IL-5: $CD8^+$

polyp, 2.1% ± 0.6 vs. $CD4^+$ polyp, 5% ± 1.7; p=0.09, IL-13: $CD8^+$ polyp, 2.3% ± 0.5 vs. $CD4^+$ polyp, 5.5% ± 1.6; p=0.07) (Figure 3.13c, d). IL-9 was significantly lower in $CD8^+$ polyp-derived cells compared to $CD4^+$ polyp-derived cells (Figure 3.13e). In addition, although expression levels were lower than for $CD4^+$ T cells, the pattern of higher percentages of $CD8^+$ IL-5 and IL-13 producing cells in polyp-derived explants vs. blood-derived cultures was maintained (IL-5: $CD8^+$ polyp, 2.1% ± 0.6 vs. $CD8^+$ blood, 0.6% ± 0.2; $p<0.05$, IL-13: $CD8^+$ polyp, 2.3% ± 0.5 vs. $CD8^+$ blood, 0.7% ± 0.2; $p<0.05$).

Percentages of IL-5, IL-13 and IL-9 positive cells were significantly higher in $CD4^+$ polyp-derived cells vs. $CD4^+$ blood-derived cells (IL-13: polyp, 5% ± 1.7 vs. blood, 0.9% ± 0.2; $p<0.01$, IL-13: polyp, 4.6% ± 1.1 vs. blood, 1.6% ± 0.4; $p<0.05$, IL-9: polyp, 1.8% ± 0.3 vs. blood, 0.8% ± 0.2; $p<0.05$) akin to patterns seen with IL-25R expression (Figure 3.5e). Furthermore, expression of IL-5 and IL-13 was positively correlated to IL-25R expression in $CD4^+$ cells but not with CRTH2 (IL-5, r=0.85, $p<0.001$; IL-13, r=0.82, $p<0.01$) (Figure 3.13j). Analysis of IL-5 and IL-13 co-producing cells showed that these were also significantly higher in $CD4^+$ polyp vs. blood-derived cells (polyp, 4.7% ± 1.6 vs. blood, 0.4% ± 0.2; $p<0.01$) (Figure 3.13i). No difference was observed in the percentage of IL-10 producing cells (Figure 3.13g). However, production of the pro-inflammatory cytokine TNFα (Figure 3.13f) was significantly higher in $CD4^+$ polyp vs. blood-derived cells (polyp, 58.3% ± 6.2 vs. blood, 30.3% ± 8; $p<0.01$) reflecting the inflammatory phenotype of nasal polyposis.

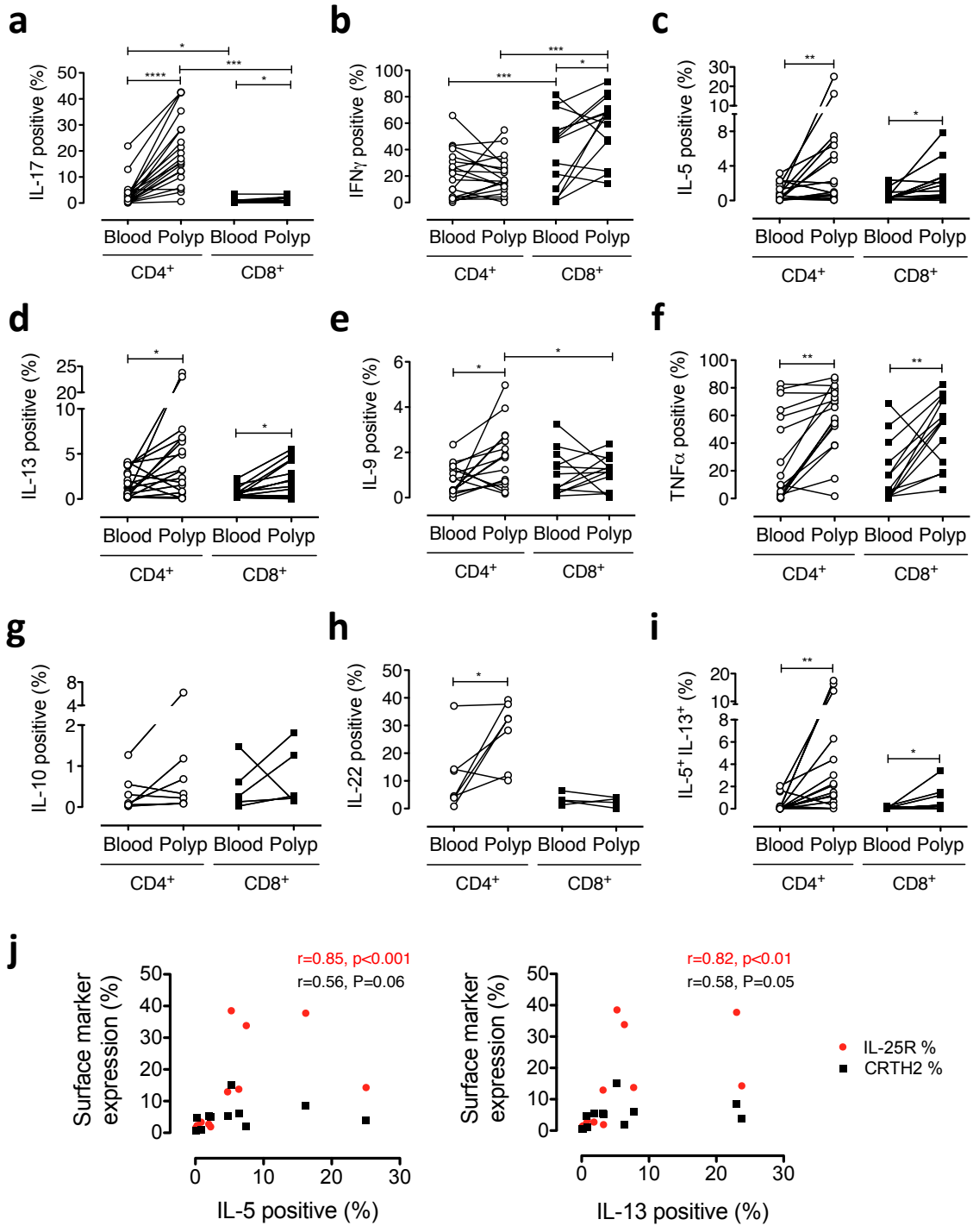


Figure 3.13 Th2 cytokines IL-5 and IL-13 as well as Th17 cytokines IL-17 and IL-22 are highly expressed by polyp but not blood-derived T cells.

Flow cytometry staining for (a) IL-17, (b) IFNγ, (c) IL-5, (d) IL-13, (e) IL-9, (f) TNFα, (g) IL-10, (h) IL-22 and (i) IL-5 and IL-13 co-producing cells is shown for $CD4^+$ (n=16-18; IL-22, n=6; IL-10, n=7) and $CD8^+$ T cells (n=12-14; IL-22, n=4; IL-10, n=5) from blood and polyp explants. Wilcoxon matched-pairs signed rank test. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$, ****, $p<0.0001$. (j) Spearman rank correlation of IL-25R and CRTH2 expression vs. IL-15 and IL-13 in $CD4^+$ polyp-derived T cells (n=12).

3.1.5.2 Profile of cytokine production by polyp-derived cells *ex vivo* is similar to explant-derived cells.

To determine if the cytokine profile of blood and polyp explant-derived cells were affected by culture conditions or the cell culture process per se, cytokine production was also analysed *ex vivo* in a small number of cases. Polyp tissue was digested with collagenase overnight and PBMC isolated from peripheral blood and incubated overnight at 37°C in parallel. Dissociated cells and PBMC were either activated with PMA/ionomycin or remained under resting condition. Cells were analysed by intracellular cytokine staining after 4 hours as previously.

Figure 3.14 shows that a smaller percentage of $CD4^+$ cells produced cytokines *ex vivo* compared to after culture. However, although percentages were lower, the pattern of higher percentages of cytokine producing cells in polyp vs. blood cells was still observed, except for TNFα. In particular, the percentage of $CD4^+IL\text{-}5^+$ cells was significantly higher in polyp cells compared to peripheral blood (polyp, 2.1% ± 0.4 vs. blood, 0.5% ± 0.2; $p<0.05$) (Figure 3.14b) correlating to IL-25R expression *ex vivo* (Figure 3.7f). Short-term culturing of cells in IL-2 supplemented complete medium therefore does not affect the phenotype of T cells.

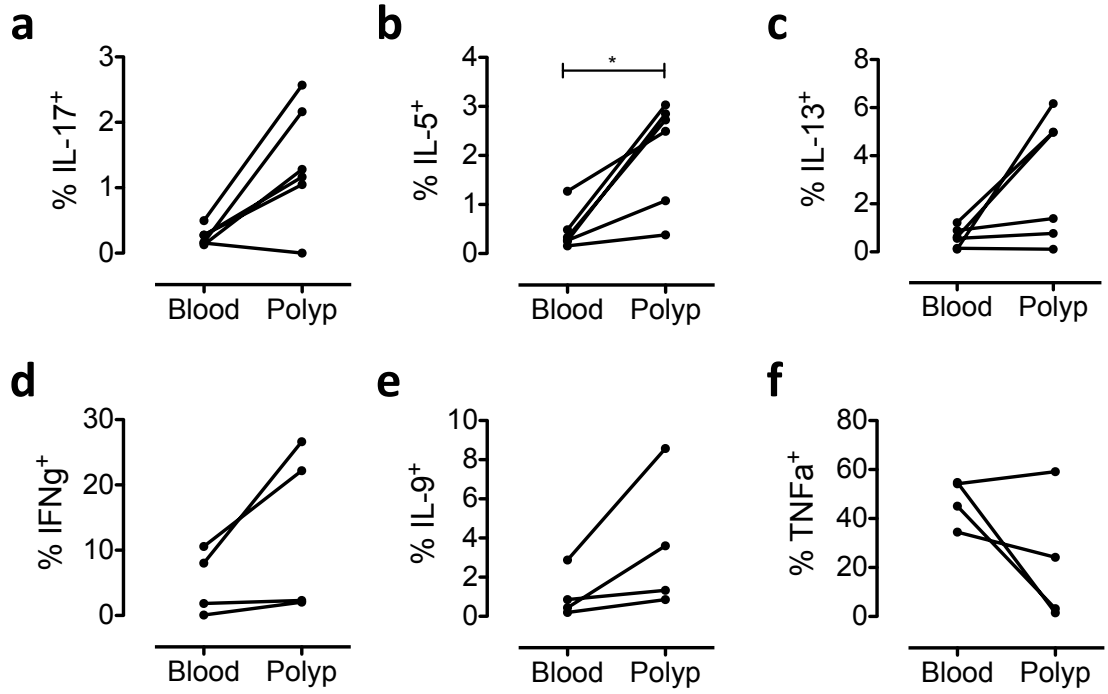


Figure 3.14 *Ex vivo* analysis of polyp tissue and peripheral blood show similar cytokine expression profiles to cultured explant-derived CD4$^+$ cells.

Polyp tissue was digested with Liberase collagenase overnight. CD4$^+$ cells dissociated from tissue and from PBMC were analysed for expression of (a) IL-17, (b) IL-5, (c) IL-13, (d) IFNγ, (e) IL-9 and (f) TNFα following PMA/ionomycin activation (4 hours). Wilcoxon matched-pairs signed rank test. *, $p<0.05$. (IL-17, IL-5, IL-13: n=6; IFNγ, IL-9, TNFα: n=4).

3.1.6 Analysis of cytokines produced in the polyp nasal mucosa vs. normal nasal mucosa

3.1.6.1 Production of IL-17 is specific to nasal tissue but production of IL-13 and IL-9 are specific to nasal polyposis

The next step was to compare the cytokine profile of T cells in nasal polyps, healthy nasal tissue and peripheral blood. Due to the limited yield of T cells from culture of healthy nasal biopsies, it was decided to focus on the expression of IL-17, IL-13 and IL-9 as these represented the Th17 and Th2 cytokines showing the largest differential expression in blood vs. polyp $CD4^+$ T cells.

The percentage of IL-17^+ cells was significantly higher in the normal nasal mucosa compared to the periphery (biopsy, 25.4% ± 8.1 vs. blood, 3.2% ± 1.1; $p<0.05$). This pattern was the same as that observed for polyp vs. blood cells from CRSwNP patients (Figure 3.15a). Hence, these data suggest that IL-17 is produced by T cells in the nasal mucosa in health and disease but is not expressed at significant levels by blood T cells. This again correlates with CCR6 expression as shown in Figure 3.9b and suggests that $CCR6^+$ Th17 cells are tissue-specific.

In contrast, no difference in the percentage of IL-13^+ cells (Figure 3.15b) was found between blood and nasal biopsies from healthy volunteers (biopsy, 4.8% ± 0.8 vs. blood, 3.8% ± 0.7). IL-9 also did not show elevated expression in healthy nasal mucosal biopsy T cells compared to peripheral blood cells (Figure 3.15c). This therefore suggests that Th2 cytokines are produced by polyp-derived cells only, correlating with the low expression of IL-25R in normal biopsies (Figure 3.5d). This indicates that IL-$25R^+$ Th2 cells are not a feature of the general nasal mucosa but are disease-specific.

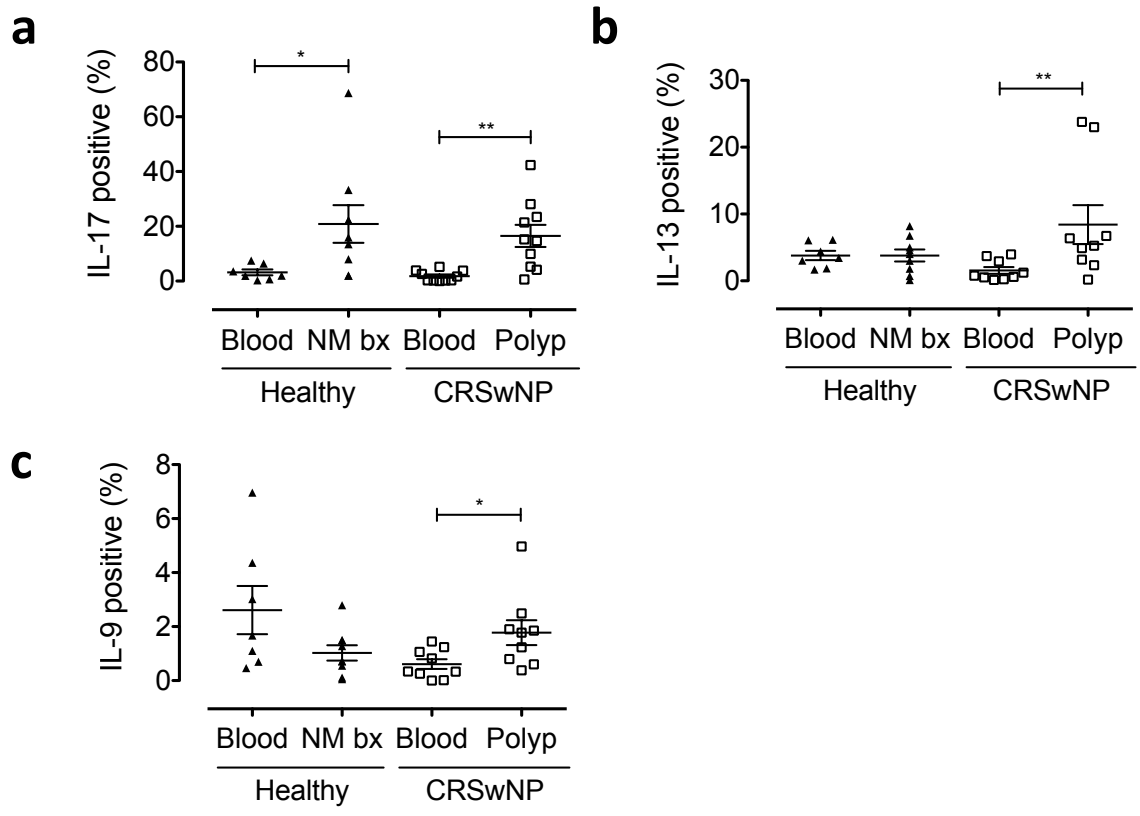


Figure 3.15 IL-17 is expressed by CD4$^+$ T cells in both normal nasal mucosa and diseased polyp but Th2 cytokines IL-13 and IL-9 are detected only in polyp tissue.

Flow cytometry staining for cytokines (a) IL-17, (b) IL-13 and (c) IL-9 in normal nasal mucosa biopsy (NM bx) and blood from healthy volunteers (n=7) vs. polyp tissue and blood from CRSwNP patients (n=9, n=10 for IL-17). Wilcoxon matched-pairs signed rank test. *, $p<0.05$, **, $p<0.01$.

3.1.6.2 Co-production of IL-17 and IL-13 is not observed in nasal mucosal T cells

Having established that IL-17 and IL-13 were both produced by polyp-derived cells, the next question addressed was whether $CD4^+$ T cells producing these cytokines localised to the same or different T cell populations.

Figure 3.16 shows flow cytometry staining data presented as histograms and dot plots for polyp and normal biopsy-derived cells. Cells under resting conditions do not produce cytokines and only express cytokines upon PMA/ionomycin activation. For normal nasal biopsies, only IL-17 was produced by $CD4^+$ T cells and thus, no co-production with IL-13 was observed. In polyp-derived T cells, IL-17 and IL-13 were clearly expressed by distinct populations (Figure 3.16b) with minimal co-production observed (<1%).

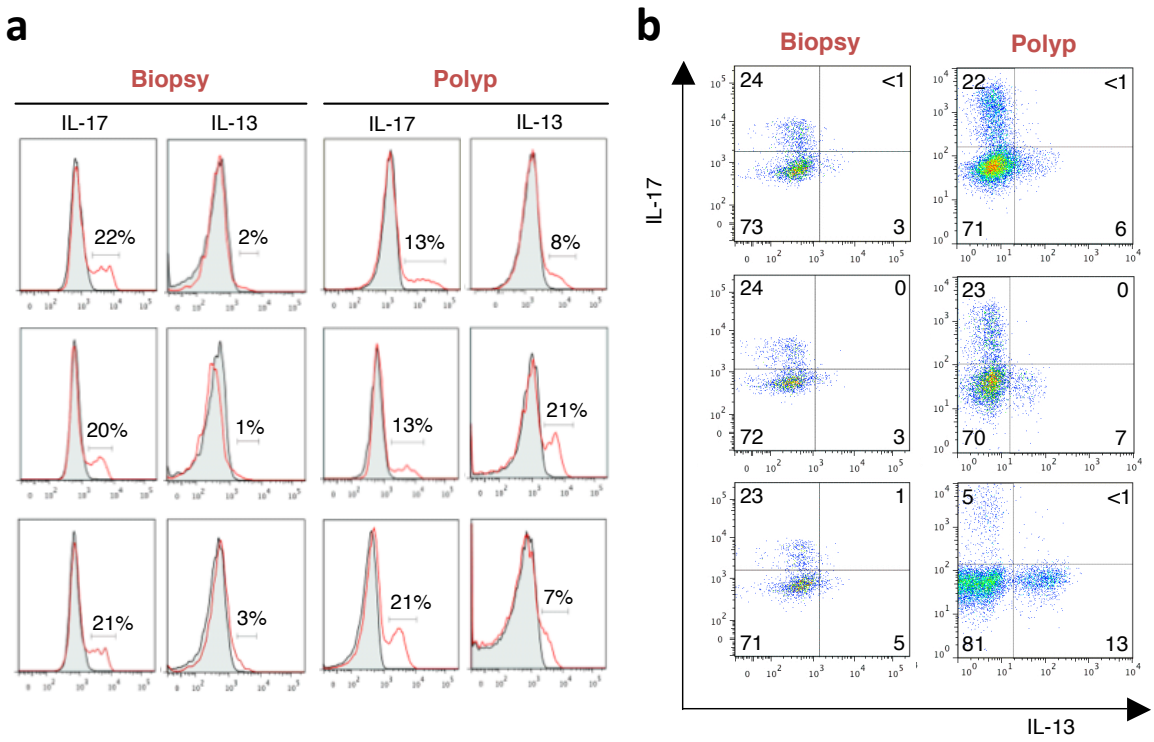


Figure 3.16 IL-17 and IL-13 are produced by distinct T cell populations.

(a) Flow cytometry staining for IL-17 and IL-13 from $CD4^+$ T cells derived from normal nasal biopsy and polyp explants. Representative data is shown (n=3 for each tissue, each specimen on separate row). Percentages of positive cells are shown. (Grey solid histogram, resting condition; red open histogram, activated condition). (b) IL-17 and IL-13 co-production by $CD4^+$ T cells in healthy nasal biopsies and diseased polyp was analysed by flow cytometry (n=3).

3.1.6.3 Protein levels of cytokines in culture supernatants correlate with intracellular cytokine staining

Cells from normal nasal biopsy and polyp explants were restimulated with anti-CD3/CD28 on the same day as flow cytometric analysis (day 7-post stimulation) to induce cytokine secretion. Cell free culture supernatants were then harvested 48 hours later. Levels of cytokines in supernatants were analysed by cytometric bead array to allow direct comparison of cytokine levels in healthy normal nasal mucosa vs. diseased nasal polyp cultures.

Levels of the Th2 cytokines IL-13, IL-5 and IL-9 (Figure 3.17a-c) were significantly higher in polyp supernatants compared to normal biopsy supernatants, in accordance with intracellular cytokine staining data. Furthermore, another prototypical Th2 cytokine IL-4 (Figure 3.17f) also showed the same response with a significant 9-fold increase in levels detected in polyp supernatant vs. supernatant from normal biopsies. In contrast, IFNγ and IL-17 showed no differences in protein levels (Figure 3.17h, i) in agreement with previous results. In addition, IL-22 levels in culture supernatants were examined by ELISA as the cytometric array beads for this cytokine were unavailable (Figure 3.17j). Levels of IL-22 detected in culture supernatants from polyp and normal nasal mucosa explants were not significantly different.

Examining other cytokines, IL-10 was found to be significantly higher (15-fold) in polyp supernatant compared to normal biopsies (Figure 3.17d). A 2-fold increase in the pro-inflammatory cytokine IL-6 (Figure 3.17e) was also detected in polyp supernatants compared to normal biopsy supernatants. However, in contrast, levels of TNFα were significantly higher in supernatant from normal biopsy cultures compared to polyp cultures (Figure 3.17g).

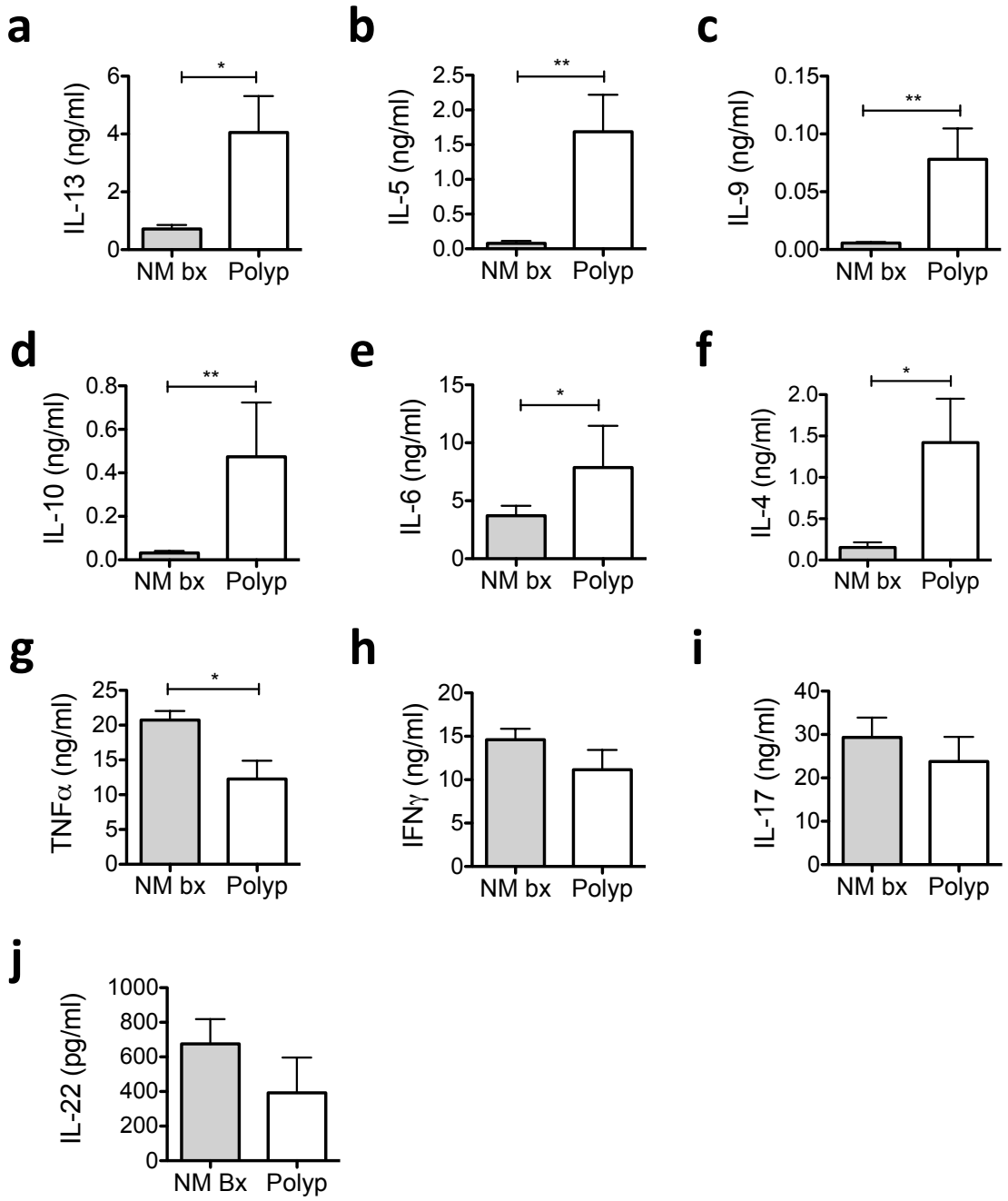


Figure 3.17 Cytokine levels in polyp and normal nasal mucosa culture supernatants show similar patterns to that observed with intracellular cytokine staining.

Levels of (a) IL-13, (b) IL-5, (c) IL-9, (d) IL-10, (e) IL-6, (f) IL-4, (g) TNFα, (h) IFNγ and (i) IL-17 was analysed in supernatants from polyp (n=8) and normal nasal mucosa (n=7) explant cultures 48 hours post anti-CD3/CD28 stimulation on day 7 of culture by CBA. IL-22 levels (j) were measured in supernatants from polyp (n=4) and normal nasal mucosa (n=7) explant cultures by ELISA. Mann Whitney test. *, $p<0.05$, **, $p<0.01$.

3.1.6.4 Th17 and Th2 cytokine levels are not affected by aspirin sensitivity, atopic status or steroid use

CRSwNP patients recruited in this study showed some clinical heterogeneity in parameters such as aspirin sensitivity, use of the oral steroid prednisolone and atopic status (determined with a skin prick test) (Table 3.2). Results for IL-17, IL-13 and IL-5 intracellular T cell staining from polyp and blood-derived cultures were therefore analysed according to the patient's aspirin status (Figure 3.18a), oral steroid use (Figure 3.18b) or atopic status (Figure 3.18c). No significant differences were observed in the percentage of IL-17, IL-13 or IL-5 producing blood cells when any of the three factors were considered. However, although not significant, a trend was observed for higher expression of the Th2 surface marker IL-25R by polyp-derived cells in aspirin sensitive (AS) vs. aspirin tolerant (AT) patients (AS, 20.3% ± 4.2 vs. AT, 6.7% ± 3.9; p=0.07) (Figure 3.18d). CRTH2 also showed a slight elevated expression in AS vs. AT patients (AS, 5.9% ± 1.5 vs. AT, 3% ± 1.2; p=0.24). Finally, increases in expression of CRTH2 and IL-25R by polyp-derived T cells were also detected in non-atopic compared to atopic patients (CRTH2: SPT+, 2.3% ± 0.7 vs. SPT-, 6.6% ± 1.8; p=0.06, IL-25R: SPT+, 11.4% ± 3.8 vs. SPT-, 21.6% ± 6; p=0.18) (Figure 3.18e).

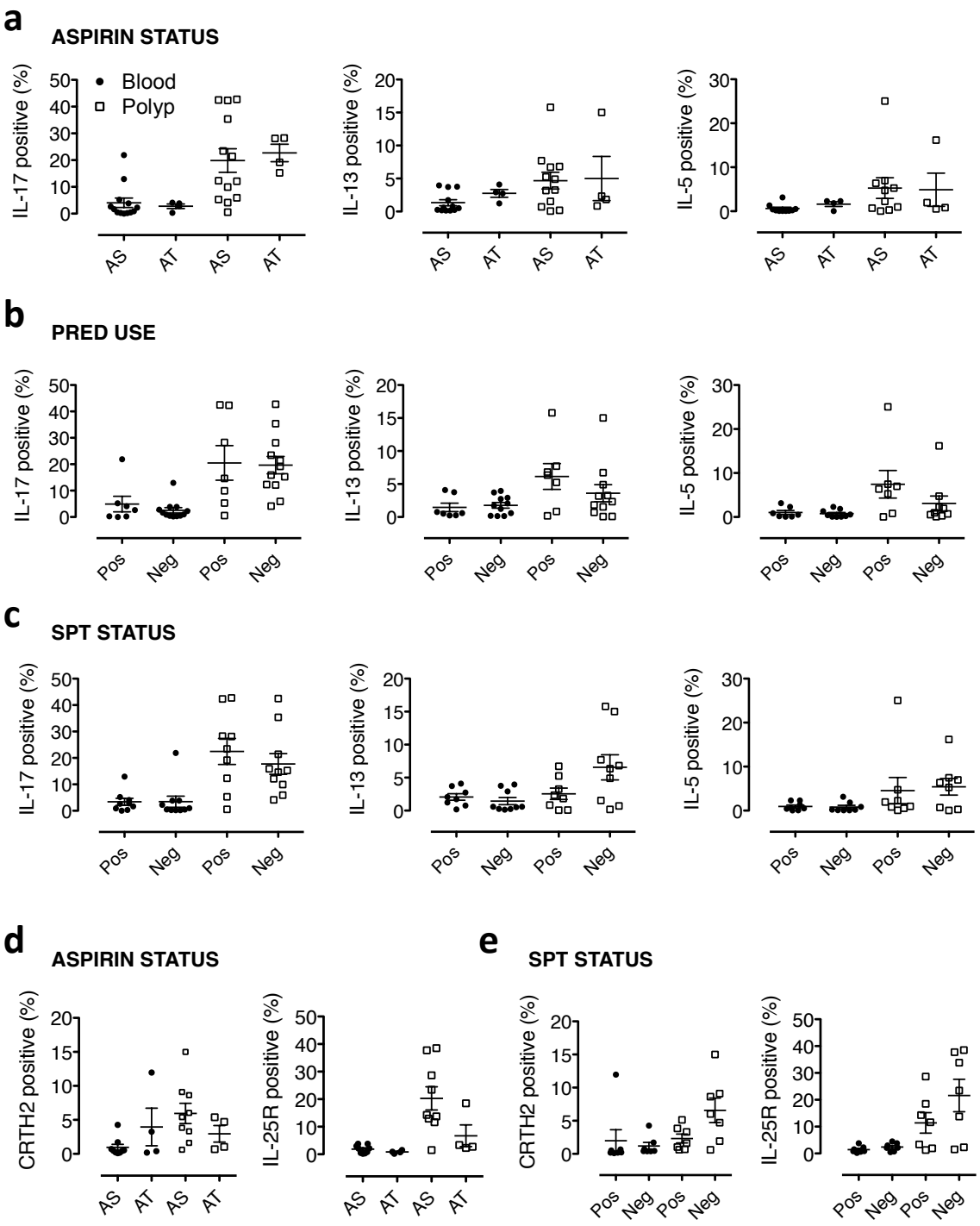


Figure 3.18 Aspirin sensitivity, steroid use and atopic status on cytokine expression profile of CD4$^+$ T cells derived from polyp explants.

Data for percentages of IL-17, IL-13 and IL-5 positive cells detected in nasal polyp explants and blood was analysed according to the patient's (a) aspirin status (AS, aspirin sensitive; AT, aspirin tolerant), (b) use of the oral steroid prednisolone (Pos, positive; Neg, negative) and (c) skin-prick test result (Pos, positive; Neg, negative). Data for CRTH2 and IL-25R expression was grouped according to (d) aspirin status and (e) skin-prick test result. Mann Whitney test.

3.1.7 Skin biopsies show high expression of IFNγ and IL-17 following allergen challenge

To compare cytokine expression by $CD4^+$ T cells in the nasal mucosa and skin, cutaneous biopsies from PollenLITE trial participants receiving placebo treatment were analysed by intracellular cytokine staining following activation with PMA/ionomycin. Unfortunately, the limited number of cells recovered from diluent challenged biopsies precluded analysis in most cases and sufficient cells for analysis were only recovered from 4 allergen challenged skin biopsies.

Figure 3.19a shows that a large number of IFNγ producing cells are found in the skin correlating with high CXCR3 expression observed (Figure 3.10b). Moreover, the percentage of $IFN\gamma^+$ cells in the skin was comparable to expression in both polyp and blood-derived cells (allergen challenged skin, 25.5% ± 5; polyp, 23.6% ± 4.6; blood 19.3% ± 4.7). $IL\text{-}17^+$ cells also followed the same pattern as CCR6 expression (Figure 3.10a) with significantly higher percentages observed in the skin and polyp compared to peripheral blood (blood, 1.6% ± 0.4 vs. allergen challenged skin 12.7% ± 2.8; $p<0.05$, vs. polyp 16.5% ± 2; $p<0.001$) (Figure 3.19b). Examination of IL-17 co-localisation further confirmed this with the majority of $IL\text{-}17^+$ cells co-expressing CCR6 (Figure 3.19d). This was significantly higher than the percentage of cells co-expressing IL-17 and IL-25R ($p<0.05$) and suggests that Th17 cells may be a feature of both skin and nasal mucosa.

Similar numbers of $IL\text{-}5^+$ $CD4^+$ T cells were observed from explants of allergen challenged skin and nasal polyps (Figure 3.19c). Although no matched peripheral blood samples were available, this percentage was significantly higher in the skin than in the periphery of CRSwNP patients (allergen challenged skin, 8.7% ± 4.7 vs. blood, 0.9% ± 0.2; $p<0.05$). Of the cytokines examined, the highest percentage of co-expression with

IL-25R was observed for IL-5 with the lowest co-expression observed for IFNγ (Figure 3.19d). Thus, Th2 cytokine-expressing IL-25R^{+} T cells appear to characterise both allergen challenged skin and nasal polyps.

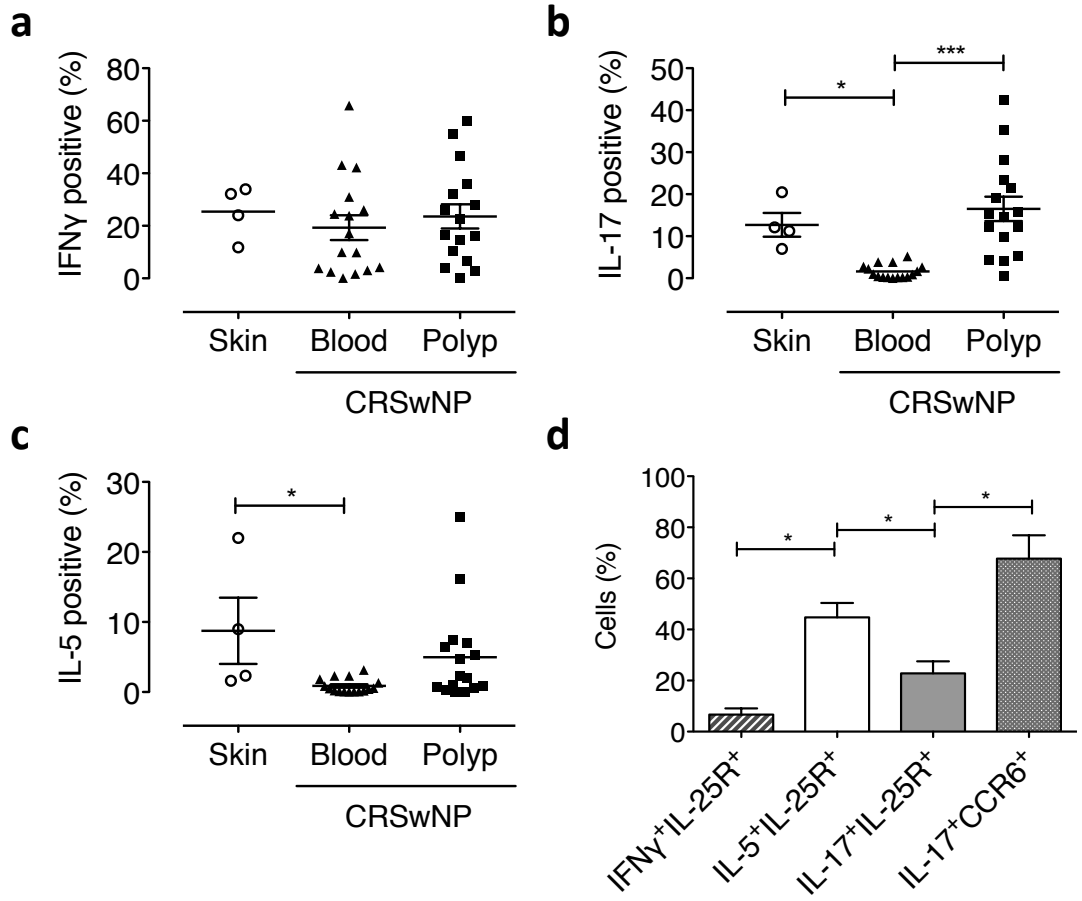


Figure 3.19 T cells cultured from allergen challenged skin biopsies show higher expression of IFNγ and IL-17 compared to IL-5.

Allergen challenged skin biopsies (n=4) vs. polyp and blood (n=16) cultures from CRSwNP patients was analysed on day 7 of culture by flow cytometry for production of (a) IFNγ, (b) IL-17 and (c) IL-5. Kruskal-Wallis test with Dunn's post hoc test. *, $p<0.05$. (d) Co-expression of IFNγ, IL-5 and IL-17 with IL-25R or CCR6 by T cells from allergen challenged skin biopsies was analysed by flow cytometry (n=4). Mann Whitney test. *, $p<0.05$.

3.2 Discussion

Recent studies have identified the presence of novel T helper cell subsets, innate lymphoid cells and epithelial cell-derived cytokines in promoting the allergic/ inflammatory immune response. However, limited literature exists on the role of such cells in the local nasal mucosal environment. The aim of this chapter was to characterise the phenotype of T cells in the nasal mucosa versus the periphery, in health and in CRSwNP. Although CRSwNP represents an important disease in its own right, it is also a useful model for studying tissue eosinophilic inflammation. Comparison of T cell phenotypes between the nasal mucosa and skin was also performed. However, prior to characterisation, optimisation of the tissue culture protocol was required.

3.2.1 Explant model vs. collagenase digest

Methods for preparation of single cell cultures from tissue samples include mechanical disruption, enzymatic digestion as well as explant cultures. In this thesis, nasal polyps were processed under collagenase digested and explant (undigested) conditions for comparison. Cells were analysed following:

1) *In vitro* expansion from explant cultures
2) *In vitro* expansion from collagenase digested tissue
3) *Ex vivo* from collagenase digested tissue

Following short-term expansion, undigested explants yielded higher numbers of viable cells compared to collagenase digested tissue (Figure 3.7). Furthermore, cell yields were highest from explant cultures out of the 3 tissue culture protocols used in this thesis. This method was therefore selected as large cell numbers were required for subsequent experiments. Moreover, explant cultures involved the initial expansion of cells in the

presence of the whole tissue with an intact extracellular matrix, thus mimicking the tissue architecture and *in vivo* environment more closely compared to digested cultures.

Collagenase digestion is widely used as a rapid method to obtain single cell suspensions. However, collagenase digestion is associated with cell death due to the disruption of cell membranes. The digestion protocol used in this thesis was optimised to include the use of purified collagenase enzymes in order to reduce the number of proteolytic enzymes present (trypsin, pepsin) that are presumed to be responsible for this effect. Digestion did not appear to significantly impact the phenotype of tissue-derived cells. Analysis of digested and undigested polyp cells showed comparable expression of the T cell surface markers IL-25R, CRTH2, CCR6 and CXCR3 (Figure 3.7). However, low cell yields were obtained for digested tissue at the end of the 7 day short-term culture period and an additional round of anti-CD3/CD28 stimulation was necessary to boost cell numbers to sufficient levels for analysis. Thus, polyp T cells were analysed from undigested polyp explants in further experiments, as this allowed maximal cell recovery and also reduced the length of the *in vitro* culture required to obtain sufficient T cell numbers.

Ex vivo analysis of tissue-derived cells represented the most physiologically relevant method of analysis used in this thesis due to the short time frame between cell isolation and analysis. However, *ex vivo* analysis first required collagenase digestion of polyp tissue which has been reported to cleave surface molecules such as CD4, thus making flow cytometric analysis difficult (Mulder *et al.*, 1994, Abuzakouk *et al.*, 1996). In addition, low cell numbers were obtained without the *in vitro* expansion step, therefore limiting the number of subsequent experiments. The short-term culture period for cell expansion however, is one possible limitation of this study. To address this, IL-25R expression and Th2 cytokine production from T cells was examined *ex vivo* in a limited number of cases.

T cells analysed *ex vivo* displayed a similar pattern to T cells from expanded explant cultures with higher percentages of IL-25R^{+} cells in the tissue compared to peripheral blood (Figure 3.7). However, the overall percentage expression was lower in *ex vivo* analysed cells compared to expanded explant cultures, possibly due to the lack of anti-CD3/CD28 antibody stimulation and/or cleavage of surface molecules limiting detection by flow cytometry. Nonetheless, IL-5, IL-9, IL-13 and IL-17 producing cells were elevated in T cells from collagenase digested polyp compared to peripheral blood (Figure 3.14).

Finally, explant cultures involved the sole addition of the non-polarising cytokine IL-2 to stimulate T cell growth. Therefore, no or limited skewing of the T cell phenotype should occur, at least in theory. Overall, these data indicate that minimal differences in cell phenotype occurred when analysis of tissue was performed *ex vivo* or after *in vitro* culture. Thus, results observed with the explant model appeared reliable and not due to any experimental artefacts as a consequence of the short-term culture period.

3.2.2 General T cell phenotypic markers in nasal polyps

Polyp-derived T cells were overwhelmingly of a $CD45RO^+$ memory phenotype, consistent with that observed in the lung (Purwar *et al.*, 2011). Memory T cells can be further divided into central (T_{CM}) and effector memory (T_{EM}) subsets based on expression of CD62L and CCR7 (Sallusto *et al.*, 1999, Galkina *et al.*, 2003). T_{CM} express CD62L and CCR7, enabling the trafficking of these cells to peripheral lymph nodes via high endothelial venules expressing their respective ligands, PNAd and CCL21 (Sallusto *et al.*, 1999, Islam *et al.*, 2012). In contrast, T_{EM} are generally considered to be $CD62L^{lo}$ and CCR7 negative (Sallusto *et al.*, 2004). A significantly higher percentage of T_{EM} was observed from polyp-derived cells compared to peripheral blood-derived cells with the converse true for $T_{CM.}$ This was also observed in the normal nasal mucosa.

Sathaliyawala *et al.* (2013) recently showed that tissue-resident memory cells can be identified based on CD69 expression, in conjunction with effector or central memory phenotype determined by CCR7 expression (tissue-resident $T_{CM:}$ $CD69^+CCR7^+$; tissue-resident $T_{EM:}$ $CD69^+CCR7^-$). The majority of T cells derived from polyps were $CD69^+$, indicative of tissue-resident memory cells. However, whether they were tissue-resident effector or tissue-resident central memory cells was not determined in this study as no co-staining of CCR7 with CD69 for flow cytometric analysis was performed. Nevertheless, the significantly higher percentages of T_{EM} in nasal polyps and the normal nasal mucosa compared to the periphery, along with elevated CD69 expression, suggests that these cells may be resident in nasal tissue rather than circulating in the periphery. Furthermore, over 50% of cells in the lung are tissue-resident effector memory cells (Purwar *et al.*, 2011, Sathaliyawala *et al.*, 2013) lending support to this hypothesis. The large T_{EM} population in polyps and the normal nasal mucosa possibly reflects the essential function of long-lived memory T cells in tissues where an

immediate effector response is required upon recognition of a previously encountered antigen for protective immunity.

Examination of the adhesion molecule PSGL1 showed that it was detected on nearly all T cells. This was expected for a molecule that has been shown to be expressed by the majority of lymphocytes (Vachino *et al.*, 1995) and responsible, along with CD62L, for the adhesion of T cells to the nasal polyp endothelium (Symon *et al.*, 1999). The integrin CD49a showed higher expression in polyp vs. blood-derived cells. Chapman *et al.* (2010) have reported that the presence of $CD4^+$ cells in the murine airway following secondary influenza infection was dependent on CD49a expression. Furthermore, Purwar *et al.* (2011) observed that CD49a expression was relatively lung-specific. Less than 50% of gut-resident memory cells and only 14% of skin-resident memory cells expressed CD49a. Thus, CD49a may be a potential marker of effector memory T cells in the airways.

Although many studies on the role of CLA and $\alpha_4\beta_7$ on skin and gut homing respectively have been published (Fuhlbrigge *et al.*, 1997, Rott *et al.*, 1997), specific homing markers for other tissues, including nasal tissue, are lacking. $\alpha_4\beta_7$ expression was not detected in polyp explant-derived cells. However, the skin homing receptor CLA, was expressed by a higher percentage of polyp and normal nasal mucosa-derived cells compared to the periphery. This was unexpected since cells from the lower respiratory tract reportedly do not express CLA (Purwar *et al.*, 2011, Ainslie *et al.*, 2002). Although the underlying mechanism of CLA expression on polyp T cells is unknown, it has been proposed that antigen-specific T cells may become 'trapped' in tissues upon antigen recognition (Marelli-Berg *et al.*, 2010, Fu *et al.*, 2013). Although speculative, since both nose and skin surfaces are colonised by the commensal bacterium *S. aureus*, it is possible that a proportion of circulating *S. aureus*-specific, CLA expressing T cells may accumulate in the nasal mucosa due to antigen recognition.

Moreover, a significantly larger percentage of CLA^+ cells was detected in polyps compared to the normal nasal mucosa, possibly reflecting the higher *S. aureus* colonisation rate in CRSwNP patients compared to healthy individuals (Van Zele *et al.*, 2004, Gevaert *et al.*, 2013b). Furthermore, recurrent antigen exposure may facilitate long-lived antigen-specific tissue-resident memory cells at the site of antigen exposure (Vezys *et al.*, 2009) with expansion of the CLA^+ population through local proliferation. However, further studies will be needed to characterise CLA^+ nasal polyp T cells in detail, including their cytokine expression profile.

3.2.3 Th17 cells as a tissue-specific T cell phenotype

The phenotype of cytokines expressed by polyp and blood-derived cells correlated with results obtained from surface phenotypic analysis. For example, CCR6 is recognised as a marker of Th17 cells (Acosta-Rodriguez *et al.*, 2007, Wan *et al.*, 2011) and high percentages of $CCR6^+$ cells have been reported in nasal tissue of healthy and allergic patients as well as in healthy skin and lung biopsies compared to peripheral blood (Francis *et al.*, 2008). Consistent with this, CCR6 expression was significantly higher in polyp and normal nasal mucosa-derived cells compared to the periphery. Furthermore, CCR6 expression was significantly higher in the normal nasal mucosa compared to the diseased nasal polyp although this may be due to a 'dilution effect' as $IL\text{-}25R^+$ T cells expressed low levels of CCR6. Intracellular cytokine staining for IL-17 was in accordance with CCR6 expression with the detection of a large percentage of $IL\text{-}17^+$ cells in polyps as well as in the normal nasal mucosa but not in the periphery. Thus, Th17 cells are tissue-specific, reflective of the capacity of CCR6 bearing cells to traffic to tissues (Francis *et al.*, 2008, Wang *et al.*, 2009).

The involvement of Th17 cells in CRSwNP, specifically in western patients, is currently the subject of debate with several conflicting studies published. In contrast to the data presented in this thesis, Peters *et al* (2010) were unable to detect IL-17 production in nasal polyps or in control tissue homogenates. This disparity may be explained by the different methods of tissue preparation utilised in the two studies. Whilst Peters and colleagues analysed expression in tissue homogenates, this study utilised the explant model with the addition of IL-2 to promote T cell growth. IL-2 via PI-3 kinase signalling is able to induce IL-17 production from $CCR6^+$ cells in culture previously determined to be $IL\text{-}17^-$ *ex vivo* (Wan *et al.*, 2011) although IL-2 alone does not differentiate uncommitted Th cells to Th17 cells. Although this may have played a role in boosting the number of Th17 cells detected in the explant model, $IL\text{-}17^+$ cells were

still observed in nasal polyps analysed following overnight tissue digestion in the absence of IL-2, albeit at a lower frequency. Thus, this indicates that local IL-17^+ cells are present *in vivo* in nasal polyps and in the normal nasal mucosa and these results agree with the proposal by Wan and colleagues that *ex vivo* analysis may under-represent the frequency of Th17 cells present in tissue.

Th17 cells are also capable of co-expressing IL-22 (Liang *et al.*, 2006). IL-22 producing cells were detectable in polyp-derived cells only and not in the periphery, further corroborating the existence of a large Th17 population in nasal polyps. Although expression of IL-22 was not analysed by flow cytometry in normal nasal mucosal biopsies due to the limited number of cells available, ELISA analysis in culture supernatants showed that IL-22 was present at comparable levels to that in polyp explant cultures. Furthermore, microarray analysis (Chapter 5) showed that the gene for IL-22 was preferentially expressed by normal nasal biopsy T cells vs. peripheral blood T cells. The potential role of Th17 cells in the nasal mucosa will be further discussed in Chapter 5.

3.2.4 Th2 cells as a CRSwNP disease-specific T cell phenotype

Cells producing the Th2 cytokines IL-5 and IL-13 were elevated in polyp vs. blood-derived cells. As with CCR6 and IL-17, this was in agreement with the cell surface marker expression, with IL-25R (Angkasekwinai *et al.*, 2007, Wang *et al.*, 2007) and CRTH2 (Nagata *et al.*, 1999, Cosmi *et al.*, 2000) representing Th2 cells. Indeed, numbers of cells expressing IL-25R also positively correlated with Th2 cytokine expression. In addition, the percentages of IL-13 and IL-9 producing cells, detected by flow cytometry, were higher in polyp-derived cells but not in nasal mucosal biopsies compared to peripheral blood from healthy volunteers. Thus, these results suggest that IL-25R may be a marker of Th2 cells in nasal polyps and expression of IL-25R is CRSwNP-disease specific, agreeing with previous reports of Th2 cytokine expression and eosinophilic infiltration in Caucasian CRSwNP patients (Van Zele *et al.*, 2006, Zhang *et al.*, 2008). The possible role of IL-25R$^+$ cells in CRSwNP will be discussed in more detail in Chapter 4.

Polyp-derived cells producing IL-17 and IL-13 were distinct cell populations, as evidenced by minimal co-localisation of expression. Although subsequent experiments showed that the Th17 response was not disease specific, Th17 and Th2 responses have the potential to interact in CRSwNP. For example, Wakashin *et al.* (2008) reported that adoptive transfer of a mixture of ovalbumin (OVA)-specific Th2 and Th17 cells resulted in synergistic effects on eosinophil recruitment to the murine lung. Furthermore, Esnault *et al.* (2012) have shown that co-culture of CD4$^+$ T cells with eosinophil culture supernatants results in increased IL-17 production mediated by IL-1β released from eosinophils.

Although Th2 cells produce IL-4, this cytokine was not routinely measured by intracellular cytokine analysis due to constraints with cell numbers, fluorophore

availability and the lower expression of IL-4, compared to IL-5 and IL-13. Cell numbers recovered from nasal biopsy explants also precluded the assessment of the whole cytokine profile measured in polyp explant samples by flow cytometry. However, CBA analysis was used as an efficient method to simultaneously measure a large number of cytokines in cell culture supernatants. IL-4 protein levels were higher in polyp culture supernatants compared to normal nasal biopsy supernatants, in parallel with the other Th2 cytokines IL-5 and IL-13 (CBA and flow data). In addition, IL-17 levels in culture supernatants did not differ between polyp and normal nasal biopsies when measured by CBA.

Although no significant difference in IL-10 levels was observed between polyp and blood-derived cells by flow cytometry, CBA analysis showed a significant increase in IL-10 levels in polyp culture supernatants compared to normal nasal biopsies. Li *et al.* (2008) have previously shown that the concentration of IL-10 is significantly decreased in nasal polyp homogenates compared to control healthy nasal mucosa. However, this experiment was performed on unstimulated polyp tissue homogenates. Recent studies have shown that IL-10 production is significantly increased from polyp tissue homogenates upon stimulation with aeroallergens (Faith *et al.*, 2012) and *Staphylococcus aureus* enterotoxin B (SEB) (Derycke *et al.*, 2014). These data agree with the results presented in this thesis as CBA analysis was performed on supernatants from cell cultures 48 hours post anti-CD3/CD28 stimulation. The higher level of IL-10 could be consistent with the presence of regulatory T cells in nasal polyp tissue, possibly as a response to inflammation (Faith *et al.*, 2012). In contrast, the minimal concentrations of IL-10 in normal nasal biopsies may reflect the relatively non-inflammatory state of the healthy nasal mucosa. The concentration of the pro-inflammatory cytokine TNFα, together with IL-6, was also higher in supernatants from polyp cultures. TNFα has been shown to upregulate the expression of eotaxin (CCL11)

and RANTES (CCL5) from polyp fibroblasts (Saji *et al.*, 2000, Yoshifuku *et al.*, 2007). Thus, these studies raise the possibility that non-Th2 pro-inflammatory cytokines present in nasal polyps may also promote the chronic inflammation observed in CRSwNP.

3.2.5 Relationship between CRSwNP clinical status and T cell phenotype

CRSwNP patients showed some heterogeneity in clinical parameters such as atopy, aspirin sensitivity and steroid use. Analysis of IL-13 and IL-5 production according to clinical status showed no significant differences. However, a trend was observed for higher percentages of cells expressing IL-25R and CRTH2 in AS patients vs. AT patients. Nasal polyposis is a common feature in aspirin-exacerbated respiratory disease (AERD) and the latter may represent the most severe cases of nasal polyposis (Kim *et al.*, 2007). AERD is also associated with high baseline levels of the pro-inflammatory prostaglandin PGD_2 (Bochenek *et al.*, 2003, Pierzchalska *et al.*, 2003) which is chemotactic for Th2 cells as well as eosinophils and ILC2 via the receptor CRTH2 (Hirai *et al.*, 2001, Shiraishi *et al.*, 2005, Xue *et al.*, 2014). Thus, the high levels of CRTH2 expressing Th2 cells in AS patients suggests that these cells have the potential to be involved in the severe clinical phenotype of AERD (aspirin-sensitive) compared to aspirin-tolerant patients. In contrast, it is not currently known if IL-25 is chemotactic for Th2 cells.

Several epidemiology studies have reported that there is a higher prevalence of CRSwNP and also increased disease severity in non-atopic asthmatics compared to atopic asthmatics (Settipane, 1996, Robinson *et al.*, 2006, Pearlman *et al.*, 2009). Consistent with this, there was a trend for higher expression of CRTH2 and IL-25R in non-atopic CRSwNP patients compared to atopic CRSwNP patients in this study. This suggests that local Th2 responses are not aeroallergen driven and a different mechanism such as aspirin sensitivity could potentially be responsible for polyposis in atopic CRSwNP patients. Indeed, in CRSwNP patients whose atopic and aspirin statuses were both known, 6 out of 9 atopic patients also displayed aspirin sensitivity.

Nanzer *et al.* (2013) previously reported that the steroid dexamethasone is able to increase IL-17 production in a dose-dependent manner by PBMC from healthy donors. In this study, no significant association was observed between IL-17 expression and oral steroid (prednisolone) use although at least 80% of CRSwNP patients recruited in this study used topical nasal steroids only, at the time of surgery. However, the presence of a significant IL-17 signature in the normal nasal mucosa of medication-free healthy volunteers indicates that steroids administered orally or as a nasal spray, are unlikely to be entirely responsible for the Th17 response in CRSwNP patients.

3.2.6 Comparison of nasal mucosal and skin T cells

T cells in the nasal mucosa were compared with the skin as phenotypic differences between T cells between the nasal mucosa and gut mucosa have previously been reported (Jahnsen *et al.*, 1998). The presence of a large $CXCR3^+$ Th1 population in both the nasal mucosa and skin is suggestive of a role for Th1 cells in the local host defence against bacterial infection (Schroder *et al.*, 2004, D'Elios *et al.*, 2011). Both these surfaces are exposed to the external environment and the propensity for a Th1 response in the normal nasal mucosa has been previously demonstrated (Till *et al.*, 2001). Expression of CXCR3 was higher in diluent challenged vs. allergen challenged skin-derived cells although this may represent a 'dilution effect' due to the recruitment of Th2 cells. The significantly higher CXCR3 expression observed in both allergen and diluent challenged skin-derived T cells compared to polyp-derived T cells suggests that Th1 cells may have a more prominent role in host defence in the skin compared to the nasal mucosa, although the percentages of IFNγ-producing cells were in fact similar in allergen challenged skin and polyp cultures. More cases will be required to validate this observation, particularly as relatively few skin biopsies yielded sufficient cells for experimentation. The limited number of cells obtainable from a skin biopsy precluded the complete analysis of surface markers and intracellular cytokines assessed for in nasal polyp samples. Cell numbers from diluent challenged skin were particularly limiting, possibly because cells were in a non-activated state in the absence of allergen challenge and thus were less poised for proliferation (Frew *et al.*, 1988). This, and the lack of matched blood specimens, hampered the general phenotypic assessment of skin T cells in this study.

The data presented in this thesis suggests that CRTH2 and IL-25R expression by skin-derived T cells is similar to that by polyp-derived T cells and this was higher than in blood cultures from CRSwNP patients. The same pattern of expression was observed

for IL-5 producing cells. Expression of IL-25R and CRTH2 were both significantly higher in T cells derived from nasal polyps vs. healthy normal nasal mucosa. In the skin, only IL-25R$^+$ cells, and not CRTH2$^+$ cells, increased following grass allergen challenge. This suggests that IL-25R may be a superior marker to CRTH2 for Th2 cells. A higher percentage of IL-25R$^+$ cells than CRTH2$^+$ cells were detected in nasal polyp explant cultures in this study and a significant positive correlation was observed between IL-25R expression – but not CRTH2 – and Th2 cytokine production.

The high percentage of CCR6 expressing Th17 cells in both diluent and allergen challenged skin biopsy cultures was also similar to that seen with nasal polyp explants. This supports the observation of Wang *et al.* (2009) that CCR6 may be a tissue-homing receptor and a marker of Th17 cells. The findings presented here agree with the previously published report by Francis *et al.* (2008) who reported that CCR6$^+$ T cells are present in cultures from skin biopsies but not peripheral blood. Thus, the presence of Th17 cells in both skin and nasal mucosal tissues under normal, homeostatic conditions suggests that these cells may represent a default T helper cell phenotype involved specifically in host defence at external surfaces.

3.3 Summary

In this chapter, the expression profiles of T cell subset surface markers and cytokines were examined in nasal polyps and in the healthy normal nasal mucosa. Local nasal T cells responses were also compared to the periphery. Lastly, T cells from skin biopsies were examined to compare the phenotype of T cells from the nasal mucosa to a different organ. The data presented in this chapter indicates that:

1. IL-17$^+$ and CCR6$^+$ cells are present in the healthy and diseased nasal mucosa but not in the periphery.
2. An abundant IL-25R$^+$ population exists in polyp-derived T cells but not in healthy nasal mucosa-derived or blood-derived T cells. This population correlates with Th2 cytokine expression.
3. Higher expression of IL-25R and CRTH2 may exist in polyp-derived T cells from aspirin sensitive vs. aspirin tolerant patients and non-atopic vs. atopic patients.
4. The phenotype of T cells in the skin, following intradermal allergen challenge, shares features of the T cell response seen in CRSwNP patients.

Chapter 4 Characterisation of nasal IL-25R^{+} T cells

4.1 Introduction

Since its discovery, the epithelial cell-derived cytokine IL-25 has been shown to promote Th2-type responses in animal models of allergic airway inflammation (Fort *et al.*, 2001, Tamachi *et al.*, 2006, Angkasekwinai *et al.*, 2007, Ballantyne *et al.*, 2007). In humans, several studies have shown the association of IL-25 with disease. IL-25 levels are significantly higher in serum from active Churg-Strauss syndrome patients compared to inactive or healthy controls (Terrier *et al.*, 2010). Elevated numbers of IL-25^+ cells are also present in lesional skin versus non-lesional skin from chronic spontaneous urticaria patients (Kay *et al.*, 2015). Furthermore, IL-25 and IL-25R mRNA transcripts are increased upon allergen challenge in human asthmatic bronchial mucosa and in atopic dermatitis skin lesions (Corrigan *et al.*, 2011a, Wang *et al.*, 2007).

IL-25 has been demonstrated *in vitro* to increase collagen production by stimulated human fibroblasts (Gregory *et al.*, 2012) and to enhance secretion of CCL11 and CCL5 (RANTES) from lung fibroblasts to initiate and maintain eosinophilia in allergic inflammation (Letuve *et al.*, 2006). TSLP-activated dendritic cells have also been shown to upregulate expression of IL-25R on memory Th2 cells with IL-25 prolonging the expression of Th2 transcription factors (GATA-3, c-MAF and JunB) to potentiate the type 2 immune response (Wang *et al.*, 2007). Furthermore, IL-25 is able to promote angiogenesis in endothelial cells from asthmatic patients through upregulation of VEGF (Corrigan *et al.*, 2011b). Thus, these studies demonstrate the ability of IL-25 to regulate and promote the Th2 response in allergic inflammation. Notably however, few studies exist on the role of IL-25/IL-25R in CRSwNP disease or in the nasal mucosa.

In addition, the epithelial cell-derived cytokine IL-33 and its receptor, ST2, are also associated with type 2 immune responses (Schmitz *et al.*, 2005, Kurowska-Stolarska *et al.*, 2008). In human disease, elevated levels of both IL-33 and ST2 mRNA are

expressed in the nasal epithelium of allergic rhinitis patients compared to healthy controls (Kamekura *et al.*, 2012). Higher ST2 mRNA expression, but not IL-33 mRNA, was also detected in polyps from CRSwNP patients vs. healthy control nasal tissue (Baba *et al.*, 2014). Furthermore, expression of IL-33 mRNA was significantly greater in epithelial cell cultures derived from recalcitrant CRSwNP compared to treatment-responsive CRSwNP patients (Reh *et al.*, 2010). ILC2s have also been demonstrated to respond to both IL-25 and IL-33 to potentiate IL-13 production in CRSwNP (Neill *et al.*, 2010, Mjosberg *et al.*, 2011, Shaw *et al.*, 2013, Miljkovic *et al.*, 2014).

Despite these numerous studies, only a limited literature exists on the relevance of the IL-25/IL-33 axis and their interactions with the local T cell response, particularly in the nasal mucosa. *In vitro* differentiated human Th2 lines and human peripheral blood-derived memory Th2 cells have previously been demonstrated to express the IL-25 receptor and display a Th2 phenotype (Wang *et al.*, 2007, McDonald *et al.*, 2008). However, the identification of an abundant IL-25R expressing population in nasal polyp explant-derived cells (Chapter 3) presented a valuable opportunity to further examine these cells, particularly since they were derived directly from human tissue associated with chronic eosinophilic Th2-type inflammation. Hence, an in-depth characterisation of the phenotype and gene expression profile of IL-25R$^+$ cells was performed.

The antigen involved in CRSwNP has not been identified and the aetiology of the disease remains unknown (Pawankar, 2003, Tomassen *et al.*, 2011). Data presented in the preceding chapter in this thesis showed that IL-25R$^+$ cells were detected in polyp-derived cultures only and displayed a Th2 phenotype that is associated with pathology in eosinophilic CRSwNP. From this, it was hypothesised that IL-25R$^+$ cells recognise a common antigen that could potentially be involved in CRSwNP pathogenesis. Thus, in this chapter, the TCR repertoire of IL-25R$^+$ cells was also analysed to address this hypothesis.

In summary, the aims of the work presented in this chapter were to further characterise $CD4^+IL\text{-}25R^+$ cells identified in polyp explant cultures by examining:

1. The phenotype of $IL\text{-}25R^+$ vs. $IL\text{-}25R^-$ cells
2. The gene expression profile of these two populations
3. The effects of IL-25 and IL-33 on $IL\text{-}25R^+$ cells
4. The TCR Vβ repertoire of $IL\text{-}25R^+$ vs. $IL\text{-}25R^-$ cells.

4.2 Results

4.2.1 IL-25R expressing cells derived from polyp explants are not invariant NKT cells.

Invariant NKT (iNKT) cells express the helper T cell marker CD4 and several studies in the literature have reported the existence of a subset of iNKT cells that also express the IL-25 receptor (Terashima *et al.*, 2008, Stock *et al.*, 2009, Watarai *et al.*, 2012). $CD45^+$ polyp-derived cells were therefore examined to exclude the possibility that the $CD4^+$IL-$25R^+$ population detected in polyp explants cultures were iNKT cells. Expression of the invariant T cell receptor (TCR) alpha chain, Vα24-Jα18, is restricted to iNKT cells and expression was analysed by flow cytometry. No detectable expression of Vα24-Jα18 was found in either blood or polyp-derived $CD45^+$ cells (Figure 4.1). This was observed in five CRSwNP patients examined and confirms that the large IL-$25R^+$ cell population present in polyp explants were $CD4^+$ T cells and not iNKT cells.

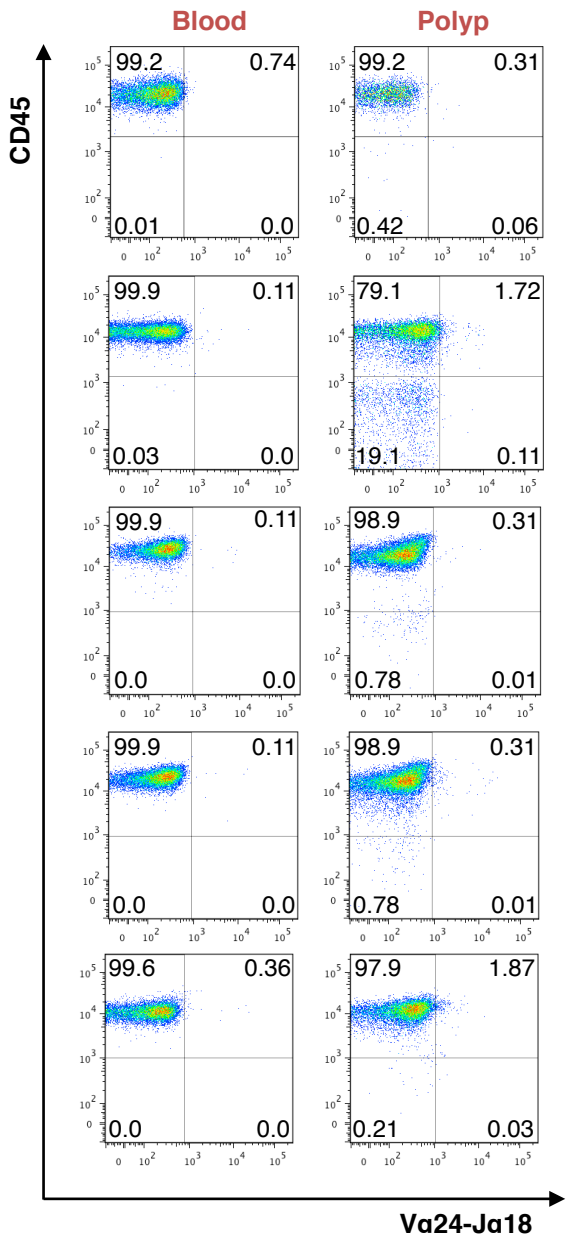


Figure 4.1 IL-25R$^+$ cells are not invariant NKT cells.

CD45$^+$ polyp-derived cells were analysed for expression of the invariant Vα24-Jα18 TCR alpha chain expressed exclusively by iNKT cells. Representative flow cytometry staining for matched blood and polyp samples from 5 individual patients is shown.

4.2.2 The T helper type 2 phenotype of IL-25R$^+$ cells

To further characterise the phenotype of CD4$^+$IL-25R$^+$ cells, expression of general T cell and phenotypic surface markers was examined on day 7 post anti-CD3/CD28 stimulation. CD45RO$^+$ memory cells constituted the entirety of the IL-25R$^+$ population and the overwhelming majority were TCRαβ$^+$ cells (Figure 4.2c), in line with data presented in the previous chapter.

4.2.2.1 IL-25R expression does not co-localise to non-Th2 surface markers

Polyp and blood-derived cells were analysed for co-expression of IL-25R with the Th17 marker CCR6, Th1 marker CXCR3 and Th2 marker CRTH2. Minimal IL-25R expression was detected in blood-derived CD4$^+$ cells. The majority of IL-25R$^+$ polyp-derived cells were CCR6 negative with a similar pattern observed for CXCR3 (Figure 4.2a, b). Moreover, significantly lower percentages of CCR6$^+$ cells and CXCR3$^+$ cells were found amongst IL-25R expressing T cells compared to cells lacking IL-25R (CCR6: IL-25R$^+$, 7.5% ± 2.3 vs. IL-25R$^-$, 92.5% ± 2.3; $p<0.01$, CXCR3: IL-25R$^+$, 6.5% ± 2 vs. IL-25R$^-$, 93.5% ± 1.8; $p<0.001$). In contrast, although not significant, over 50% of CRTH2$^+$ cells co-localised to IL-25R (IL-25R$^+$, 52.1% ± 8.5 vs. IL-25R$^-$, 46.8% ± 8.8). Taken together, these data show that IL-25R is neither expressed by CCR6-associated Th17 nor CXCR3-associated Th1 cells (i.e. non-Th2 cells) but a degree of co-localisation is observed with the Th2 marker CRTH2.

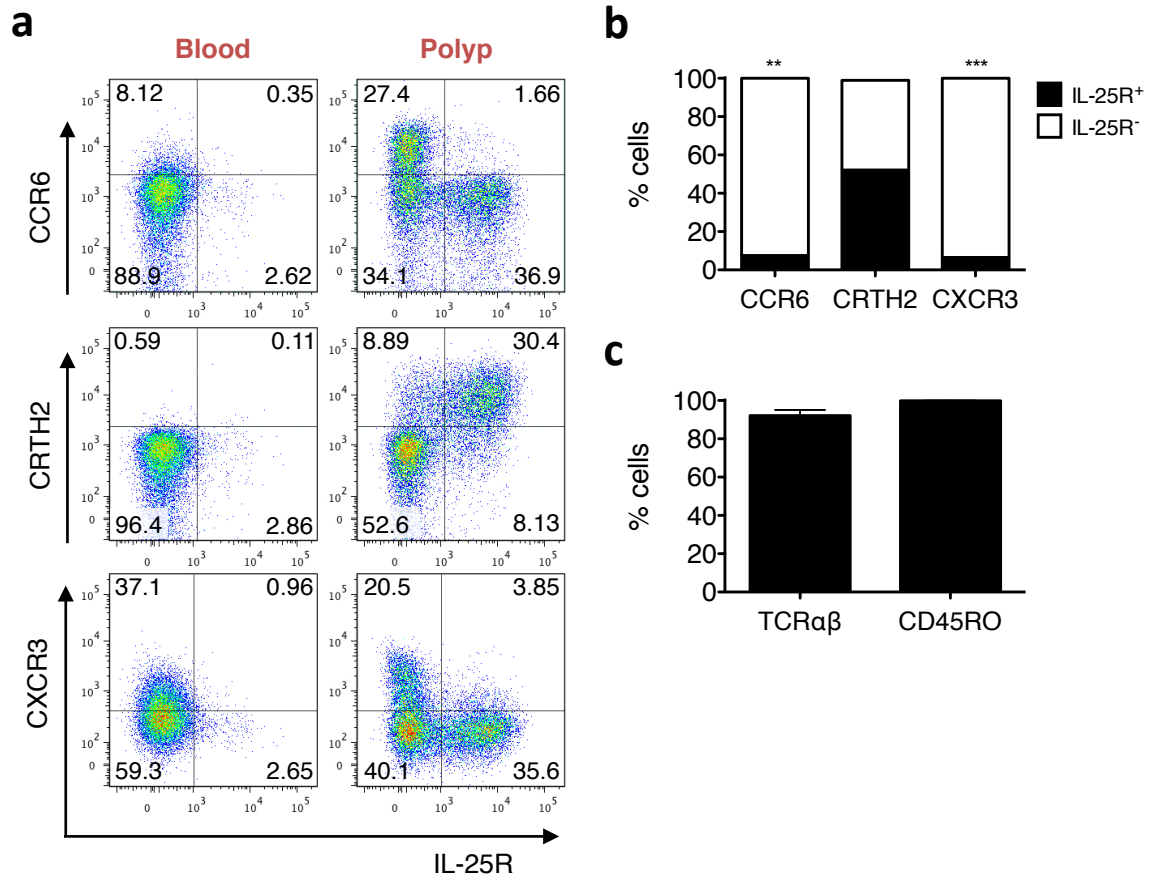


Figure 4.2 IL-25R is present on memory TCRαβ cells and co-localises with CRTH2.

(a) Representative flow cytometry staining for IL-25R and CCR6 (Th17 marker), CXCR3 (Th1 marker) and CRTH2 (Th2 marker) from $CD4^+$ polyp-derived (results representative of n=10 individual experiments). (b) CCR6, CRTH2 and CXCR3 co-expression with IL-25R (n=10). (c) $TCR\alpha\beta^+$ and $CD45RO^+$ cells were examined for co-expression of IL-25R (n=10). Wilcoxon matched-pairs signed rank test. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$.

4.2.2.2 IL-25R expressing cells produce Th2 cytokines

As IL-25R$^+$ cells show a Th2 surface phenotype, IL-25R$^+$ cells were evaluated next for Th2 cytokine production. Blood and polyp-derived cells were analysed for intracellular cytokine production on day 7 post anti-CD3/CD28 stimulation. Representative intracellular cytokine staining data for CD4$^+$ T cells derived from blood and polyp explant cultures is shown in Figure 4.3.

The majority of the Th2 cytokine producing cells were observed in the IL-25R$^+$ population. Over 60% of IL-13$^+$ cells (Figure 4.4b) and IL-5$^+$ cells (Figure 4.4c) co-expressed IL-25R (IL-5: IL-25R$^+$, 64.4% ± 7.9 vs. IL-25R$^-$, 35.6% ± 7.9; IL-13: IL-25R$^+$, 60.2% ± 8.3 vs. IL-25R$^-$, 39.8% ± 8.3). The percentage of IL-5/IL-13 double positive cells was also significantly higher (approximately 2.5-fold) in the IL-25R$^+$ population compared to the IL-25R$^-$ population (Figure 4.4h) (IL-25R$^+$, 72.2% ± 5 vs. IL-25R$^-$, 27.8% ± 5; $p<0.05$). However, no difference was observed for IL-9 producing cells (Figure 4.4d).

Notably, the overwhelming majority of IFNγ$^+$ cells did not express IL-25R (IL-25R$^-$, 90.7% ± 2.8 vs. IL-25R$^+$, 9.3% ± 2.8; $p<0.01$). Furthermore, the majority of CD4$^+$ polyp-derived cells producing IL-17 (Figure 4.4e) and IL-22 (Figure 4.4f) were also IL-25R negative in accordance with the lack of CCR6 and IL-25R co-expression by polyp-derived cells. IL-17$^+$ cells were 4-times more abundant in the IL-25R$^-$ cell population compared to the IL-25R$^+$ population (IL-25R$^-$, 80.2% ± 5.2 vs. IL-25R$^+$, 19.8% ± 5.2; $p<0.05$) with the overwhelming majority of IL-22$^+$ cells detected in the IL-25R$^-$ population (IL-25R$^-$, 96.2% ± 1.4 vs. IL-25R$^+$, 3.8% ± 1.4; $p=0.06$). Moreover, cells producing TNFα were also 6-fold more abundant in the IL-25R$^-$ population compared to the IL-25R$^+$ population (Figure 4.4g) (IL-25R$^-$, 85.7% ± 6.4 vs. IL-25R$^+$, 14.3% ± 6.4; $p=0.06$). Overall, these results suggest that CD4$^+$IL-25R$^+$ cells predominantly and

selectively produce Th2 cytokines and are largely responsible for the Th2 signature in nasal polyp T cell cultures.

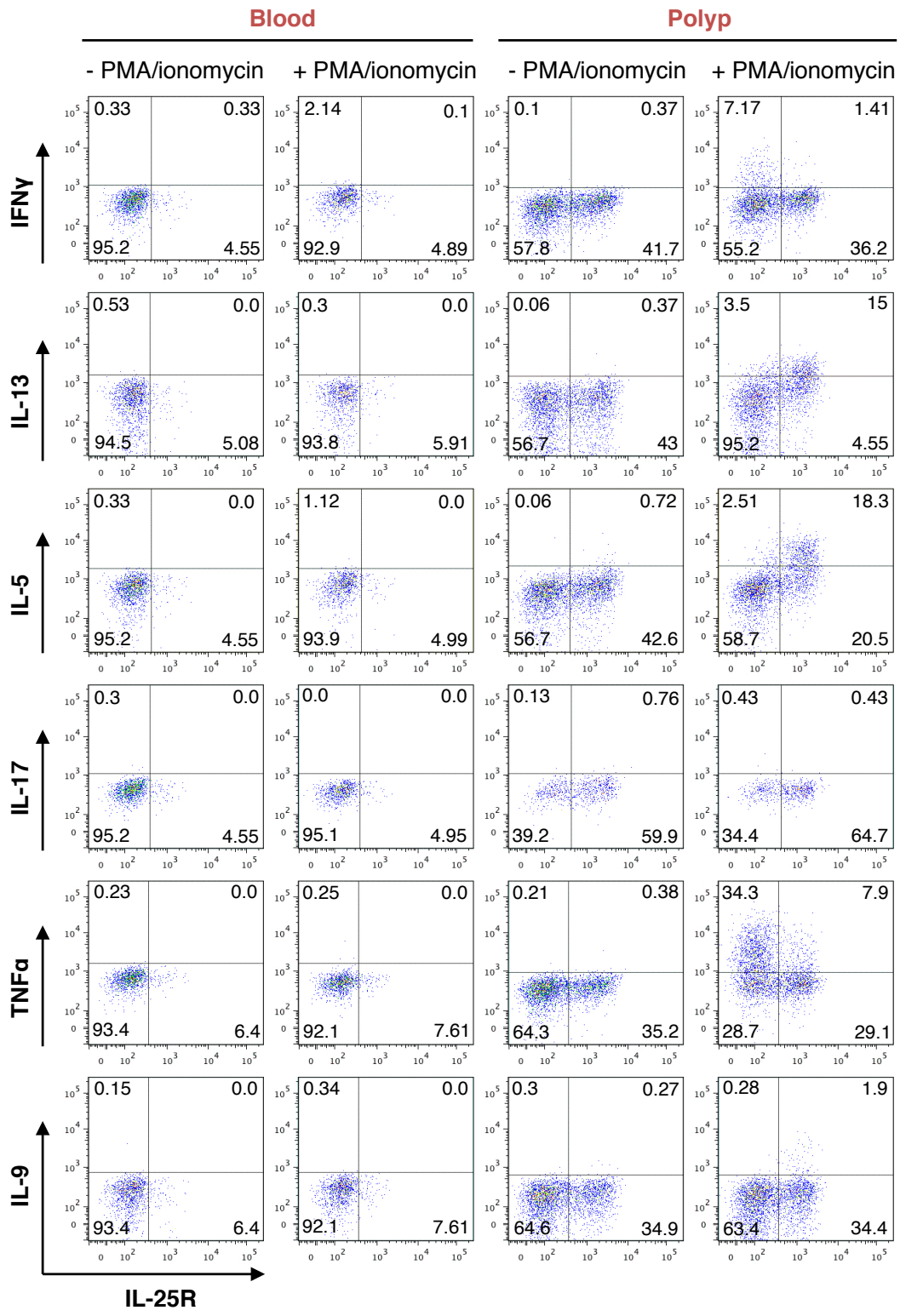


Figure 4.3 Cytokine expression profile by CD4$^+$IL-25R$^+$ T cells from polyp and blood-derived cultures.

T cells were analysed on day 7 following anti-CD3/CD28 stimulation and expansion. Cells were activated with PMA/ionomycin for 4 hours or cultured in medium only before intracellular cytokine analysis. Representative flow cytometry staining for blood- and polyp-derived CD4$^+$IL-25R$^+$ T cells under resting or activated state is shown.

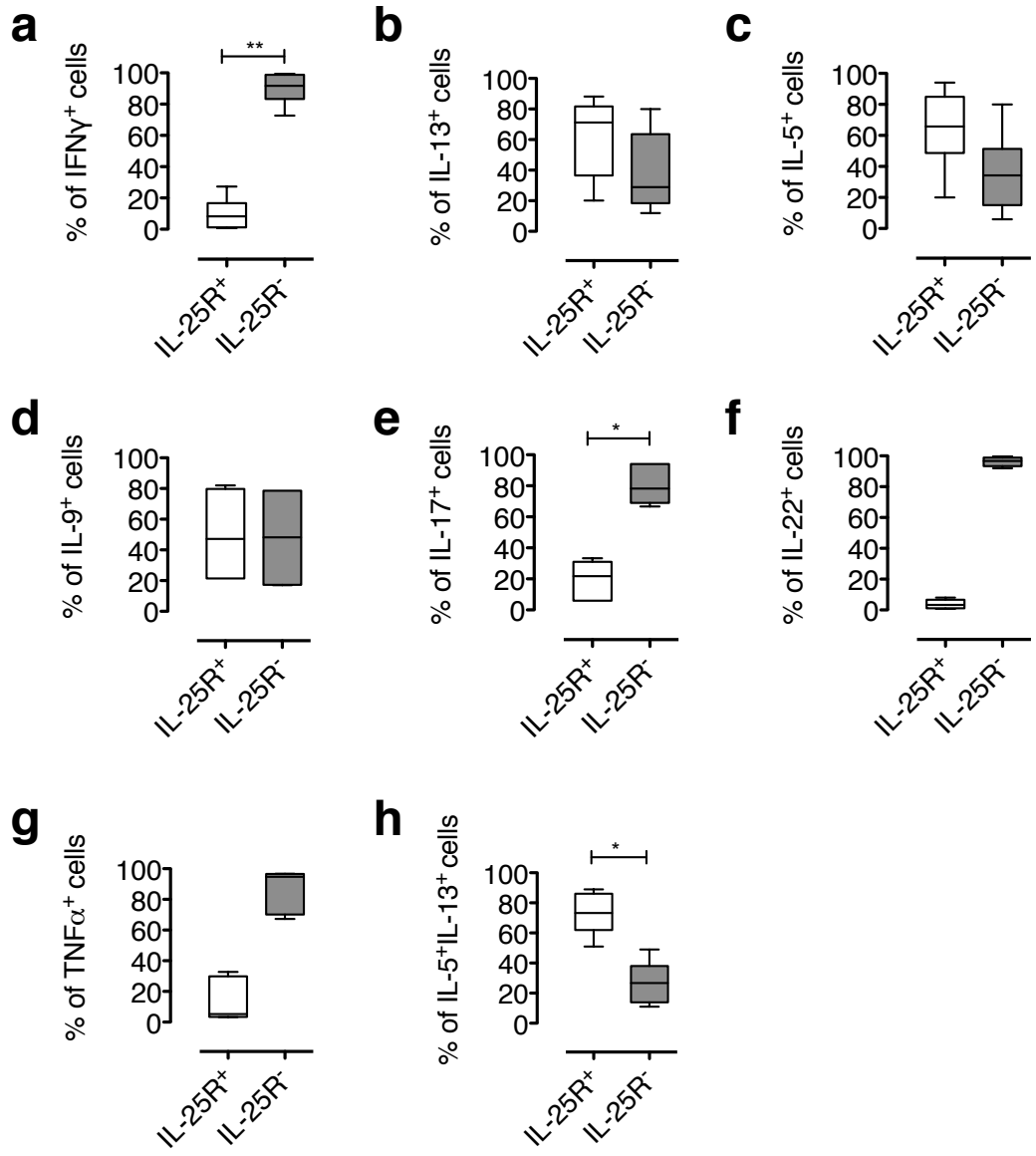


Figure 4.4 IL-25R expression co-localises to Th2 cytokines but not to Th1 or Th17 cytokines in CD4$^+$ cells.

IL-25R expression by polyp CD4$^+$ cells expressing (a) IFNγ, (b) IL-13, (c) IL-5 (n=13), (d) IL-9, (e) IL-17 (n=9), (f) IL-22 (n=5), (g) TNFα and (h) IL-5/IL-13 (n=9) is shown. Wilcoxon matched-pairs signed rank test. *, $p<0.05$, **, $p<0.01$.

4.2.3 Gene expression analysis of IL-25R$^+$ cells

4.2.3.1 Fluorescence-activated cell sorting for IL-25R$^+$ cells

Having determined the surface phenotype and cytokine expression profile of CD4$^+$IL-25R$^+$ cells, the gene expression profile was examined next. CD4$^+$IL-25R$^+$ polyp explant-derived T cells were sorted on day 7 post anti-CD3/CD28 stimulation by fluorescence-activated cell sorting (FACS). Cells were first gated on the lymphocyte population (based on forward scatter and side scatter) followed by gating on the singlet population (Figure 4.5). Viable CD4$^+$ cells were then selected before IL-25R$^+$ as well as IL-25R$^-$ cells were collected for comparison.

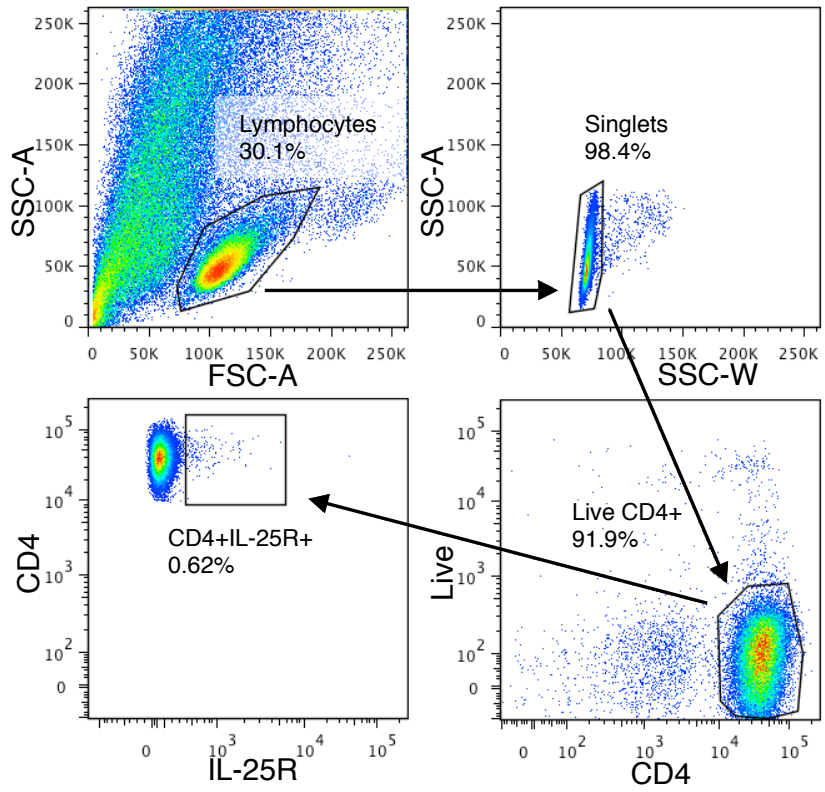


Figure 4.5 Cell sorting strategy for $CD4^{+}IL\text{-}25R^{+}$ cells from polyp explant cultures.

Cells derived from polyp explant cultures were sorted by FACS for $CD4^{+}IL\text{-}25R^{+}$ and $CD4^{+}IL\text{-}25R^{-}$ populations on day 7 post anti-CD3/CD28 restimulation. The percentage of cells in each gate is indicated. Live cells were detected with the eFluor780 viability dye.

4.2.3.2 Genome wide expression profiling by microarray analysis

The gene expression profiles of $CD4^+IL\text{-}25R^+$ and $CD4^+IL\text{-}25R^-$ polyp-derived cells were compared. Sorted cells from three CRSwNP patients were activated with PMA/ionomycin for 4 hours before cells were harvested and prepared for microarray analysis using the Illumina Human HT-12 v4 Expression BeadChip as described in Chapter 2, page 89.

Variation between each individual sample was analysed by performing principal component analysis (PCA). Each sample on the array chip is represented as a dot in the PCA plot (Figure 4.6) with the distance between any pair of dots related to their similarities in a number of variables. PCA analysis showed that samples within each group ($IL\text{-}25R^+$ resting, $IL\text{-}25R^+$ activated, $IL\text{-}25R^-$ resting and $IL\text{-}25R^-$ activated) generally clustered together indicating that expression profiles were broadly similar. The major factor responsible for variation between samples was the activation status of the cells, with IL-25R expression representing the second largest source of variation.

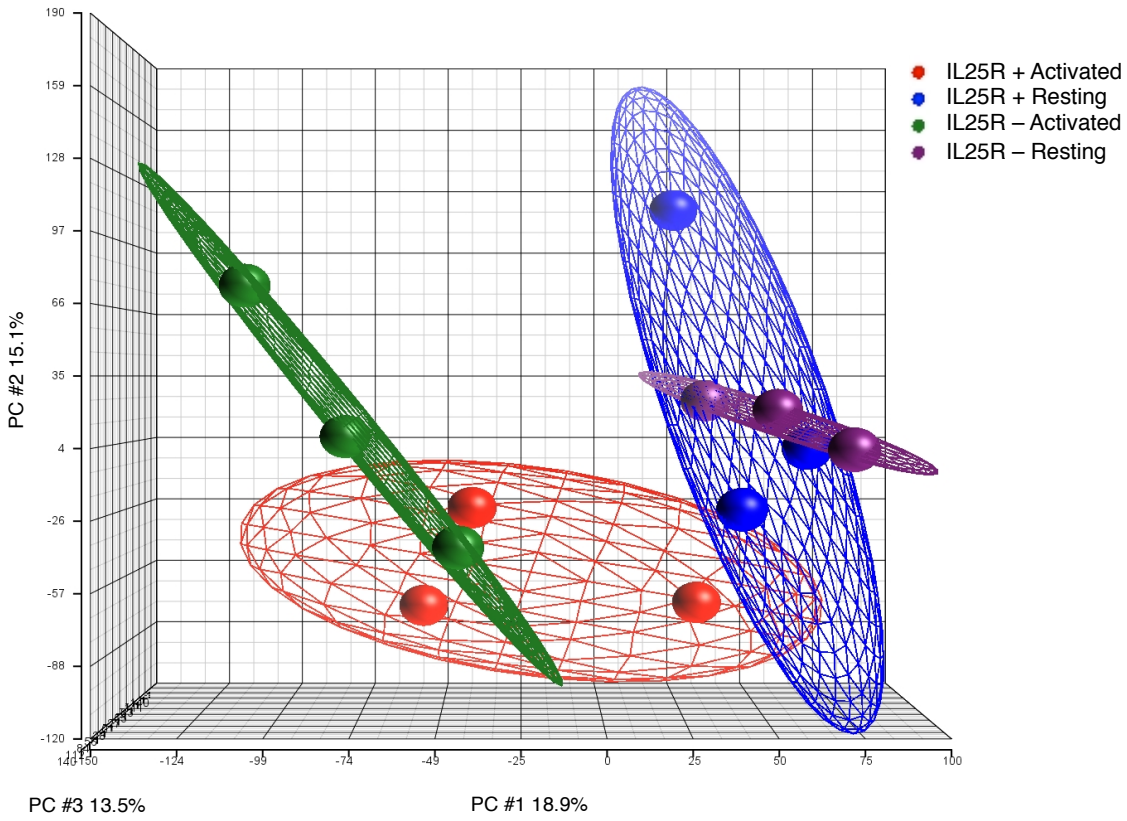


Figure 4.6 Cell activation status is the largest variable observed followed by expression of IL-25R.

$CD4^{+}$ $IL\text{-}25R^{+/-}$ polyp-derived cells were sorted by FACS and activated with PMA/ionomycin or cultured in medium only for 4 hours. Gene expression was analysed with an Illumina Human HT-12 v4 Expression BeadChip. Principal component analysis is shown, performed in Partek Genomics Suite (n=3 per group).

Comparison of activated IL-25R$^+$ and activated IL-25R$^-$ samples identified 42 genes that demonstrated differences in expression of 2-fold or greater (Figure 4.7). Of the 42 differentially expressed genes, 34 were statistically significant ($p<0.05$) (Table 4.1). The gene for IL-25R, *IL17RB*, was the second most upregulated in activated CD4$^+$IL-25R$^+$ compared to activated CD4$^+$IL-25R$^-$ cells, confirming the validity of the FACS sort strategy (IL-25R$^+$ vs. IL-25R$^-$, fold change 5.2; $p<0.01$).

Hierarchical clustering of the differentially expressed genes showed that activated CD4$^+$IL-25R$^+$ cells consistently showed upregulation of Th2-associated genes compared to activated CD4$^+$IL-25R$^-$ cells (Figure 4.7). *IL5* exhibited the largest fold-difference in expression with IL-25R$^+$ cells expressing 6.5-times the amount of *IL5* transcript compared to IL-25R$^-$ cells (Table 4.1). This was statistically significant ($p<0.01$) and the same pattern was observed for the genes encoding GATA3 (fold change 2.6; $p<0.05$) and IL-9 (fold change 3.6; $p<0.05$). Furthermore, a trend for upregulated expression was also observed for *IL4* (fold change 2.5; $p=0.06$) and *IL13* (fold change 3; $p=0.05$). Expression of the gene for pro-melanin-concentrating hormone (*PMCH*) showed significant upregulation (fold change 2.4; $p<0.05$) correlating with previously published data from *in vitro*-derived Th2 cultures (Sandig *et al.*, 2007). Preferential expression of the prostaglandin-endoperoxide synthase 2 gene (*PTGS2*), also known as cyclooxygenase-2 (COX-2), was similarly observed in IL-25R$^+$ cells vs. IL-25R$^-$ cells (fold change 2.9; $p<0.01$).

In addition to the Th2 gene signature, IL-25R$^+$ cells showed a parallel downregulation of the Th1/Th17-associated genes *IFNG* (fold change -3; $p=0.06$), *LTA* (lymphotoxin-α) (fold change -2.2; $p=0.2$) and *PRF1* (perforin-1) (fold change -2.5; $p<0.01$) compared to IL-25R$^-$ cells. Furthermore under resting conditions, the upregulation of Th2-related genes (*GATA3*, fold change 2; *IL5*, fold change 2.7; *IL13*, fold change 2.7) was also observed in IL-25R$^+$ cells. This was accompanied by preferential expression of *PRF1*

(fold change 2.3) in resting IL-25R$^-$ cells. Thus, these data show that differential gene expression is observed between IL-25R$^+$ and IL-25R$^-$ cells under both resting and activated conditions.

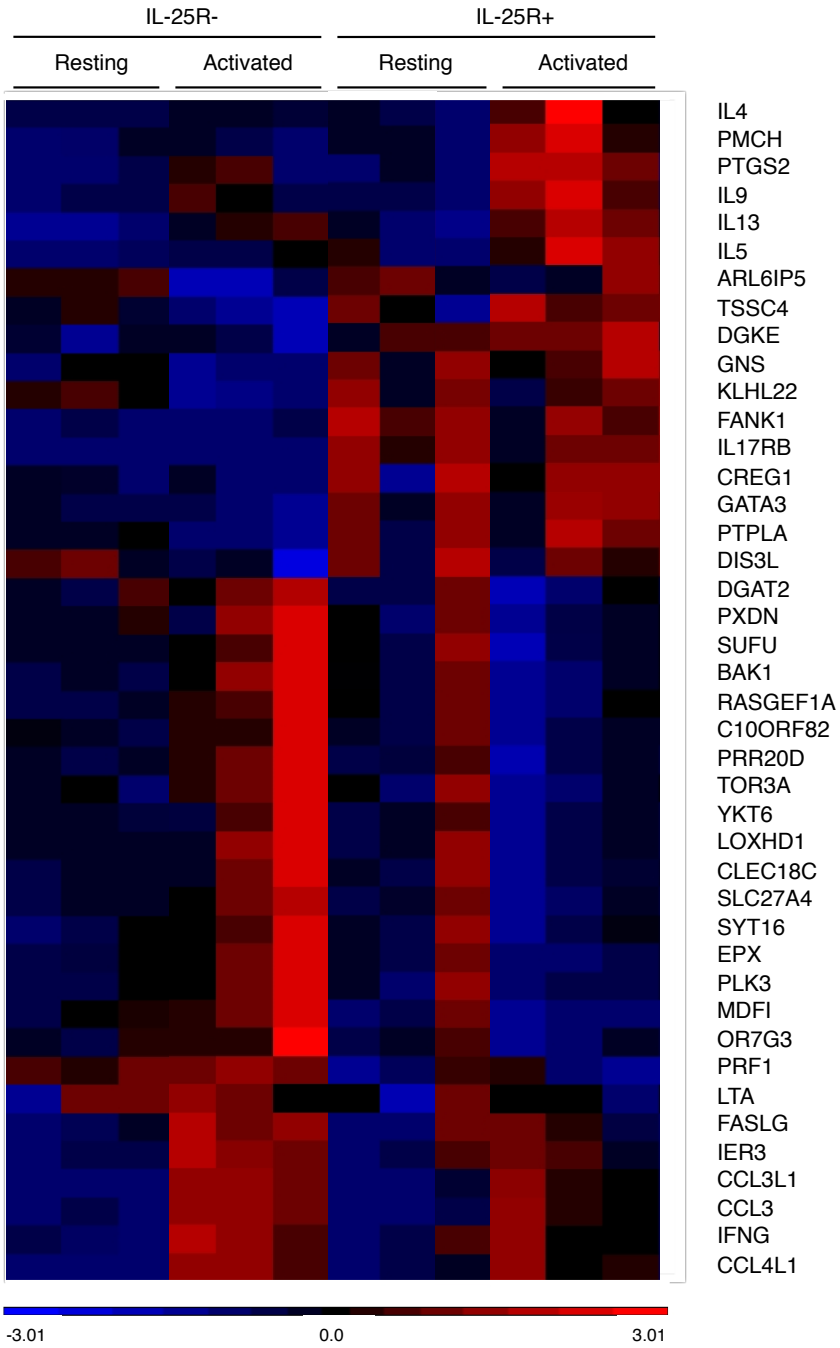


Figure 4.7 Differentially expressed genes by IL-25R^{+} and IL-25R^{-} cells.

Data analysis was performed in Partek Genomics Suite using a 3 way-ANOVA model. Data is presented as a heatmap showing hierarchical clustering of genes with differential expression (fold change >2 or <-2). Comparison of activated IL-25R^{+} and activated IL-25R^{-} samples (n=3 per group) identified 42 genes that were differentially expressed. Of these, 34 genes were significant ($p<0.05$).

Table 4.1 Differentially expressed genes in activated polyp-derived $CD4^+$ IL-$25R^+$ and IL-$25R^-$ populations.

Gene	p value	Ratio	Fold-difference
IL-25R- (activated) down vs. IL-25R+ (activated)			
IL5	0.008 **	0.152	-6.562
IL17RB	0.006 **	0.191	-5.225
IL9	0.017 *	0.278	-3.601
IL13	0.051	0.325	-3.075
GNS	0.001 **	0.335	-2.989
PTGS2	0.008 **	0.343	-2.917
FANK1	0.007 **	0.346	-2.889
DGKE	0.003 **	0.349	-2.867
GATA3	0.015 *	0.389	-2.568
IL4	0.062	0.398	-2.511
CREG1	0.055	0.407	-2.457
PMCH	0.011 *	0.422	-2.372
PTPLA	0.009 **	0.440	-2.274
TSSC4	0.001 **	0.469	-2.133
DIS3L	0.075	0.473	-2.116
ARL6IP5	0.005 **	0.484	-2.067
KLHL22	0.014 *	0.495	-2.018
IL-25R- (activated) up vs. IL-25R+ (activated)			
IFNG	0.055	3.032	3.032
IER3	0.017 *	2.700	2.700
CCL3L1	0.015 *	2.595	2.595
PRF1	0.005 **	2.489	2.489
TOR3A	0.012 *	2.411	2.411
SUFU	0.004 **	2.372	2.372
SLC27A4	0.009 **	2.343	2.343
YKT6	0.034 *	2.283	2.283
MDFI	0.004 *	2.258	2.258
CCL3	0.054	2.203	2.203
LTA	0.196	2.193	2.193
EPX	0.004 **	2.139	2.139
FASLG	0.021 *	2.100	2.100
BAK1	0.004 **	2.099	2.099
CCL4L1	0.180	2.095	2.095
DGAT2	0.003 **	2.078	2.078
CLEC18C	0.008 **	2.065	2.065
PRR20D	0.002 **	2.056	2.056
PXDN	0.003 **	2.049	2.049
LOXHD1	0.018 *	2.032	2.032
C10orf82	0.011 *	2.027	2.027
SYT16	0.002 **	2.025	2.025
OR7G3	0.002 **	2.020	2.020
PLK3	0.012 *	2.016	2.016
RASGEF1A	0.003 **	2.001	2.001

Genes are listed in order according to magnitude of difference in mRNA expression between IL-$25R^+$ vs. IL-$25R^-$ T cells. P values were calculated using a 3 way-ANOVA model. *, $p<0.05$, **, $p<0.01$.

4.2.3.3 qRT-PCR confirmation of microarray results

Quantitative real time-PCR (qRT-PCR) was performed to validate the differentially expressed genes detected by microarray. FACS sorted cells ($CD4^{+}IL\text{-}25R^{+/-}$) were activated with PMA/ionomycin for 4 hours before cells were harvested and processed as detailed in Chapter 2, page 87.

All genes examined by qRT-PCR showed the same significant differential expression detected by microarray (Figure 4.8). The highest relative expression of Th2-associated genes (IL17RB, IL4, IL13, IL5, GATA3, PMCH, PTGS2) was still detected in activated IL-$25R^{+}$ cells compared to IL-$25R^{-}$ cells. Moreover, highest relative expression of Th1-associated genes (IFNG, LTA) was observed in activated IL-$25R^{-}$ cells compared to IL-$25R^{+}$ cells.

Collectively, these results confirm the microarray data, namely that *ex vivo* IL-$25R^{+}$ cells derived from nasal polyps exhibit a Th2 gene expression profile which is not observed in IL-$25R^{-}$ cells. On the contrary, microarray data suggested that *ex vivo* IL-$25R^{-}$ cells display a Th1/17-associated gene expression profile, which was validated by qRT-PCR analysis.

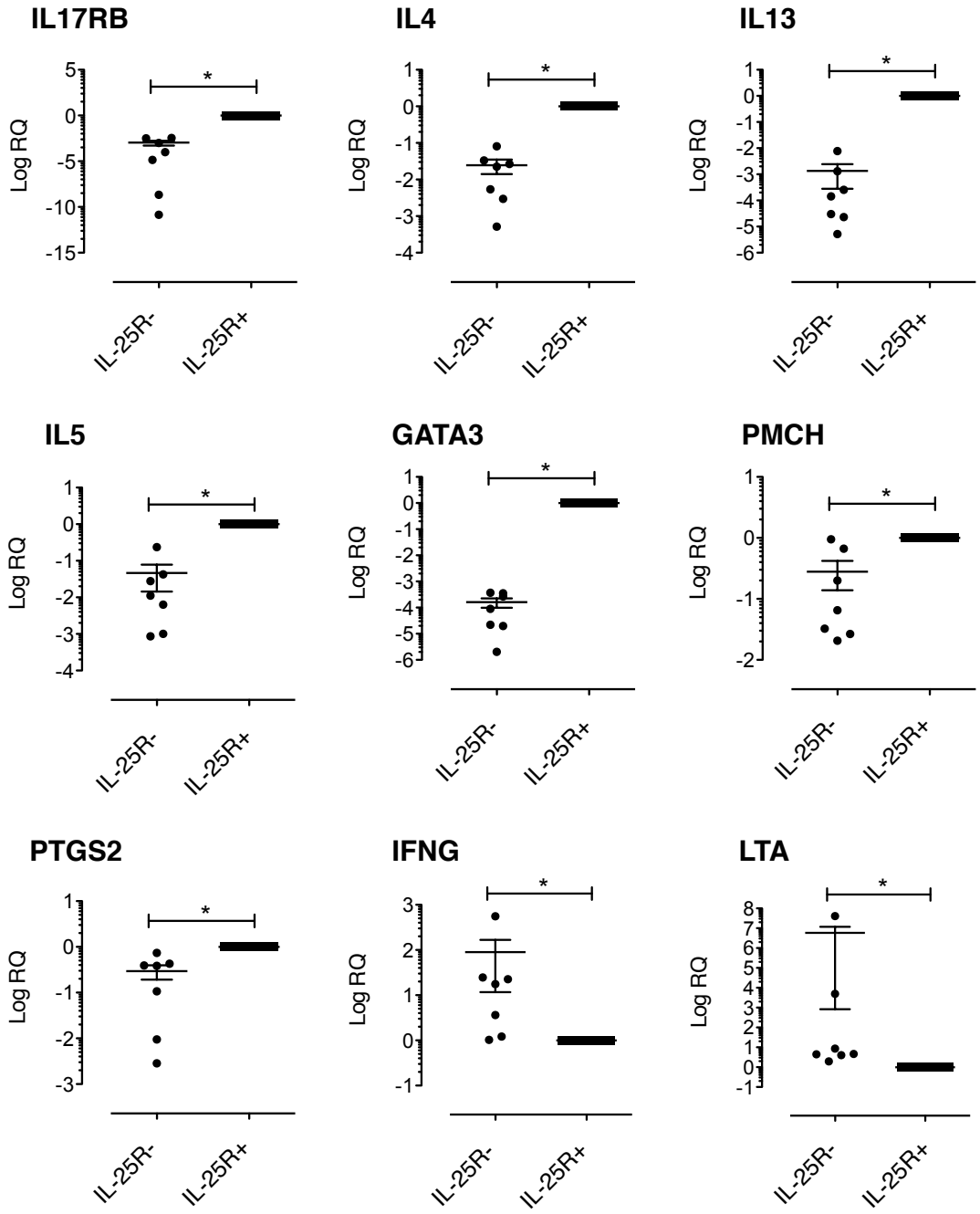


Figure 4.8 RT-PCR validation of gene expression array data.

RNA was extracted from resting or activated IL-25R$^{+/-}$ cells. Expression of Th1-related genes (*IFNG*, *LTA*) and Th2-related genes (*IL17RB*, *IL4*, *IL13*, *IL5*, *GATA3*, *PMCH*, *PTGS2*) was examined with TaqMan primer/probe sets (n=7 per group). Wilcoxon matched-pairs signed rank test. *, $p<0.05$.

4.2.4 Expression of the IL-33 receptor

Expression of the IL-25 receptor in ILC2s is associated with expression of ST2, the receptor for IL-33 (Mjosberg *et al.*, 2011, Shaw *et al.*, 2013). In addition, ST2 has been reported to be important for Th2-type immune responses (Xu *et al.*, 1998, Schmitz *et al.*, 2005). As IL-25R^{+} polyp-derived cells were of a Th2 phenotype, the expression of ST2 was also examined in resting and activated CD4^{+}IL-25R$^{+/-}$ T cells sorted from polyp explant cultures. ST2 is expressed in transmembrane (TM) and soluble isoforms (sST2) and both transcripts were examined by qRT-PCR.

The highest relative expression of transmembrane ST2 and sST2 was detected in activated IL-25R^{+} cells. Minimal or no expression was detected in resting IL-25R^{-} or resting IL-25R^{+} cells (Figure 4.9a). Furthermore, expression of transmembrane ST2 was significantly higher in activated IL-25R^{+} cells compared to activated IL-25R^{-} cells ($p<0.05$). This pattern was also observed for the sST2 isoform ($p<0.05$).

Relative amounts of TM ST2 and sST2 transcripts were determined by expressing detected mRNA levels relative to the amount of 18s ribosomal RNA, the endogenous control (Figure 4.9b). Analysis of the two transcripts showed that sST2 was the more abundant of the two ST2 isoforms and sST2 mRNA expression was significantly higher compared to TM ST2 mRNA ($p<0.05$, Wilcoxon matched-pairs signed rank test). Collectively, these data show that ST2 is expressed predominantly by IL-25R^{+} T cells derived from polyp explants and thus, ST2 and IL-25R co-expression is observed not only in ILC2s but also in Th2 cells.

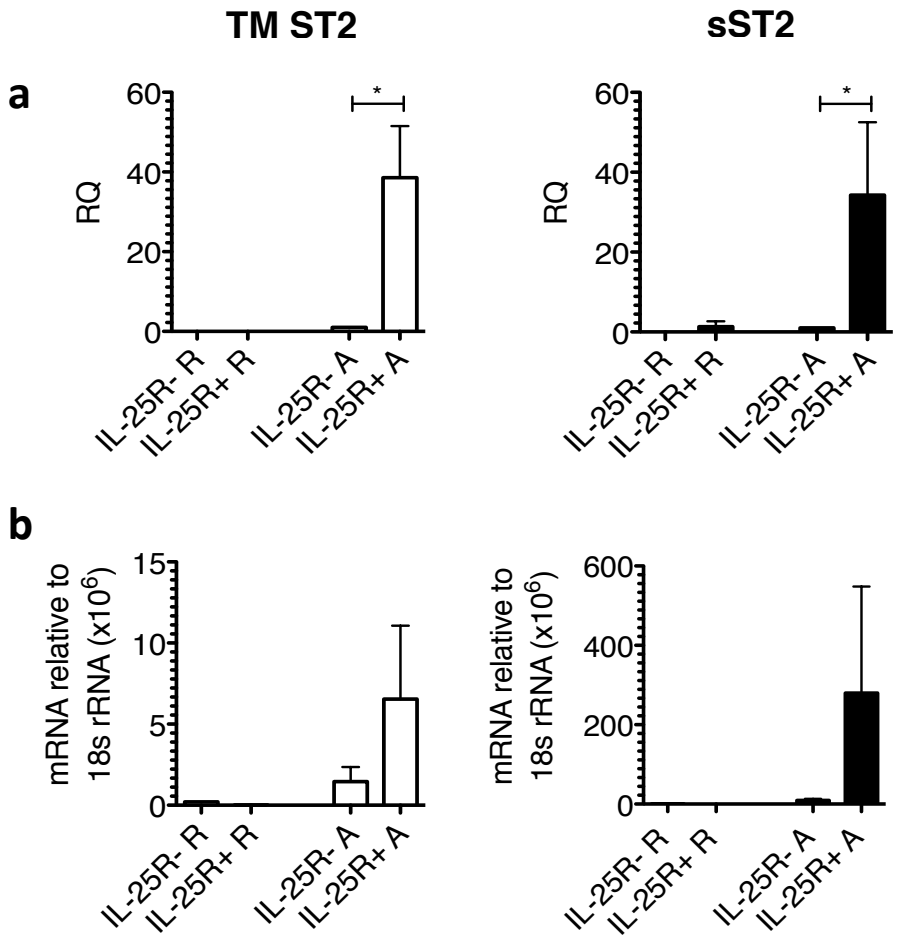


Figure 4.9 Cells expressing IL-25R also express the IL-33 receptor ST2.

(a) Expression of the transmembrane (TM ST2) and soluble (sST2) forms of ST2 was analysed with TaqMan primer/probe sets (n=4). (b) Relative amounts of ST2 mRNA relative to 18s rRNA for TM ST2 and sST2 is shown. Wilcoxon matched-pairs signed rank test. *, $p<0.05$.

4.2.4.1 IL-25R and ST2 expressed by polyp-derived cells are functional

To test the functionality of IL-25R and ST2, polyp-derived cells were cultured and expanded in the presence of anti-CD3/CD28 antibodies. Recombinant human (rh) IL-25 was added to T cell cultures on day 7 post stimulation, which were then cultured for a further 7 days in the presence of anti-CD3/CD28 antibodies before intracellular cytokine staining analysis. Blood-derived cells were also analysed in parallel for comparison.

Representative intracellular cytokine staining data for $CD4^{+}IL\text{-}25R^{+}$ T cells derived from blood and polyp explant cultures is shown in Figure 4.10. Addition of rhIL-25 increased the percentages of IL-5 and IL-13 producing $IL\text{-}25R^{+}$ cells from both polyp explant and blood-derived cultures. The overall percentages of IL-5 and IL-13 producing $IL\text{-}25R^{+}$ cells observed in blood-derived cells were approximately 10-fold lower than polyp-derived cells in accordance with the low IL-25R expression detected in peripheral blood. No difference was found in the percentage of IL-17 producing cells indicating that the response of $IL\text{-}25R^{+}$ cells to rhIL-25 is limited to Th2 cytokines.

As $IL\text{-}25R^{+}$ cells expressed ST2, the ability of rhIL-33 to potentiate Th2 cytokine production was also investigated. Furthermore, because initial experiments involved the addition of rhIL-25 at a late stage of short-term culture, the experiment was repeated with recombinant cytokine supplementation either on day 0 or on day 7. Intracellular cytokine staining analysis was subsequently performed 7 days post recombinant cytokine addition.

The percentage of $IL\text{-}5^{+}IL\text{-}25R^{+}$ cells in polyp-derived cells showed a significant increase of approximately 1.5-fold upon rhIL-25 addition compared to polyp-derived cells cultured in the presence of medium alone ($p<0.01$) (Figure 4.11a). The proportion of IL-5 producing cells was also significantly elevated in the presence of rhIL-33 (1.3-

fold; $p<0.05$). Furthermore, the percentages of IL-13^{+}IL-$25R^{+}$ cells were similarly increased in the presence of rhIL-25 and rhIL-33 by 1.4-fold ($p<0.01$) and 1.2-fold ($p<0.01$) respectively. Blood-derived cells showed the same pattern of expression with significant increases in the number of IL-5^{+}IL-$25R^{+}$ cells by approximately 1.4-fold upon the addition of rhIL-25 ($p<0.05$) and rhIL-33 ($p<0.05$). A trend increase was observed for IL-$25R^{+}$ IL-13 producing cells (rhIL-25; $p=0.08$, rhIL-33; $p=0.16$). The similar fold changes induced by rhIL-25 and rhIL-33 suggested that both cytokines had comparable effects on Th2 cytokine potentiation. Moreover, addition of rhIL-25 was also able to potentiate Th2 cytokine production in the small number of IL-$25R^{+}$ cells observed in the periphery.

The time of recombinant cytokine addition did not appear to affect the ability of rhIL-25 or rhIL-33 to potentiate Th2 cytokine production (Figure 4.11b). Analysis of intracellular cytokine staining data solely from cultures with rhIL-25 addition on day 7 showed that the enhanced percentages of Th2 cytokine producing cells was maintained in both blood and polyp-derived cells (IL-5: polyp; $p<0.01$, blood; $p<0.05$, IL-13: polyp; $p<0.01$, blood; $p<0.05$). Analysis of data exclusively from cultures with rhIL-33 addition on day 7 showed that trend increases were still observed in the percentages of IL-5 producing cells from blood ($p=0.06$) and polyp cultures ($p=0.09$). Furthermore, a significant increase in the percentage of IL-13 producing cells derived from polyp explants was observed upon rhIL-33 addition ($p<0.05$).

Taken together, these results indicate that IL-25R and ST2 are functional and are able to respond to their respective cytokine ligand *in vitro* to potentiate Th2 cytokine production.

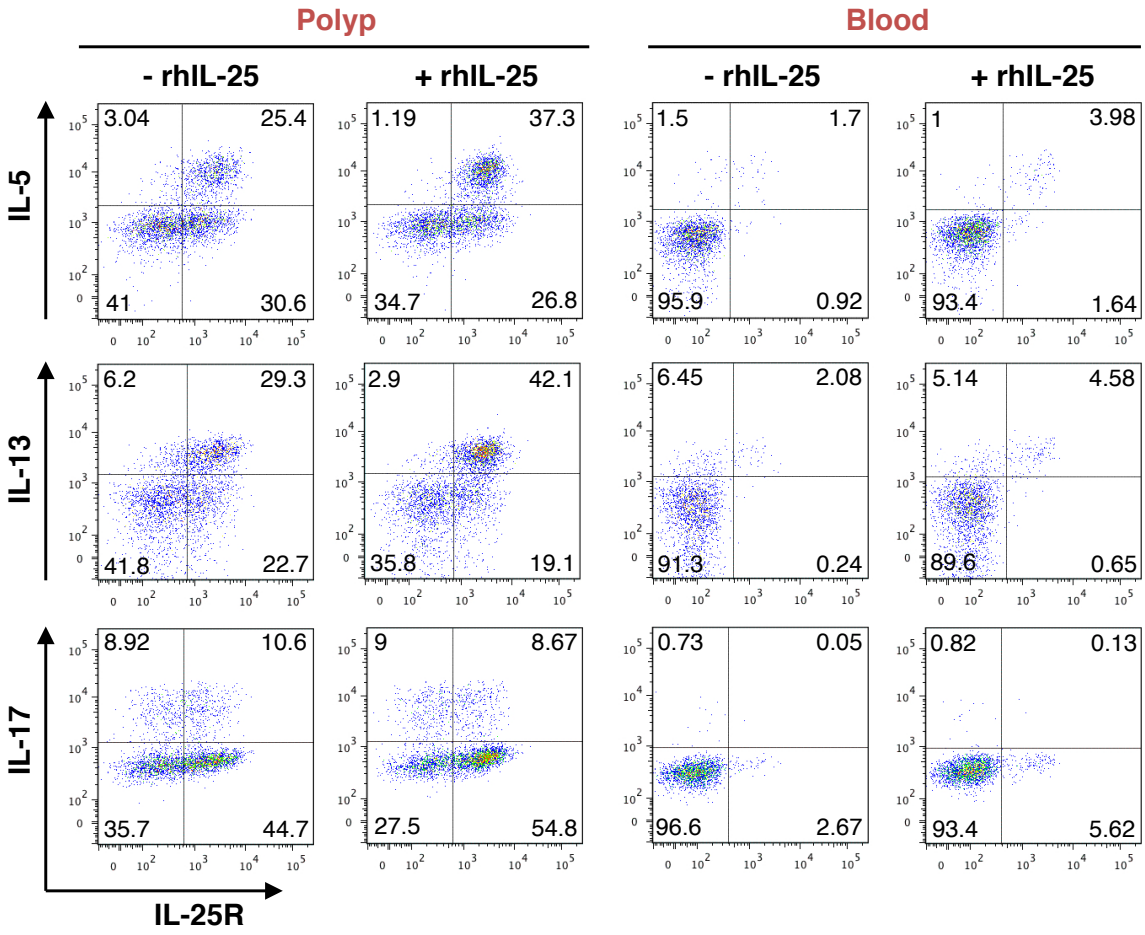


Figure 4.10 IL-25R$^+$ cells are functional and respond to recombinant IL-25 *in vitro*.

Polyp- and blood-derived cells were restimulated with anti-CD3/CD28 on day 7 of culture in the presence/absence of recombinant human IL-25 (125 ng/ml). Cells were cultured for a further 7 days before analysis of intracellular cytokine production. Representative flow cytometry staining data for 8 individual experiments is shown.

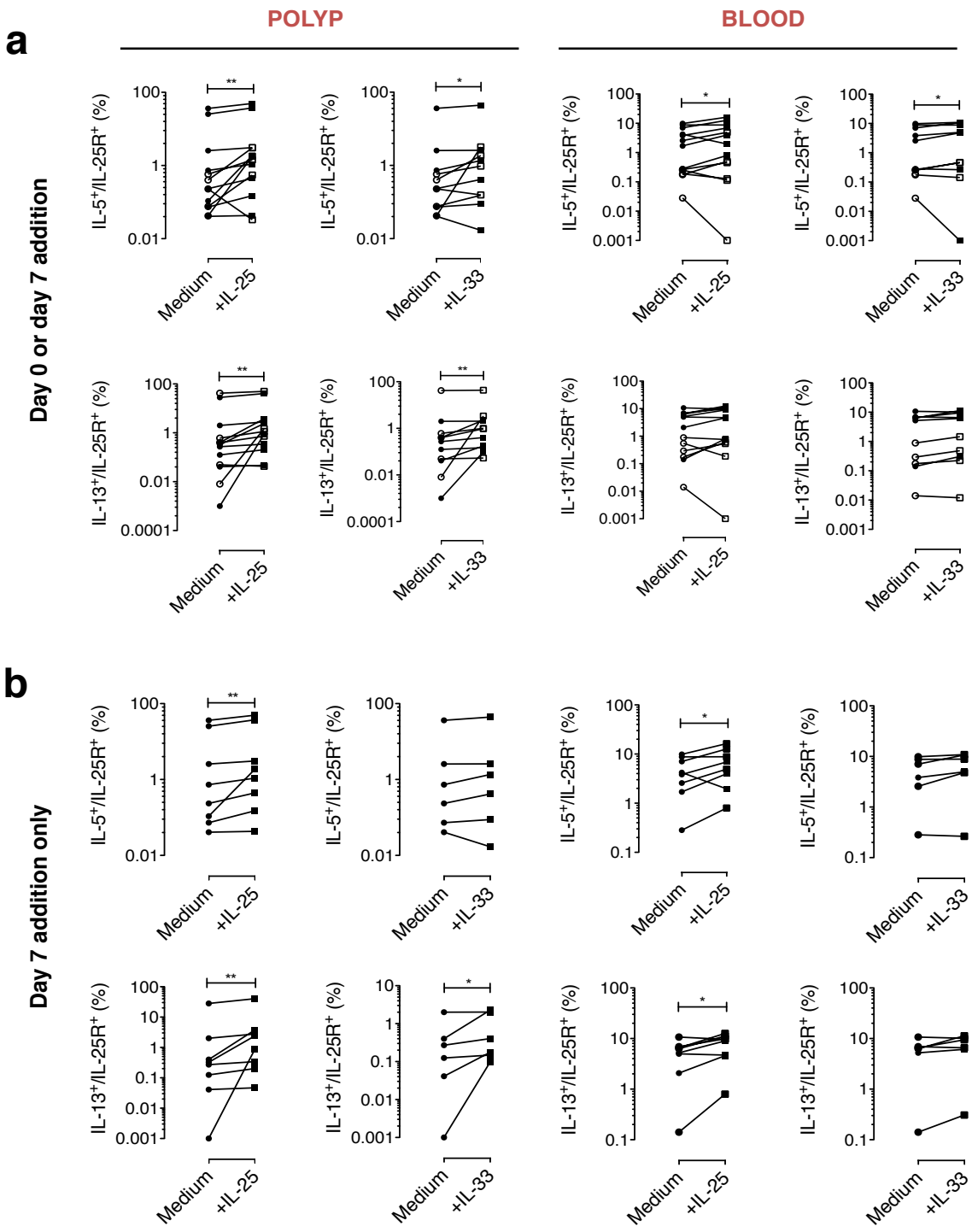


Figure 4.11 Addition of IL-25 or IL-33 potentiates Th2 cytokine production.

Recombinant human IL-25 or IL-33 was added to explant cultures on day 0 (IL-25, n=5; IL-33, n=6) or on day 7 post anti-CD3/CD28 stimulation (IL-25, n=8; IL-33, n=5). Analysis was performed 7 days post cytokine addition. Percentages of IL-5^+IL-$25R^+$ cells or IL-13^+IL-$25R^+$ in presence/absence of IL-25/IL-33 added on day 0 (open symbols) and on day 7 post anti-CD3/CD28 stimulation (closed symbols) is shown in (a). Data for IL-25/IL-33 addition on day 7 only is shown in (b). Wilcoxon matched-pairs signed rank test. *, $p<0.05$, **, $p<0.01$.

4.2.5 Endogenous sources of IL-25 and IL-33

4.2.5.1 Nasal polyp epithelium and eosinophils express IL-25

The expression of IL-25 in nasal polyps was examined by immunohistochemical analysis. Healthy normal nasal biopsies were also immunostained for IL-25 to compare IL-25 expression in CRSwNP and healthy tissue. Tissue was fixed in 4% PFA and immunohistochemistry performed as described in Chapter 2, page 78.

IL-25 was expressed in the epithelium of nasal polyps (Figure 4.12a) with no expression observed in polyp tissue immunostained with isotype control. IL-25 expression was more abundant in nasal polyp epithelium, with strong staining observed in 7 out of 9 nasal polyp specimens. In contrast, weak staining was detected in the epithelium of 4 out of 9 healthy normal nasal biopsy specimens with no IL-25$^+$ cells detected in the epithelium for the remaining 5 healthy nasal biopsy specimens (Figure 4.13a). However, the diffuse nature of IL-25 staining in the epithelium precluded precise quantification for statistical analysis.

IL-25$^+$ cells were also detected in the polyp submucosa (Figure 4.12c). Cell counts of IL-25$^+$ cells in the submucosa of nasal polyp tissue and healthy normal nasal biopsies showed that nasal polyps had a significantly higher number of cells expressing IL-25 (polyp, 14.2 cells/mm^2 ± 5.3 vs. healthy biopsy, 1.7 cells/mm^2 ± 1.1; $p<0.001$) (Figure 4.12b). The cell morphology of IL-25$^+$ cells indicated that these cells were largely eosinophils (Figure 4.12c) in line with the eosinophilic Th2 phenotype observed in CRSwNP patients in western countries.

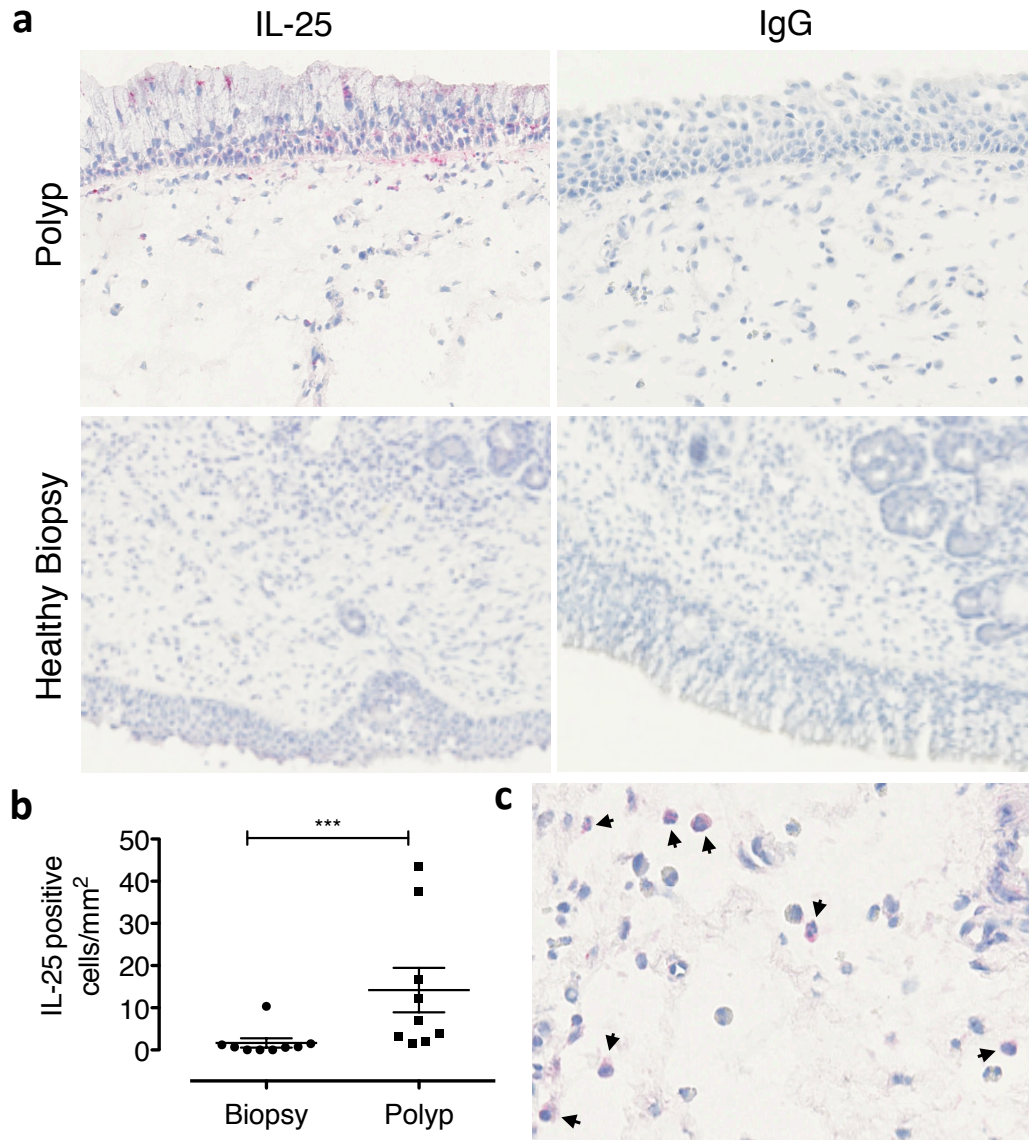


Figure 4.12 IL-25 is expressed in polyps by epithelium and submucosal eosinophils.

(a) Representative immunohistochemical staining for IL-25 in 4% PFA-fixed polyp and healthy nasal mucosal biopsies (magnification x20). Isotype control (IgG) is also shown. (b) Number of IL-25^{+} cells in the submucosa of polyp vs. normal nasal biopsies (n=9 per group). Mann Whitney test. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$. (c) Representative immunohistochemical staining for IL-25^{+} eosinophils in the submucosa of polyp tissue (magnification x40).

4.2.5.2 IL-33 is expressed by both nasal polyp and healthy nasal tissue epithelium and endothelial cells

Since polyp-derived cells expressed ST2 and responded to IL-33 *in vitro,* expression of IL-33 in nasal polyp tissue was also examined by immunohistochemistry. Healthy normal nasal biopsy tissue was stained in parallel for comparison.

IL-33 immunostaining was observed in nasal polyp epithelium, which was not observed with an IgG isotype control (Figure 4.13a). In addition, IL-33 immunostaining was also detectable in the epithelium of healthy normal nasal biopsies. Cell counting and statistical analysis was once again hindered by the diffuse nature of staining. However, positive IL-33 immunostaining in the epithelium was observed in 6 out of 9 nasal polyp specimens and 8 out of 9 healthy normal nasal biopsy specimens.

Besides epithelial expression of IL-33, expression of IL-33 was also detected in endothelial cells of both nasal polyp and healthy nasal biopsy tissue (Figure 4.13b). This was observed in 6 out of 9 healthy normal nasal biopsies and 5 out of 9 nasal polyp specimens. Overall, these data suggest that expression of IL-25 in eosinophils and the epithelium is specific to CRSwNP whilst IL-33 is expressed constitutively in the epithelium and endothelium by healthy nasal tissue.

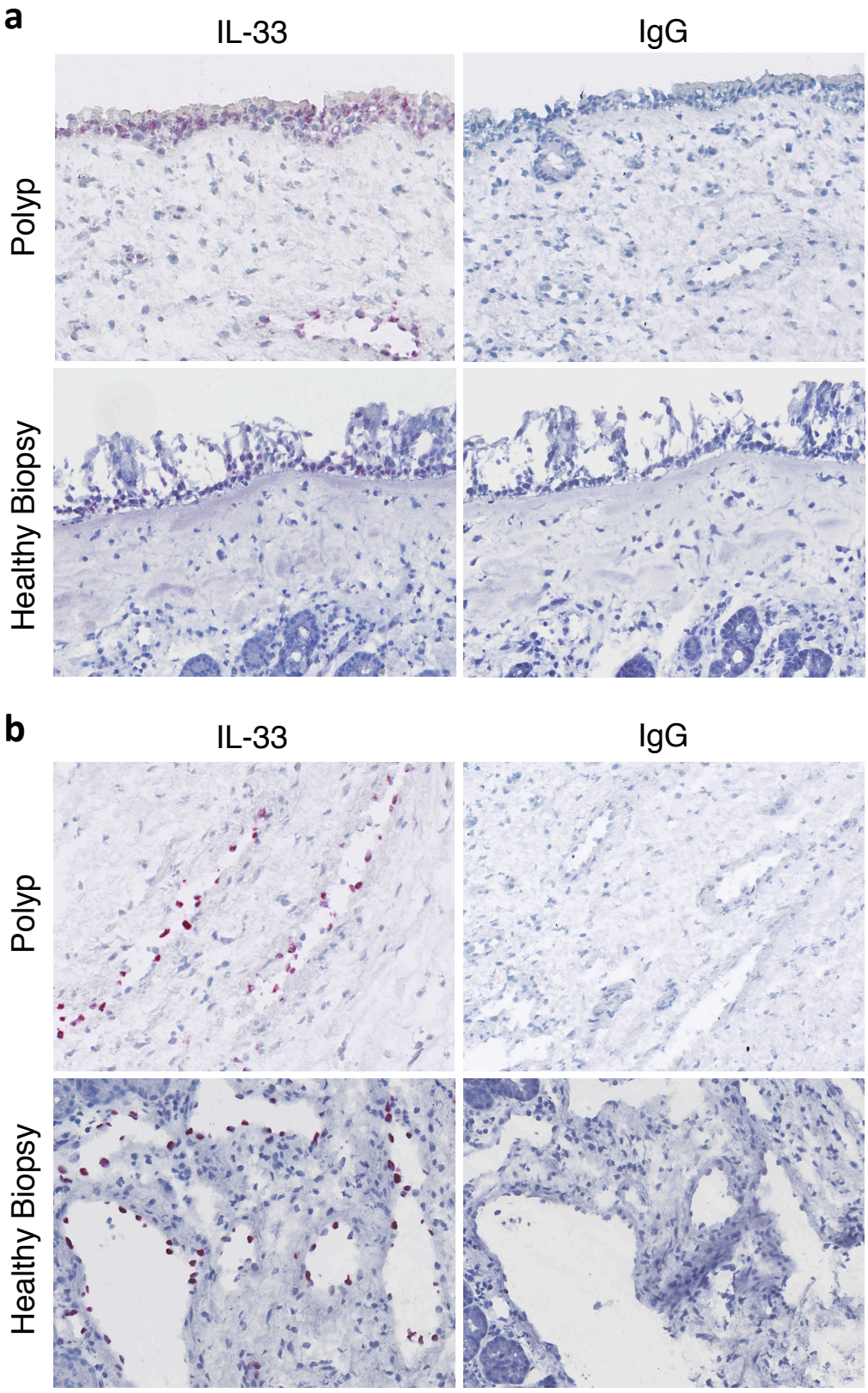


Figure 4.13 IL-33 is expressed by epithelial and endothelial cells.

Representative immunohistochemical staining for IL-33 in (a) epithelium and (b) endothelial cells of nasal polyp and normal nasal mucosal biopsies (n=9). Control IgG staining is also shown (magnification x20).

4.2.6 IL-25R$^+$ cells exhibit common TCR clones

Data presented in this thesis suggests that CD4$^+$IL-25R$^+$ cells are an abundant population in polyp explant cultures and display a pathogenic Th2 phenotype. The TCR repertoire of IL-25R$^+$ cells was therefore examined to determine if this cell population recognised a common antigen that could potentially be involved in CRSwNP pathogenesis. Analysis of the TCR variable beta (Vβ) repertoire of CD4$^+$IL-25R$^{+/-}$ cells was performed with the immunoSEQ assay (Chapter 2, page 90). The usage of TCR Vβ families, as a measure of TCR diversity, and individual nucleotide sequences of the CDR3 regions were obtained.

Skewing of the TCR Vβ repertoire was not detected with low clonality scores reported for both IL-25R$^+$ and IL-25R$^-$ cells from the four CRSwNP patients analysed (Table 4.2). However, sequencing of the CDR3 region revealed that IL-25R$^+$ cells had a smaller number of unique clones compared to IL-25R$^-$ cells in all four CRSwNP patients analysed (Figure 4.14a). In addition, the percentages of shared sequences were minimal at 1% or less for all samples (Figure 4.14b).

Examination of the unique clones detected in the IL-25R$^+$ population revealed that although a smaller number of unique clones were present compared to IL-25R$^-$ cells, two common CDR3 sequences could be identified (Table 4.2). The two sequences (CASSLNTGYEQYF and CASSYPGEAFF) were present in three out of four IL-25R$^+$ samples analysed. Furthermore, the two clones were not present in the same three individuals. Expression of one of the sequences, CASSLNTGYEQYF, was detected in a single IL-25R$^-$ sample. Together, these results raise the possibility that IL-25R$^+$ T cells in polyp explants could be recognising common epitopes on antigen(s) that may potentially be involved in CRSwNP pathogenesis.

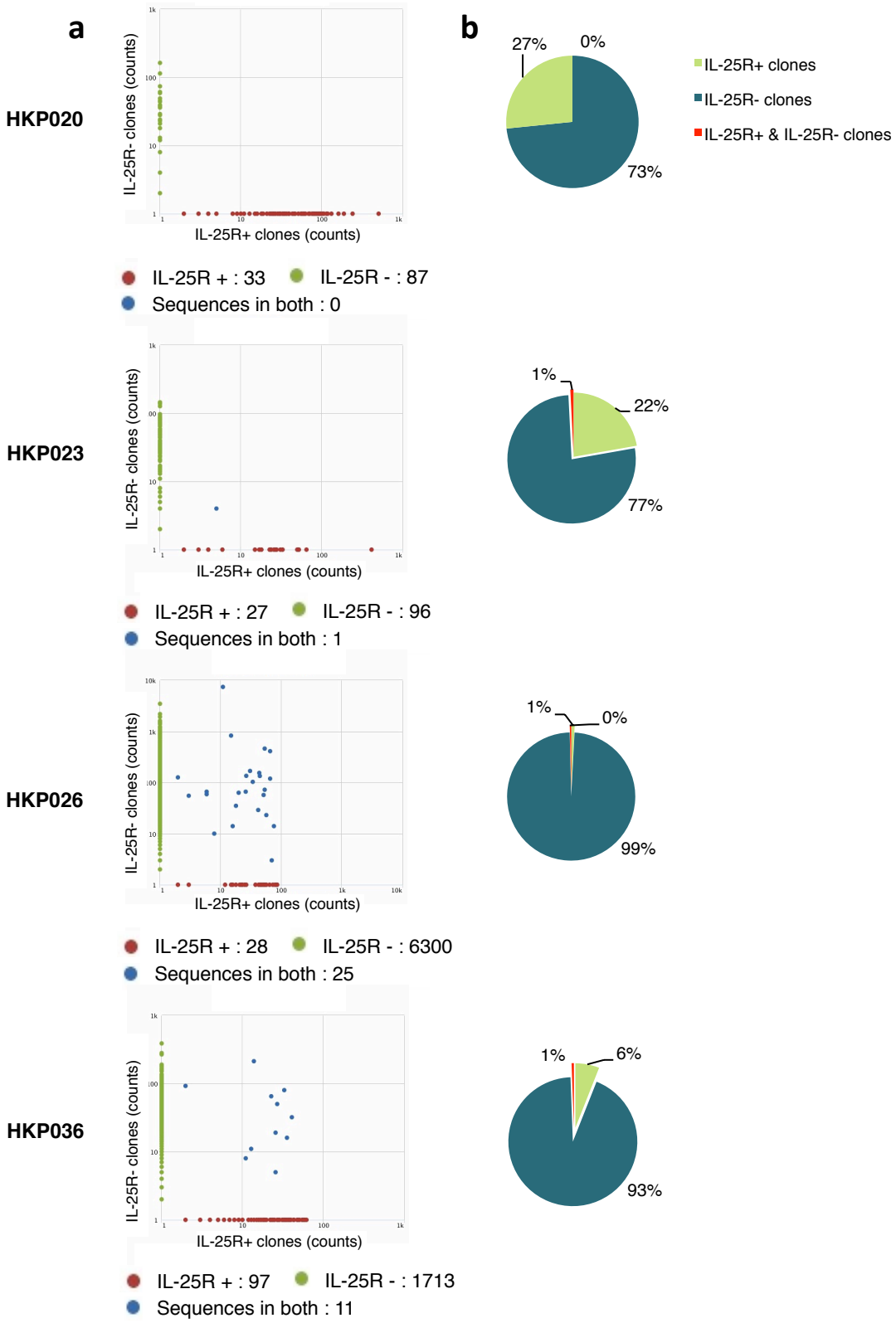


Figure 4.14 IL-25R$^+$ and IL-25R$^-$ cells have a diverse TCR Vβ repertoire.

DNA from sorted CD4$^+$IL-25R$^+$ and CD4$^+$IL-25R$^-$ polyp-derived cells was isolated and TCR Vβ repertoire analysed by immunoSEQ assay (n=4). (a) Number of unique CDR3 sequences in the IL-25R$^+$, IL-25R$^-$ or shared by both populations is shown for each CRSwNP patient. (b) The percentage of sequences in each population is shown.

Table 4.2 Clonality and common clones in IL-25R$^+$ and IL-25R$^-$ populations.

	HKP020		HKP023		HKP026		HKP036	
	IL-25R+	IL-25R-	IL-25R+	IL-25R-	IL-25R+	IL-25R-	IL-25R+	IL-25R-
Clonality	0.105	0.124	0.294	0.071	0.073	0.079	0.054	0.048
Total clones (productive)	4871	1146	969	3801	1896	443183	2435	47486
Common clones								
CASSLNTGYEQYF	+	-	+	+	+	-	-	-
CASSYPGEAFF	+	-	+	-	-	-	+	-

The immunoSEQ analyser software assigned a clonality value for each sample. Clonality was expressed on a scale from 0, diverse repertoire, to 1, clonal repertoire. The total number of productive clones detected is shown. Two common clones were identified in the IL-25R$^+$ cell population in three out of four CRSwNP patients.

4.3 Discussion

Although the presence of IL-25R$^+$ cells in CRSwNP has been previously reported, these studies have focused on the ILC2 population (Mjosberg *et al.*, 2011, Miljkovic *et al.*, 2014). The aim of this chapter was to perform an in-depth characterisation of the phenotype and gene expression profile of IL-25R$^+$ T cells derived from nasal polyps compared to IL-25R$^-$ T cells. Furthermore, as limited literature exists on the interaction of the epithelial cell-derived cytokines IL-25 and IL-33 with local nasal mucosal T cells, this was also explored. The TCR Vβ repertoire of IL-25R$^+$ cells derived from polyp explants was also compared to that of IL-25R$^-$ cells.

4.3.1 Distinct expression of Th2-associated surface markers and cytokines by IL-25R$^+$ cells

At the time of experimental work, the data presented in this thesis was the first known demonstration of *ex vivo* IL-25R$^+$ Th2 cells from human nasal tissue associated with active disease. Subsequently, two studies have been published which demonstrate the existence of IL-25R$^+$ T cells in nasal polyps. Th2 cells and purported Th9 cells (defined as IL-5$^-$IL-9$^+$ cells), as well as an IL-25R$^+$CD4$^+$ population, were described in eosinophilic CRSwNP tissue homogenates by Iinuma *et al.* (2015). The authors also showed that IL-25R mRNA correlated with eosinophil counts. However, direct co-localisation of IL-25R with Th2 or Th9 cells was not presented. Moreover, although another study by Shin *et al.* (2015) similarly demonstrated the existence of IL-25R$^+$ cells in Asian CRSwNP patients, the cell type and phenotype were not examined. The data presented in this thesis is therefore novel and distinct in that an in-depth characterisation of the IL-25R$^+$ cell population derived from polyp explants was performed.

The lack of Th1 cytokine production by $CD4^{+}IL\text{-}25R^{+}$ polyp-derived cells is reflective of the minimal co-localisation observed between the Th1 surface marker CXCR3 and IL-25R (Figure 4.2). Minimal co-localisation was also observed for polyp-derived IL-$25R^{+}$ cells with the Th17 marker CCR6. Notably, over 50% of the IL-$25R^{+}$ population showed co-expression with the Th2 marker CRTH2. This Th2 specific phenotype was also reflected in the cytokine expression profiles of IL-$25R^{+}$ vs. IL-$25R^{-}$ cells. However, not all IL-$25R^{+}$ cells co-expressed CRTH2 and IL-$25R^{+}$ cells did not account for all Th2 cytokines produced by T cells; approximately 40% of IL-5 and IL-13 producing cells were IL-25R negative. These may have been $CRTH2^{+}IL\text{-}25R^{-}CD4^{+}$ T cells although this was not evaluated. However, data presented in the previous chapter showed that the percentages of Th2 cytokine expressing T cells were more strongly correlated with IL-25R than CRTH2 expression (Figure 3.13j). Expression of CRTH2 was also lower, on average, compared to IL-25R expression in polyp-derived cells (Figure 3.5) tentatively suggesting that IL-$25R^{+}$ cells may play a more central role in the pathogenesis of CRSwNP than $CRTH2^{+}$ cells. Further work will be required to validate this observation.

ILC2 are capable of producing IL-13 and also express the CRTH2 receptor (Mjosberg *et al.*, 2011, Shaw *et al.*, 2013). The ILC2 population may therefore also contribute to the Th2 cytokine response in CRSwNP. However, the relative contributions of ILC2 and Th2 cells to the Th2 cytokine response remains unknown as the majority of studies in the literature focus on either the ILC2 or Th2 cell population but rarely both in conjunction. Indeed, studies have only recently begun to address the distinct roles played by the two cell populations in allergic asthma (Licona-Limon *et al.*, 2013, Lambrecht *et al.*, 2015). Early attempts to identify ILC2 in this project were unsuccessful and consequently, cytokine expression by ILC2 was not examined. Further studies will be required to assess the relative roles of ILC2 and Th2 cells in CRSwNP pathogenesis.

In summary, these data indicate that IL-25R$^+$ cells are of a Th2 phenotype. IL-25R$^+$ cells selectively produced the majority of Th2 cytokines with negligible Th1/Th17 cytokine production observed. This accords with the correlation presented in Chapter 3 between Th2 cytokines and IL-25R expression and supports the hypothesis that IL-25R$^+$ cells are the pathogenic cells associated with Th2-mediated chronic inflammation in CRSwNP.

4.3.2 Gene expression in IL-25R$^+$ cells

IL-25R$^+$ cells derived from polyp explants exhibited a Th2 gene expression profile that was not detected in IL-25R$^-$ cells. The identification of IL-5 as the gene with the largest differential expression was in agreement with the eosinophilic Th2 phenotype of western CRSwNP patients. Furthermore, polyp-derived IL-25R$^+$ cells displayed a gene expression profile akin to *in vitro* polarised, highly differentiated Th2 cells (McDonald *et al.*, 2008, Parmentier *et al.*, 2012). Thus, data presented in this thesis thus far, with analysis of the surface phenotype expression, intracellular cytokine production and genome wide expression profile, all independently confirmed that IL-25R$^+$ cells derived from polyp explants were Th2 cells.

In the recently published study, Iinuma *et al.* (2015) identified a high percentage of IL-9 producing Th9 cells in eosinophilic CRSwNP, defined as IFNγ$^-$IL-5$^-$IL-9$^+$CD4$^+$ T cells. The authors showed that IL-25 was able to potentiate the amount of IL-9 mRNA and IL-9 protein detected in nasal polyp mononuclear cell cultures. However, whether this was specifically produced by Th9 cells was not determined and co-localisation of IL-9 with IL-25R was not shown. In this thesis, although the presence of a definitive Th9 subset was not explored, microarray analysis showed that expression of the IL-9 gene was significantly higher in IL-25R$^+$ Th2 cells compared to IL-25R$^-$ cells. Importantly, the canonical Th2 cytokines IL-4, IL-5 and IL-13 were also preferentially expressed by IL-25R$^+$ cells. As Th2 cells are known to also produce IL-9, the results presented in this thesis indicate that it is unlikely that Th9 cells are the subset expressing IL-25R and responding to IL-25 to potentiate IL-9 production, as postulated by Iinuma *et al.* (2015).

4.3.3 Gene expression in IL-25R$^-$ cells

The genes that were upregulated in the IL-25R$^-$ population were distinct from those upregulated in the IL-25R$^+$ population. Th1/Th17-associated genes were upregulated in

IL-25R$^-$ cells reflecting the abundant Th1 and Th17 signature observed in polyp-derived cells, as discussed in Chapter 3. Although no differential expression of *IL17A* was observed, the highest preferential expression detected in IL-25R$^-$ cells was for the *IFNG* gene. The lack of differential expression for the IL-17 gene may be due to the limited number of samples analysed by microarray (n=3). Examination of the heatmap derived from array data showed that there was variability in the expression pattern (upregulation and downregulation) of many genes between IL-25R$^+$ and IL-25R$^-$ cells across the three samples. This was reflected in the PCA plot in which variations within samples groups could be observed. Nevertheless, the patterns of gene expression for qRT-PCR data reproduced those seen in the microarray analysis. This was observed for cells under both resting and activated states and across all genes examined by qRT-PCR.

In addition to IFNγ, the gene for lymphotoxin-α (LTα) showed significant preferential expression by IL-25R$^-$ cells. LTα is a member of the TNF superfamily and expression by Th1 and Th17 cells has been demonstrated to play a role in animal models of autoimmune diseases (Gramaglia *et al.*, 1999, Chiang *et al.*, 2009). However, the potential role of LTα in the healthy nasal mucosa is less clear. LTα has been shown to have a possible role in the recruitment of lymphocytes in nasal-associated lymphoid tissue (NALT) by regulating the expression of peripheral lymph node addressin (PNAd) required for trafficking of CD62L$^+$ T_{CM} cells through high endothelial veins (Harmsen *et al.*, 2002, Ying *et al.*, 2005). This suggests that LTα expressing Th1/Th17 cells in the IL-25R$^-$ population might be involved in the trafficking of T_{CM} cells. The preferential expression of the gene for perforin-1 by IL-25R$^-$ cells is also suggestive of the presence of MHC class II-restricted cytotoxic CD4$^+$ T cells in the heterogeneous IL-25R$^-$ population. Perforin-1 is involved in mediating the actions of cytotoxic CD4$^+$ T cells and reported to provide protection against viral infections (Appay *et al.*, 2002, Brown *et al.*, 2012). Together, these studies indicate that the IL-25R$^-$ population derived from

polyp explants may be involved in host immune defence in the nasal mucosa as proposed in Chapter 3.

4.3.4 Expression of ST2 and the functional roles of IL-25 and IL-33

The ability of IL-25R to respond directly to IL-25 *in vitro* to potentiate Th2 cytokine production from peripheral blood cells has been demonstrated (Wang *et al.*, 2007, Terrier *et al.*, 2010). Furthermore, a recent publication has also shown that IL-25 is able to dose-dependently potentiate IL-5 production in nasal polyp mononuclear cells (Iinuma *et al.*, 2015). The results presented in this thesis are therefore in agreement with these published studies. However, whereas Iinuma *et al.* (2015) examined the overall expression of IL-5 mRNA and protein by nasal polyp mononuclear cells, the data presented in this thesis specifically analysed $CD4^{+}$IL-$25R^{+}$ T cells and demonstrated potentiation of Th2 cytokine production with this cell population. Moreover, although another recent study by Shin *et al.* (2015) similarly demonstrated the existence of IL-$25R^{+}$ cells in Asian CRSwNP patients, the cell type was not examined. The data presented in this thesis is therefore novel and distinct. Furthermore, the ability of IL-$25R^{+}$ cells to respond to IL-25 was found to be independent of the time of recombinant IL-25 addition to polyp explant-derived cultures. This suggests that polyp explant-derived IL-$25R^{+}$ T cells display a stable Th2 phenotype under neutral culture conditions.

The expression of both transmembrane and soluble isoforms of ST2 by activated IL-$25R^{+}$ cells is believed to be the first demonstration in human disease. Although both isoforms were elevated in activated IL-$25R^{+}$ cells, transcripts of the decoy receptor sST2 were more abundant. Elevated sST2 levels have previously been demonstrated in other Th2 mediated diseases. For example, sST2 levels were higher in the sera of asthmatic and allergic rhinitis patients compared to healthy controls and expression of sST2 correlated with the severity of asthma exacerbations (Oshikawa *et al.*, 2001, Baumann *et al.*, 2013). The results presented in this thesis are therefore consistent with these studies. Elevated sST2 expression in polyp-derived cells may indicate an anti-

inflammatory response and sST2 may act in conjunction with IL-10, of which higher levels were detected in polyp explants (Chapter 3), to counteract and inhibit the Th2 mediated inflammation in CRSwNP.

A recent publication has demonstrated that IL-33 is able to increase Th2 cytokine and ST2 mRNA expression by memory T cells from nasal polyps (Endo *et al.*, 2015). The data presented in this thesis is in agreement with the results of Endo *et al.* (2015) and expand on those findings to show that $ST2^{+}IL\text{-}25R^{+}$ Th2 cells were responsible for responding to IL-33 to potentiate Th2 cytokine production. Furthermore, similar to the results for IL-25, addition of recombinant IL-33 did not enhance the numbers of $IL\text{-}17^{+}IL\text{-}25R^{+}$ Th2 cells detected indicative of its Th2 specific effect.

4.3.4.1 Endogenous sources of IL-25 and IL-33

The numbers of $IL\text{-}25^{+}$ cells were significantly higher in the submucosa of nasal polyp tissue compared to healthy nasal mucosal tissue. The observation that IL-25 is produced by eosinophils is in agreement with several previously published studies (Wang *et al.*, 2007, Terrier *et al.*, 2010, Iinuma *et al.*, 2015). IL-25 has also been reported to be predominantly produced by epithelial cells in nasal polyps from Asian CRSwNP patients (Shin *et al.*, 2015). This finding was further extended in this thesis with the demonstration that elevated numbers of $IL\text{-}25^{+}$ cells were observed in the epithelium of polyp vs. healthy nasal mucosal tissue. Thus, these results suggest that in addition to the higher number of $IL\text{-}25R^{+}$ Th2 cells present in nasal polyps, the number of cells producing IL-25 were also elevated in nasal polyps.

IL-33 is reported to be constitutively expressed in endothelial and epithelial cells and is postulated to act as an alarmin upon cell or tissue damage (Moussion *et al.*, 2008, Küchler *et al.*, 2008). Expression of IL-33 was detected in endothelial cells of nasal polyp tissue corroborating the findings of Endo *et al.* (2015). Furthermore, IL-33

staining was also observed by immunohistochemistry in endothelial cells of healthy control nasal tissue in accordance with constitutive expression. However, these results are at best semi-quantitative and do not exclude the possibility that under pro-inflammatory stimuli IL-33 expression may be higher. Expression of IL-33 was demonstrated to be increased in bronchial endothelial cells of mice in a cigarette smoke-induced chronic obstructive pulmonary disease (COPD) model (Wu *et al.*, 2014) and expression was reported to be enhanced in $CD31^+$ endothelial cells of lesional skin from chronic spontaneous urticaria patients compared to non-lesional skin (Kay *et al.*, 2015). Thus, these studies suggest that endothelial cells in nasal polyps may also respond to allergen or stimulus challenge to increase the expression of IL-33 in CRSwNP.

The role of IL-33 as an alarmin released by epithelial cells was validated in a study by Paris *et al.* (2014) who demonstrated that DAMP molecules such as ATP and HMGB-1 were able to induce IL-33 expression from polyp epithelial cells. In this thesis, IL-33 was detected in the epithelium of both nasal polyps and healthy control nasal tissue. However, the staining of IL-33 in epithelial cells was generally more dispersed in polyp tissue compared to healthy nasal mucosal tissue - staining was observed on the apical surface of epithelial cells in addition to nuclei localisation. Furthermore, $IL\text{-}33^+$ epithelial cells were observed in a smaller percentage of nasal polyp samples compared to healthy control nasal tissue. This suggests that active IL-33 secretion may have led to the lack of positive staining for IL-33 in epithelial cells of nasal polyp tissue. Moreover, the pattern of staining observed was comparable to that of epithelial cells challenged with DAMP molecules (Paris *et al.*, 2014) lending support to this hypothesis. In addition, IL-33 mRNA levels were also reported to be higher in epithelial cells from recalcitrant polyps compared to treatment responsive polyps (Reh *et al.*, 2010). Overall, these data suggest that IL-25 and IL-33 levels are elevated in CRSwNP and $IL\text{-}25R^+ST2^+$ Th2 cells are able to respond to the higher levels of their respective cytokine

ligand to exacerbate disease. Indeed, expression of IL-25 and IL-33 mRNA were reported to be higher in sinus mucosal samples from CRSwNP patients compared to CRSsNP patients or healthy controls and IL-25 mRNA expression was also correlated with poorer CT scores (Lam *et al.*, 2013).

The exact mechanism of IL-25 and IL-33 release by epithelial cells in nasal polyps remains to be determined. As discussed above, postulated mechanisms of release by epithelial cells include tissue damage as well as allergen stimulation although this was not examined in this study due to the lack of a known antigen. Nonetheless, numerous studies published in the literature have demonstrated the allergen-induced release of IL-25 and IL-33 from epithelial cells in other diseases. Normal HBECs have been shown to upregulate the transcription and release of IL-25 upon stimulation with house dust mite in a protease-activated receptor 2 (PAR2) dependent manner (Kouzaki *et al.*, 2013). In addition, in a mouse model *Alternaria* induced the release of IL-33 from airway epithelial cells in a mechanism that appeared to be dependent on protease activity (Snelgrove *et al.*, 2014). Furthermore, *Aspergillus fumigatus* – shown to induce the release of the pro-inflammatory cytokines IL-6 and IL-8 from human airway epithelial cell lines through its serine protease activity (Tomee *et al.*, 1997) – has recently been reported to increase levels of IL-33 mRNA in epithelial cells of CRSwNP but not CRSsNP patients (Shaw *et al.*, 2013). These studies raise the possibility that the epithelium in nasal polyps from CRSwNP patients could also respond to exogenous triggers (especially proteases) in a similar manner to induce the release of IL-25 and IL-33. Indeed, *S. aureus*, the commensal bacteria with high colonisation rates in CRSwNP patients and hypothesised to be involved in CRSwNP pathogenesis (discussed in Section 4.3.5) (Van Zele *et al.*, 2004, Bachert *et al.*, 2008, Gevaert *et al.*, 2013b) contains the extracellular V8 protease (Drapeau *et al.*, 1972, Hirasawa *et al.*, 2010)

although whether this is able to interact with epithelial cells to induce IL-25 and IL-33 release is currently unknown.

Taken together, these results indicate that in addition to ILC2, Th2 cells are also able to respond to IL-25 and IL-33 to potentiate Th2 cytokine production in CRSwNP. IL-25R^{+}ST2^{+} Th2 cells and the IL-25/IL-33 signalling pathways could therefore be attractive therapeutic targets for the treatment of CRSwNP.

4.3.5 TCR Vβ repertoire analysis of IL-25R$^+$ cells

TCR Vβ repertoire analysis showed that minimal CDR3 sequences were shared between IL-25R$^+$ and IL-25R$^-$ cells, indicating that the two cell populations had distinct CDR3 regions for the recognition of distinct antigens. Clonality scores showed that skewing of the TCR repertoire was not observed however. This may be due to the limited number of samples analysed (n=4). Previous studies have shown that even in larger cohorts, skewing of Vβ domains associated with superantigens was not observed in all patients but only in a small proportion (7 out of 20) (Conley *et al.*, 2006a). The identification of common CDR3 clones in the IL-25R$^+$ population, although preliminary, raises the possibility of a superantigen and/or antigen driven inflammatory phenotype in CRSwNP.

The Vβ domain expressing the CDR3 sequence, CASSLNTGYEQYF, was reported by the ImmunoSEQ analyser software to belong to the Vβ5.2 family. CLA$^+$ cells responding to the *S. aureus* superantigen SEA have been shown to preferentially express Vβ5.2 in atopic dermatitis patients (Davison *et al.*, 2000). Furthermore, the second CDR3 sequence, CASSYPGEAFF, was reported by the ImmunoSEQ analyser to be expressed by a Vβ6 family product. This Vβ family has similarly been shown to be associated with *S. aureus* superantigens. Wang *et al.* (2008) reported that polyp samples colonised by the TSST-1 producing strain of *S. aureus* had enhanced expression of Vβ6 compared to polyp samples that were negative for superantigens. Thus, these data suggest that the Vβ domains expressing the common CDR3 clones in IL-25R$^+$ cells may be superantigen related and consequently, the preferential binding of superantigens to these Vβ domains could potentially be involved in the polyclonal activation of IL-25R$^+$ cells to induce Th2-mediated inflammation in CRSwNP. However, discrepancies in the Vβ domains preferentially bound by superantigens in CRSwNP exist in the literature (Wang *et al.*, 2008, Conley *et al.*, 2006a, Thomas *et al.*,

2009). For example, Thomas and colleagues reported that TSST-1 was associated with the Vβ2 family. This may be due to the different methods of detection used. Flow cytometry and RT-PCR were used in these earlier studies to analyse TCR repertoire but these methods are much less sensitive and identify fewer members of the Vβ family compared to the ImmunoSEQ method used in this thesis. Furthermore, the exposure of patients to different strains of *S. aureus* may also have affected the TCR repertoire as superantigens are produced in a strain-specific manner (Dinges *et al.*, 2000).

The conventional view is that superantigens bind and link MHC class II molecules with the TCR independently of the peptide-binding groove (Proft *et al.*, 2003). The identification of common CDR3 sequences in IL-25R$^+$ cells therefore suggests that the T cell response in CRSwNP involves antigen specificity. However, numerous studies in the literature have shown that superantigens interact and bind to residues of the bound peptide in the MHC/TCR complex. Kim *et al.* (1994) reported the crystal structure of the TSST-1/HLA-DR1 complex and showed that TSST-1 contacted the bound peptide over the majority of the peptide surface. The authors concluded that TSST-1 binding was partially peptide dependent. Furthermore, *Streptococcal* pyrogenic exotoxin C (SPE-C) was shown to complex with HLA-DR2a in a zinc-dependent manner with extensive contact detected between SPE-C and the bound peptide (Li *et al.*, 2001). This was also observed for SEH bound to HLA-DR1 and the binding of all zinc-dependent superantigens, including SEA, was postulated to be similar (Petersson *et al.*, 2001). Thus, these studies indicate that the preferential binding of superantigens to the MHC/TCR complex is not only dependent on the Vβ domain expressed but may also show some specificity for the bound peptide. Thus, the common CDR3 sequences observed in IL-25R$^+$ cells may be related to this superantigen/peptide interaction.

The identification of common CDR3 sequences is also suggestive of the recognition of a common antigen(s) by IL-25R$^+$ cells. Skin prick testing of the four CRSwNP patients

showed that these patients were not sensitised to the same aeroallergens, thus any shared antigen is unlikely to be a common allergen. In preliminary analysis of the common sequences performed by Dr. Anna Davies (Randall Division of Cell and Molecular Biophysics, King's College London) utilising the Protein Data Bank, no matches with known TCR/peptide complexes were identified. Extensive molecular modelling was required to solve the protein structure via crystallography, in addition to computational methods (Brusic *et al.*, 2004), to evaluate the compatibility of peptide candidates to bind to the peptide groove. This was not pursued further in this thesis due to time constraints.

Further studies will be required to determine the exact antigen and/or superantigen that are recognised by these TCR-bearing IL-25R$^+$ cells. These preliminary results suggest that IL-25R$^+$ cells may be recognising common epitopes which could in turn, drive the expansion of TCR-bearing IL-25R$^+$ cells to potentiate IL-25-mediated Th2 inflammation.

4.4 Summary

In this chapter, $CD4^+IL\text{-}25R^+$ cells derived from nasal polyp explants were characterised in detail. Co-localisation of IL-25R with T cell subset surface markers and intracellular cytokines was examined. The gene expression profile of sorted $CD4^+IL\text{-}25R^+$ cells was compared to $CD4^+IL\text{-}25R^-$ cells. Expression of ST2 and the functional effects of IL-25 and IL-33 on $IL\text{-}25R^+$ cells were also examined. Lastly, the TCR Vβ repertoire of sorted $CD4^+IL\text{-}25R^+$ cells was compared to that of $CD4^+IL\text{-}25R^-$ cells. The data presented in this chapter show that:

1. $IL\text{-}25R^+$ cells exclusively display a Th2 surface phenotype and selectively produce Th2 cytokines.
2. The gene expression profiles of $IL\text{-}25R^+$ vs. $IL\text{-}25R^-$ cells are distinct.
3. $IL\text{-}25R^+$ cells co-express ST2. Both receptors are functional and respond to IL-25 and IL-33 respectively *in vitro* to potentiate Th2 cytokine production.
4. Higher numbers of $IL\text{-}25^+$ epithelial cells and eosinophils were observed in polyp compared to normal nasal tissue. Expression of IL-33 by epithelial and endothelial cells was comparable between polyp and normal nasal tissue.
5. Common CDR3 sequences are identified in $IL\text{-}25R^+$ cells suggestive of common antigen and/or superantigen recognition.

In conclusion, local $IL\text{-}25R^+ST2^+$ Th2 cells play a pathogenic role in CRSwNP by promoting Th2-mediated inflammation. The IL-25/IL-25R and IL-33/ST2 pathways could potentially be attractive therapeutic targets in the treatment of CRSwNP.

Chapter 5 Characterisation of nasal IL-17^{+} T cells

5.1 Introduction

In 2003, Cua *et al.* established the pivotal role for IL-23 and Th17 cells in the development of experimental autoimmune encephalitis (EAE). Since then, extensive studies have shown the involvement of Th17 cells in a number of inflammatory and allergic diseases; including allergic asthma (Molet *et al.*, 2001), rheumatoid arthritis (Chabaud *et al.*, 1999) and inflammatory bowel disease (Fujino *et al.*, 2003). Indeed, IL-17 levels are significantly increased in the sputum and BAL of asthmatic patients compared to healthy controls (Molet *et al.*, 2001, Barczyk *et al.*, 2003), correlating with neutrophilia as well as disease severity (Chakir *et al.*, 2003, Zijlstra *et al.*, 2012). Elevation of IL-17 was similarly observed in allergic rhinitis and rheumatoid arthritis patients with higher IL-17 levels detected in the serum of allergic rhinitis patients (Ciprandi *et al.*, 2009) and in the synovial fluid of rheumatoid arthritis patients (Kotake *et al.*, 1999) compared to healthy volunteers.

However, primary immunodeficiency diseases such as hyper IgE syndrome (HIES) also indicate the essential role of Th17 in the host immune defence. HIES symptoms include eczema, skin abscesses, recurrent bacterial pneumonia and mucocutaneous candidiasis (Grimbacher *et al.*, 1999, Freeman *et al.*, 2008) resulting from a STAT3 loss of function mutation and defective Th17 differentiation (Holland *et al.*, 2007, Minegishi *et al.*, 2007, Milner *et al.*, 2008, Ma *et al.*, 2008). Several studies have also demonstrated that Th17 cells and the prototypical Th17 cytokines may play a non-pathogenic protective role in the immune response. Th17 cells appear to be important in regulating the expression of the neutrophil chemoattractant IL-8 for the induction of the innate immune response (Teunissen *et al.*, 1998, Ouyang *et al.*, 2008). IL-22 has been demonstrated to induce the production of a number of antimicrobial peptides (AMPs) from epithelial cells and keratinocytes including hBD2 and hBD3 (Wolk *et al.*, 2004).

The human β-defensins, first described in psoriatic skin lesions, exert their bactericidal activity through the formation of pores in the bacterial membrane or by interfering with bacterial cell wall biosynthesis (Harder *et al.*, 1997, Schroder *et al.*, 1999, Harder *et al.*, 2001, Sass *et al.*, 2010). Furthermore, IL-22 induces the expression of the S100 family of AMPs (S100A7, 8 and 9) and has been shown to be involved in epithelial repair and wound healing in a number of studies in the skin and lung (Boniface *et al.*, 2005, Aujla *et al.*, 2008, Eyerich *et al.*, 2009, Pociask *et al.*, 2013).

IL-17A and IL-17F are homologous molecules sharing 55% amino acid identity (Maggi *et al.*, 2010). Although both cytokines are involved in host immune defence and shown to play redundant roles in the immune response to *S. aureus*, IL-17A is traditionally thought to be more pro-inflammatory in nature and is more effective at inducing cytokines and inflammatory gene expression compared to IL-17F. Yang *et al.* (2008) reported that addition of IL-17F to mouse embryonic fibroblasts was able to induce expression of the pro-inflammatory genes *cxcl1*, *il6*, *ccl2* and *ccl7* although addition of IL-17A at the same concentration was able to induce higher levels of gene expression. Furthermore, Ishigame *et al.* (2009) have shown that IL-17F is not essential for induction of a number of autoimmune diseases in animal models including EAE and colitis-induced arthritis. Moreover, levels of cytokines produced by macrophages and $CD4^+$ T cells upon stimulation with IL-17F were much lower compared to stimulation with IL-17A.

In chapter 3, an abundant population of IL-17 producing cells specific to the nasal mucosa was identified. These cells were detected in healthy volunteers, suggesting that IL-17 producing cells are present in the nasal mucosa under non-inflammatory conditions. However, as previously discussed, contradictory studies exist in the literature concerning the levels of IL-17 in the nasal mucosa. Both Jin *et al.* (2014) and Shi *et al.* (2014) have reported that higher levels of IL-17 are detected in CRSwNP

patients compared to healthy controls. However, other studies have been unable to detect the presence of IL-17 in eosinophilic polyps or in healthy controls (Zhang *et al.*, 2008, Peters *et al.*, 2010). In addition, surprisingly little is known about the nasal mucosal Th17 cell response, especially in health. Hence, characterisation of these nasal T cells was performed in this chapter.

Recently, a role for inflammasomes in promoting the Th17 response has been described. Inflammasome complexes are formed by a number of scaffold proteins in response to 'danger' signals such as pathogens or cellular stress and act as molecular platforms for the subsequent recruitment and activation of caspase-1 (Schroder *et al.*, 2010, Latz *et al.*, 2013). Caspase-1 functions to cleave pro-IL-1β and pro-IL-18 to their mature forms and interestingly, these cytokines have been shown to promote Th17 responses. For example, *Mycobacterium tuberculosis* has been shown to activate the NLRP3 inflammasome in DCs and subsequent activation of caspase-1, in conjunction with IL-23, was able to promote the production of IL-17 by $CD4^+$ T cells in the absence of TCR stimulation. Production of IL-17 by γδ T cells was also detected and led to the development of EAE (Lalor *et al.*, 2011, Mills *et al.*, 2013). Furthermore, activation of the AIM2 inflammasome by cytosolic DNA is observed in keratinocytes from psoriatic lesions resulting in IL-1β production (Dombrowski *et al.*, 2011, de Koning *et al.*, 2012).

However, inflammasome activation can also be beneficial to the host. Adenylate cyclase toxin produced by *Bordetella pertussis* activates the NLRP3 inflammasome in DCs (Dunne *et al.*, 2010). Higher percentages of $IL\text{-}17^+$ T cells in the draining lymph nodes of mice immunised with adenylate cyclase toxin were observed compared to control mice. This was required for effective clearance of *B. pertussis* and IL-17 production by lymph node cells was reduced in the presence of a caspase-1 inhibitor. Notably, although the AIM2 inflammasome was discovered through its ability to detect and initiate an immune response to a number of pathogens including *Francisella tularensis*,

vaccinia virus and mouse cytomegalovirus (Hornung *et al.*, 2009, Fernandes-Alnemri *et al.*, 2009, Fernandes-Alnemri *et al.*, 2010, Rathinam *et al.*, 2010), a direct role for the AIM2 inflammasome in promoting a beneficial Th17 response has not been established. Hence, this was also examined in this chapter in the context of the nasal mucosa immune response.

In summary, the aims of the work presented in this chapter were to perform further in depth characterisation of IL-17 producing cells in the nasal mucosa by examining:

1. The surface phenotype and cytokine production profile of these cells
2. The gene expression profile of nasal vs. peripheral blood $CD4^+$ T cells
3. The possible mucosal defence role of nasal T cells via:
    a. Activation of the AIM2 inflammasome
    b. Effect on nasal epithelial cells.

5.2 Results

5.2.1 Th17 *in vitro* differentiation

Th1 and Th2 cells have been successfully differentiated *in vitro* from human naïve $CD4^+$ T cells (Cousins *et al.*, 2002). However, the conditions reportedly required for the *in vitro* differentiation of human Th17 cells are varied with several types of serum/serum-free cell culture media as well as cytokine combinations, starting cell populations and costimulation routes described in the literature (Manel *et al.*, 2008, Burgler *et al.*, 2009, Beriou *et al.*, 2010, de Wit *et al.*, 2011, Ayyoub *et al.*, 2012). As cells producing IL-17 were abundant in nasal polyp and normal nasal mucosa-derived explant cultures, the next experiments aimed to compare the phenotype of these explant-derived $IL\text{-}17^+$ cells to a highly polarised Th17 population. Preliminary experiments were therefore carried out to establish the optimal cell culture medium for the *in vitro* differentiation of Th17 cells from naïve peripheral blood $CD4^+$ T cells.

5.2.1.1 *In vitro* Th17 differentiation from naïve $CD4^+$ T cells was unsuccessful

Naïve $CD4^+$ cells were isolated from peripheral blood and cultured in serum supplemented or serum free RPMI, IMDM, X-vivo 20 and AIM-V media in the presence of Th17 polarising cytokines – IL-6, IL-1β, IL-23, TGFβ, anti-IFNγ and anti-IL-4 antibodies (Chapter 2, page 60). The combination and concentrations of Th17 polarising cytokines and antibodies used represented the most commonly described conditions for Th17 differentiation in the literature at the time of experimentation (Manel *et al.*, 2008, Burgler *et al.*, 2009, Beriou *et al.*, 2010, de Wit *et al.*, 2011). Intracellular cytokine staining analysis was performed on day 7, and every 7 days up to day 28, in order to determine the potential of T cells to produce IL-17.

Cells cultured in RPMI and IMDM media in the *absence* of serum did not proliferate and cultures were terminated on day 7 due to the high rate of cell death. Intracellular cytokine analysis showed that, even on day 28, $CD4^+$ T cells grown under the remaining culture conditions produced minimal amounts of IL-17. Figure 5.1 shows that on average, less than 2% of $CD4^+$ T cells were positive for IL-17 on day 28 and the highest percentage of IL-17^+ cells observed was only approximately 6% in the serum free AIM-V culture condition. The cytokine that was most abundantly produced was IFNγ although this still represented, on average, less than 20% of cells. Furthermore, less than 1% of cells on day 28 were IL-13^+ indicating that these cells were not of a Th2 phenotype. As Th17 cells have been shown to co-express IFNγ, the percentage of IFNγ^+IL-17^+ cells was also examined. However, IFNγ^+ cells showed no IL-17 co-production indicating that these were Th1 cells (data not shown). Moreover, further analysis revealed that on average, less than 1% of cells were positive for IL-22 with a similar percentage observed for the IL-9 producing population. Taken together, these data indicate that *in vitro* differentiation of Th17 cells using published methods was unsuccessful. Experiments to compare the phenotype of explant-derived IL-17^+ cells to highly differentiated Th17 cells were therefore not performed.

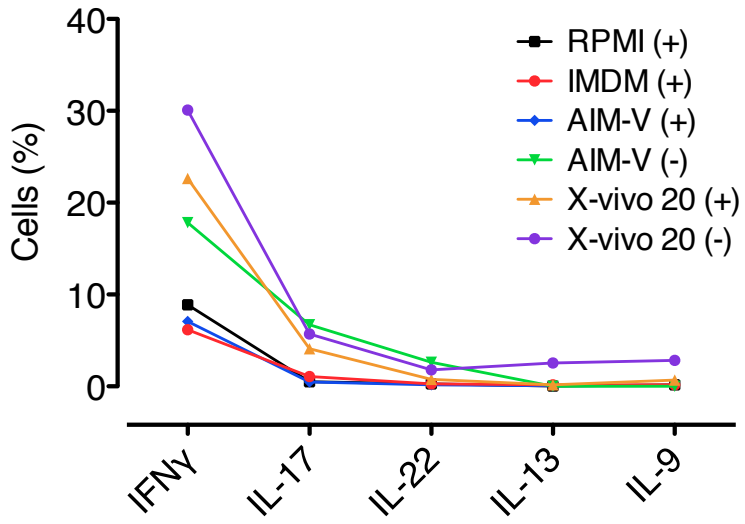


Figure 5.1 Differentiation of Th17 cells from naïve CD4$^+$ peripheral blood T cells.

Naïve CD4$^+$ T cells isolated from peripheral blood were cultured under Th17 polarising conditions (IL-6, IL-1β, IL-23, TGFβ, anti-IFNγ and anti-IL-4 antibodies) in different culture media in the presence (+) or absence (-) of serum. Percentages of cells producing IFNγ, IL-17, IL-22, IL-13 and IL-9 analysed by intracellular cytokine staining on day 28 of culture is shown for each culture condition (n=2-3).

5.2.2 Characterisation of the IL-17$^+$ T cell population in nasal polyps

5.2.2.1 Polyp-derived cells producing IL-17 are Th17 cells

To further characterise the abundant IL-17 producing CD4$^+$ T cell population observed in polyp-derived explants (Chapter 3), co-expression of IL-17$^+$ T cells with the Th17 subset surface marker CCR6 was examined by flow cytometry on day 7 post anti-CD3/CD28 stimulation.

The significant majority of IL-17$^+$CD4$^+$ T cells displayed co-localisation with CCR6 ($p<0.0001$) and notably, a similar pattern was observed for CD4$^+$ T cells producing IL-22 ($p<0.05$) (Figure 5.2a, c). Over 85% of IL-17$^+$ and IL-22$^+$ cells co-localised with CCR6 expression (IL-17: CCR6$^+$, 88% ± 2.6 vs. CCR6$^-$, 12% ± 2.6; IL-22: CCR6$^+$, 86.3% ± 3.8 vs. CCR6$^-$, 13.7% ± 3.8). Furthermore, significantly higher percentages of IL-17$^+$CCR6$^+$ cells (polyp, 16.5% ± 2.9 vs. blood, 0.9% ± 0.3; $p<0.001$) and IL-22$^+$CCR6$^+$ cells were detected in polyp explants compared to peripheral blood-derived cultures (polyp, 22.4% ± 3.9 vs. blood, 2.8% ± 1.0; $p<0.05$) (Figure 5.2b).

Analysis of the CCR6$^+$ population showed that in addition to IL-17$^+$ and IL-22$^+$ cells, IL-17/IL-22 double producing cells could also be detected (Figure 5.2a). IL-17$^+$IL-22$^+$ cells constituted, on average, 11% of the CCR6$^+$ population in polyp-derived explant cells and this was significantly higher than the percentage detected in CCR6$^+$ blood-derived cells (polyp, 11% ± 3.2 vs. blood, 4.8% ± 2.7; $p<0.05$) (Figure 5.2b). Furthermore, of the total IL-17/IL-22 producing population derived from polyp explants, the overwhelming majority showed co-localisation with CCR6 (91.8% ± 4.7) (Figure 5.2c). Thus, these data demonstrate that CCR6 is expressed by both IL-17$^+$ and IL-22$^+$ cells, as well as IL-17/IL-22 double producers, confirming that these cells are of a Th17 phenotype.

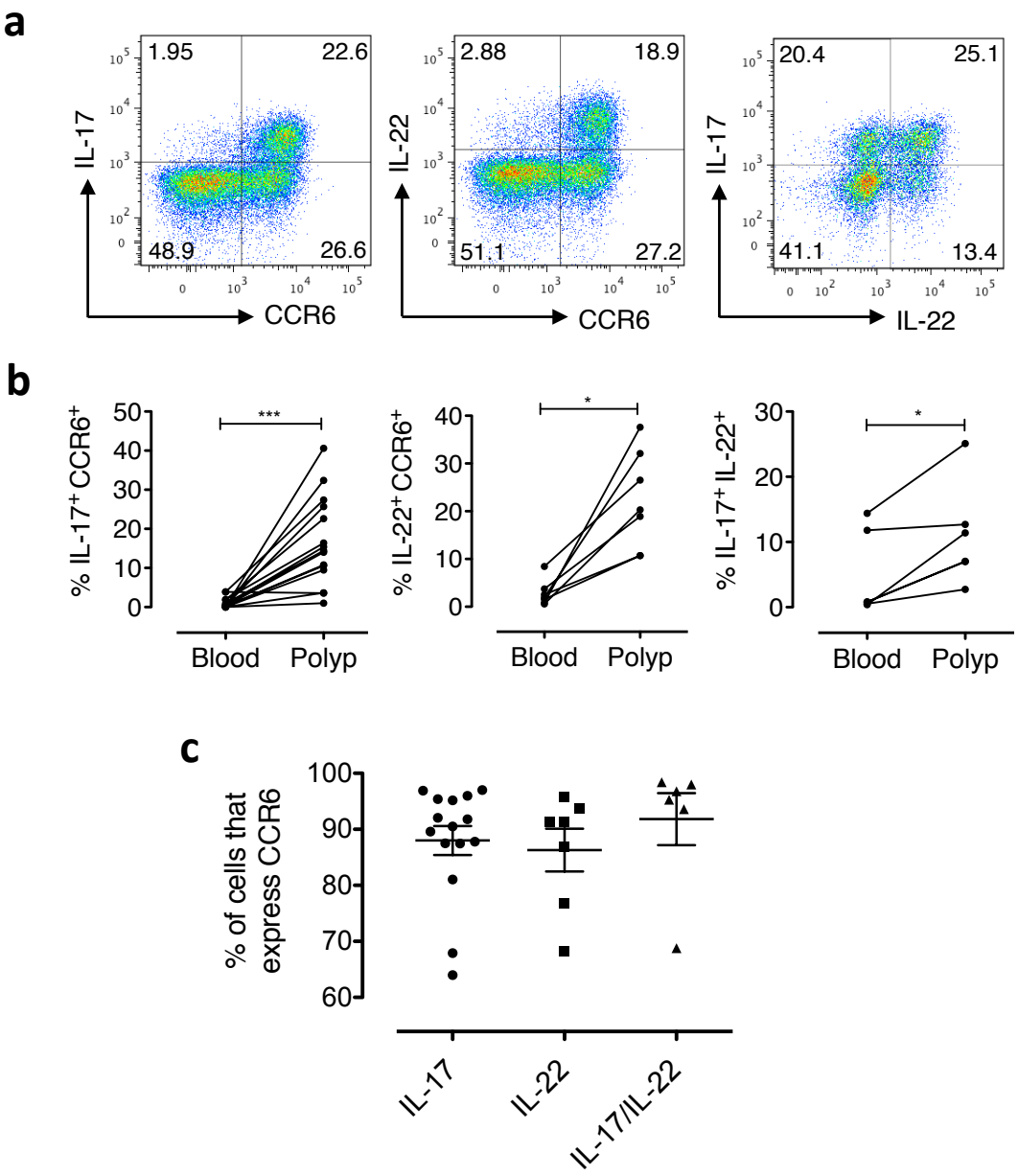


Figure 5.2 Co-localisation of CCR6 with IL-17$^+$ and IL-22$^+$ polyp-derived cells.

(a) Representative flow cytometry staining for CD4$^+$ polyp-derived cells on day 7 following anti-CD3/CD28 stimulation is shown. (b) CD4$^+$ blood and polyp-derived cells were examined for percentages of CCR6$^+$IL-17$^+$ cells (n=15), CCR6$^+$IL-22$^+$ cells (n=7) and CCR6$^+$IL-17$^+$IL-22$^+$ cells (n=7). Wilcoxon matched-pairs signed rank test. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$. (c) The percentages of IL-17$^+$, IL-22$^+$ and IL-17$^+$IL-22$^+$ cells derived from polyp explants that co-expressed CCR6 were also examined.

5.2.2.2 The Th17 phenotype displayed by polyp-derived T cells is stable

The stability of the Th17 phenotype displayed by polyp-derived $CD4^+$ T cells was examined next. Polyp explant-derived cells were cultured in RPMI supplemented with IL-2 and expression of the Th17 cytokines IL-17 and IL-22 were analysed every 7 days for 28 days, by intracellular cytokine staining. Blood-derived cells were cultured and stained for comparison.

Although cell viability affected the total number of cells that were detected, similar percentages of $IL\text{-}17^+$ and $IL\text{-}22^+$ cells from polyp explants were detected across all 4 time points with comparable IL-17 and IL-22 expression detected on day 28 as on day 7 (Figure 5.3). Little or no IL-17 and IL-22 staining was observed in T cells derived from peripheral blood in line with data presented in Chapter 3. Thus, these data indicate that the Th17 phenotype displayed by polyp explant-derived cells is stable in *in vitro* cultures.

The *in situ* expression of CCR6 and IL-17 was also examined in nasal mucosal tissue by immunohistochemical staining. CCR6 positive cells with a mononuclear morphology were detected in the submucosa of nasal polyp tissue, indicative of T cells (Figure 5.4a). Furthermore, although the majority of IL-17 positive cells in nasal polyp tissue appeared to be multi-lobed neutrophils (Figure 5.4b), mononuclear cells that stained positive for IL-17 were also detected. Hence, these results suggest that both $CCR6^+$ and $IL\text{-}17^+$ T cells are present *in vivo* in nasal polyps.

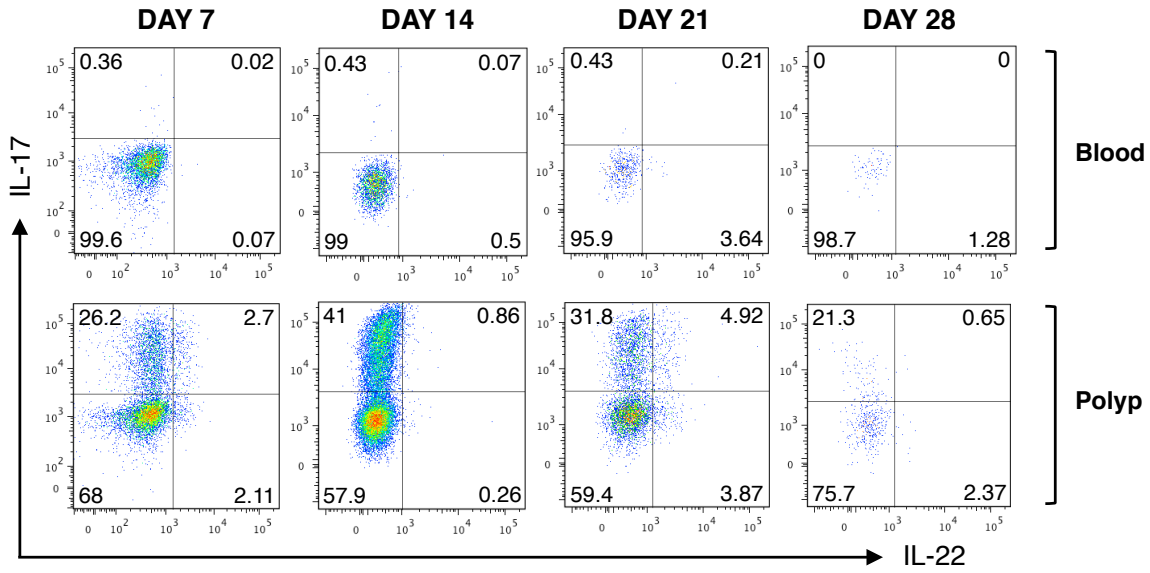


Figure 5.3 Th17 cytokine expression by blood and polyp-derived cells.

Blood and polyp-derived $CD4^+$ cells were analysed for IL-17 and IL-22 expression every 7 days for up to 28 days. Representative flow cytometry staining at each timepoint is shown for a paired blood and polyp specimen (n=3).

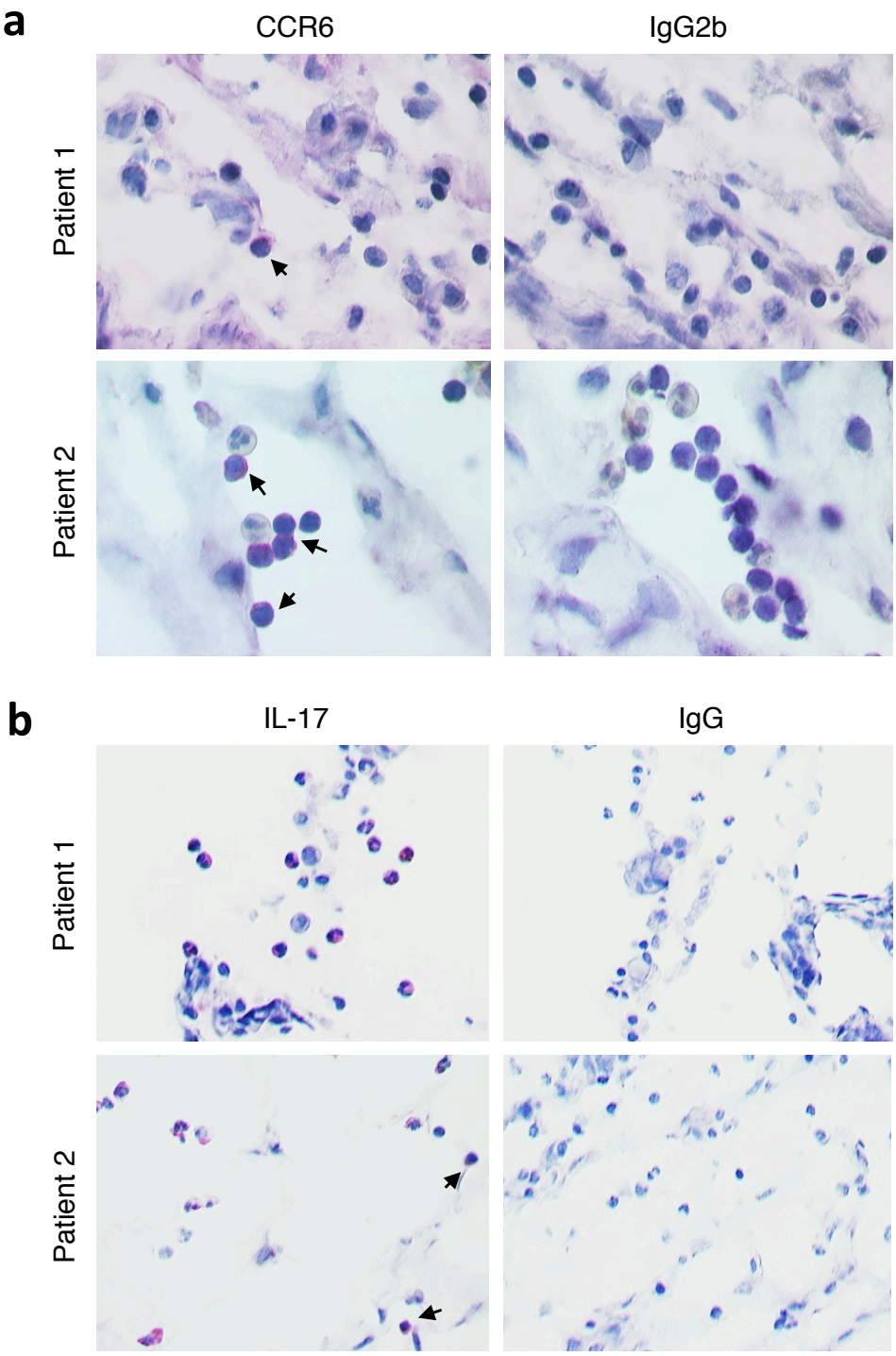


Figure 5.4 CCR6 and IL-17 positive cells are found in the polyp submucosa.

Representative immunohistochemical staining (2 independent experiments) for (a) CCR6 and (b) IL-17 in 4% PFA-fixed diseased polyp tissue from 2 CRSwNP patients (CCR6: magnification x100; IL-17: magnification x40). Respective isotype control staining (IgG2b and IgG) is also shown. Arrows indicate positive mononuclear cells.

5.2.3 Gene expression analysis of normal nasal mucosal T cells

5.2.3.1 Fluorescence-activated cell sorting for $CD4^+$ T cells

As the abundant Th17 population was detected in normal nasal biopsy explants as well as in nasal polyp explants, further characterisation of these cells by gene expression analysis was focused on cells derived from the normal nasal mucosa. This was to enable the determination of the role of T cells in the nasal mucosa compared to the periphery, without any confounding effects that may be present due to CRSwNP disease. In order to examine a pure population of $CD4^+$ T cells, unbiased for any Th cell subsets, normal nasal biopsy-derived and paired peripheral blood-derived $CD3^+CD4^+$ T cells (from healthy controls) were sorted after 7 days expansion *in vitro*. Cells were gated on the lymphocyte population, followed by gating on the singlet population (Figure 5.5). Viable cells were then sorted and collected based only on expression of the T cell surface markers CD3 and CD4.

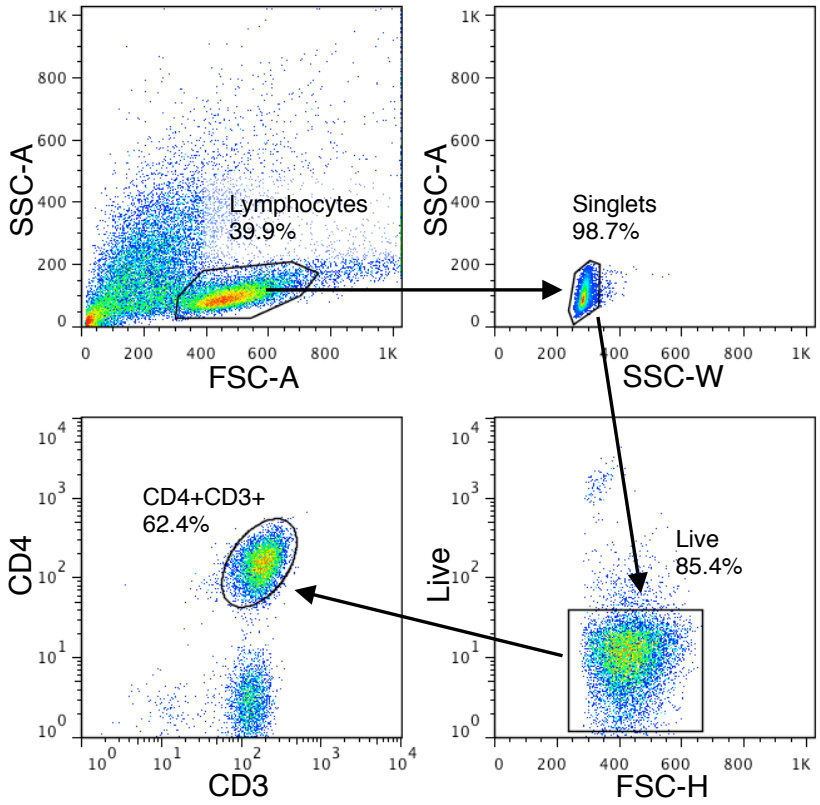


Figure 5.5 Cell sorting strategy for $CD3^+CD4^+$ cells from normal nasal mucosal and blood cultures.

Cells derived from paired normal nasal mucosal and blood cultures were sorted by FACS for the $CD3^+CD4^+$ population on day 7 post anti-CD3/CD28 restimulation. Representative flow cytometry data with the gating strategy is shown. The percentage of cells in each gate is indicated.

5.2.3.2 Genome wide expression profiling by microarray analysis

$CD3^+CD4^+$ T cells were sorted from nasal mucosal and blood-derived cultures from 3 healthy volunteers and activated with PMA/ionomycin for 4 hours. The gene expression profile of $CD3^+CD4^+$ T cells sorted from normal nasal mucosal biopsies was then compared to that from peripheral blood-derived $CD3^+CD4^+$ T cells using the Illumina Human HT-12 v4 Expression BeadChip as previously described (Chapter 2, page 89).

PCA analysis showed that samples within each group (blood, resting; blood, activated; normal nasal mucosa, resting; normal nasal mucosa, activated) clustered tightly together, indicating that samples within each group had similar gene expression profiles with little variation (Figure 5.6). The largest variable between each sample group was the cell activation status, particularly for T cells derived from the normal nasal mucosa. The specimen type i.e. the origin of the T cells, represented the second largest source of variation.

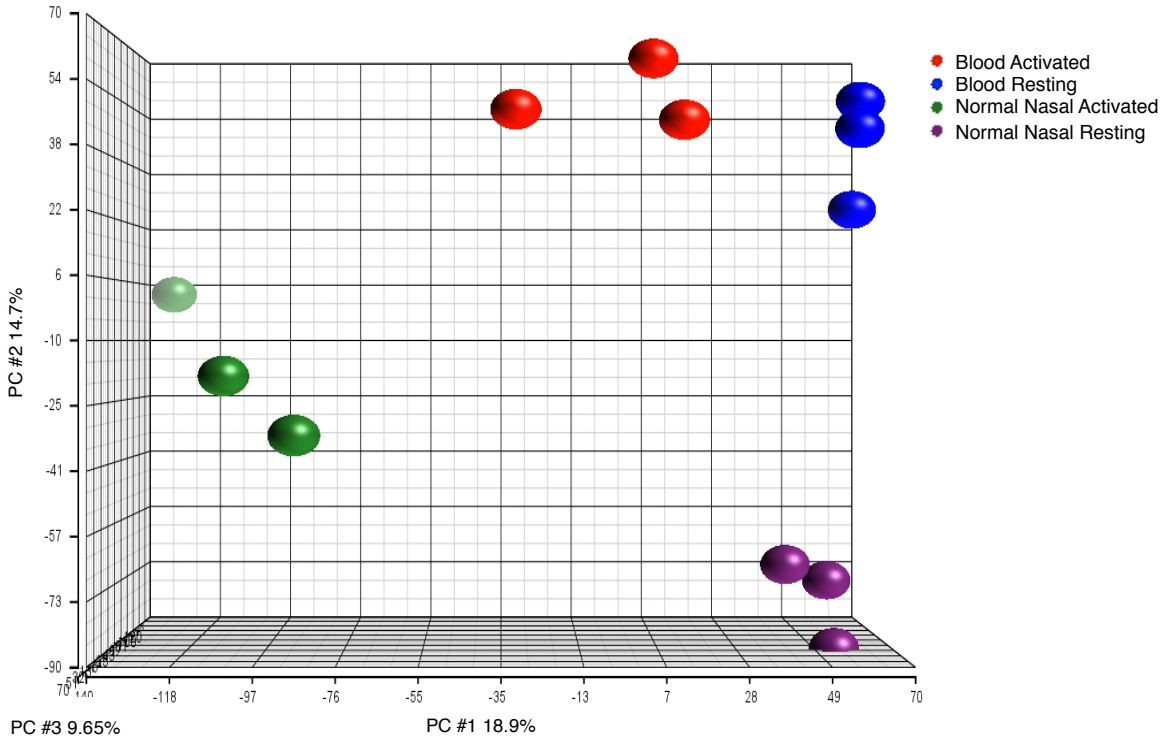


Figure 5.6 Cell activation status is the largest variable observed followed by specimen type.

$CD3^+CD4^+$ normal nasal mucosal and blood-derived cells were sorted by FACS and activated with PMA/ionomycin or left under resting state. Gene expression was subsequently analysed with an Illumina Human HT-12 v4 Expression BeadChip. Graph shows principal component analysis performed in Partek Genomics Suite (n=3 per group).

Comparison of activated blood and normal nasal mucosal $CD4^+$ T cells identified 301 genes that were significantly differentially expressed (2-fold change or greater; $p<0.05$) (Figure 5.7). Hierarchical clustering showed that activated normal nasal mucosal $CD4^+$ T cells preferentially expressed a number of Th17-related genes including the transcription factor aryl hydrocarbon receptor (*AHR*), killer cell lectin-like receptor subfamily B, member 1 (*KLRB1*) and *CCL20*, the chemoattractant for $CCR6^+$ Th17 cells which is also produced by Th17 cells themselves (Hirota *et al.*, 2007).

Examination of the genes exhibiting the largest fold increases in expression upon comparison of activated normal nasal mucosal $CD4^+$ T cells and blood $CD4^+$ T cells showed that the top 5 genes were all Th17-related (Table 5.1). *IL17F* exhibited the largest fold difference with normal nasal mucosal $CD4^+$ T cells expressing over 14-times the amount of *IL17F* transcript compared to $CD4^+$ T cells from the periphery (fold change 14.6; $p<0.001$). This magnitude of fold change was also observed for the *IL22* gene ($p<0.001$) with similar fold changes observed for *CCL20* (fold change 12; $p<0.001$) and *KLRB1* (fold change 9; $p<0.001$). Moreover, preferential expression of genes associated with other T cell subsets was not found indicating that Th17 cells are the predominant Th cell subset to display differences in gene expression between the nasal mucosa and periphery. Interestingly, the gene for absent in melanoma 2 (AIM2) which forms part of the AIM2 inflammasome was also significantly upregulated in normal nasal mucosal $CD4^+$ T cells compared to the periphery (fold change 4; $p<0.001$).

In contrast, the genes preferentially expressed by blood-derived $CD4^+$ T cells compared to normal nasal mucosa-derived $CD4^+$ T cells did not show associations with any Th cell phenotypes. The majority of upregulated genes were related to histone proteins such as *HIST1H3C* and *HIST1H2AI* (Table 5.1). Notably however, the gene that showed the largest fold increase in expression by blood vs. normal nasal mucosal $CD4^+$ T cells was *CCL22*, the ligand for CCR4 that is expressed by Th2 cells (fold change 8.2; $p<0.001$).

This differential expression was also observed when comparing blood and nasal mucosal-derived $CD4^+$ T cells in a non-activated state (fold change 3.7; $p<0.001$). Furthermore, this maintenance of differential gene expression between resting and activated conditions was also observed for genes that were upregulated by normal nasal mucosal $CD4^+$ T cells. These included *CCL20* (fold change 2.2; $p<0.01$) and *KLRB1*, which displayed the largest fold increase in expression (fold change 11.4; $p<0.001$).

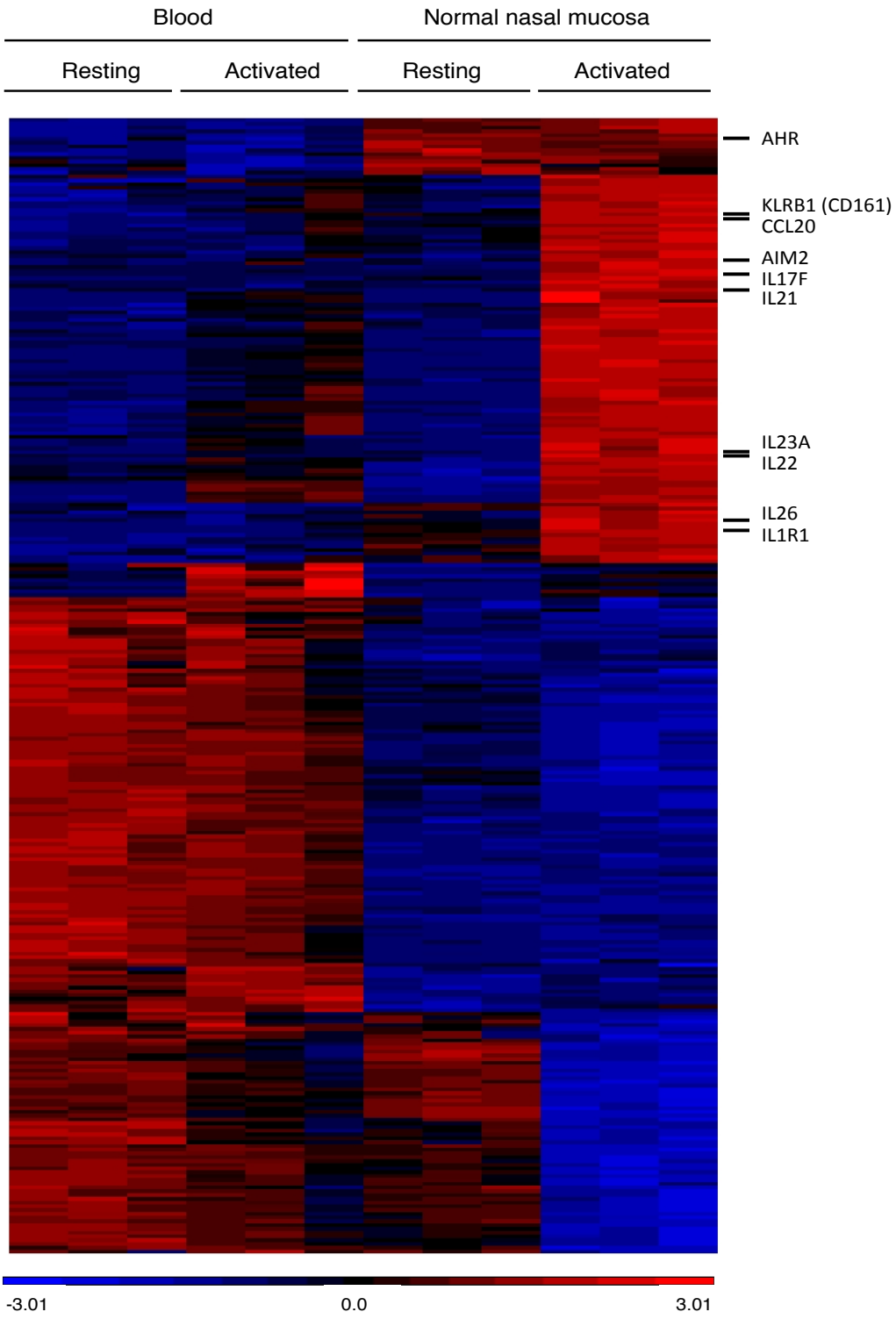


Figure 5.7 Differentially expressed genes by CD4$^+$ cells from blood and normal nasal mucosal explants.

Data analysis was performed in Partek Genomics Suite using a 3 way-ANOVA model. Data is presented as a heat map showing hierarchical clustering of genes with differential expression (fold change >2 or <-2). Comparison of activated CD4$^+$ normal nasal mucosa and activated CD4$^+$ blood samples (n=3 per group) identified 301 genes that were significantly differentially expressed ($p<0.05$).

Table 5.1 Differentially expressed genes in activated blood and normal nasal mucosal-derived $CD4^+$ cells.

Gene	p value	Ratio	Fold-difference
Blood (activated) down vs. normal nasal (activated)			
IL17F	<0.001	0.068	-14.672
IL22	<0.001	0.068	-14.622
CCL20	<0.001	0.084	-11.966
KLRB1	<0.001	0.110	-9.087
IL1R1	<0.001	0.164	-6.093
IL2	<0.001	0.189	-5.301
TNFSF8	<0.001	0.199	-5.013
PCID2	<0.001	0.211	-4.731
CDC42EP3	<0.001	0.243	-4.123
AIM2	<0.001	0.250	-4.000
TNFSF9	<0.001	0.256	-3.911
INS-IGF2	<0.001	0.260	-3.850
VPS13C	<0.001	0.260	-3.847
IL26	<0.001	0.262	-3.811
C21orf71	<0.001	0.274	-3.649
PTGER4	<0.001	0.274	-3.647
MIR155HG	<0.001	0.276	-3.620
GPR87	<0.001	0.280	-3.574
PFKF3B	<0.001	0.282	-3.547
BATF3	<0.001	0.284	-3.468
Blood (activated) up vs. normal nasal (activated)			
CCL22	<0.001	8.207	8.207
HMGB2	<0.001	6.772	6.772
HIST1H2AI	<0.001	6.545	6.545
CBX5	<0.001	6.109	6.109
ARHGAP11B	<0.001	5.304	5.304
HIST1H3C	<0.001	5.004	5.004
HIST1H1B	<0.001	4.816	4.816
HIST1H2AJ	<0.001	4.476	4.476
HIST1H1E	<0.001	4.152	4.152
HIST1H3F	<0.001	4.149	4.149
CEP55	<0.001	3.944	3.944
PRKCDBP	<0.001	3.865	3.865
FEN1	<0.001	3.850	3.850
HIST1H2BC	<0.001	3.772	3.772
FAM72D	<0.001	3.750	3.750
HIST1H2BF	<0.001	3.739	3.739
ETS1	<0.001	3.730	3.730
HIST1H2AC	<0.001	3.627	3.627
RRM1	<0.001	3.605	3.605
HIST1H2AE	<0.001	3.574	3.574

Top 20 genes for each specimen type (blood and normal nasal mucosa) are listed in order according to magnitude of difference in mRNA expression between normal nasal mucosal vs. blood $CD4^+$ T cells.

5.2.3.3 Validation of microarray results

Validation of the differential gene expression observed between blood and normal nasal mucosal-derived $CD4^+$ T cells was performed utilising qRT-PCR. Although the limited amount of cDNA from sorted normal nasal mucosal biopsies precluded the analysis of a larger number of samples and prevented statistical significance, relative expression of gene transcripts for the Th17-associated genes *IL17A*, *IL17F*, *KLRB1, CCL20* and *RORC* were all detected at higher levels by qRT-PCR in activated normal nasal mucosal-derived $CD4^+$ T cells compared to the periphery (Figure 5.8a). Furthermore, this was also observed for the *AIM2* gene. Overall, these results provide validation for the data obtained by microarray analysis and support the observation that the Th17 signature, over any other T helper cell subset, is dominant in the nasal mucosa compared to the periphery.

As the expression of IL-17F was not previously examined in this thesis and microarray data showed that nasal mucosal $CD4^+$ T cells displayed high relative expression of *IL17F* transcripts, the next step aimed to determine the expression of IL-17F protein by flow cytometry. Minimal amounts of $IL\text{-}17F^+$ cells were detected in the periphery, with expression of IL-17F observed at percentages even lower than that of IL-17A (Figure 5.8b). However, although $IL\text{-}17A^+$ cells constituted the larger percentage of the two populations, a clear population of $IL\text{-}17F^+$ cells could be detected in polyp-derived cells in line with the microarray data. Furthermore, IL-17A/F co-producing cells were also observed in polyp-derived explants with over 60% of IL-17F producing cells displaying the ability to co-produce IL-17A. Thus, these results confirm that IL-17F is a major cytokine produced by $CD4^+$ T cells in the nasal mucosa, in health and in CRSwNP disease.

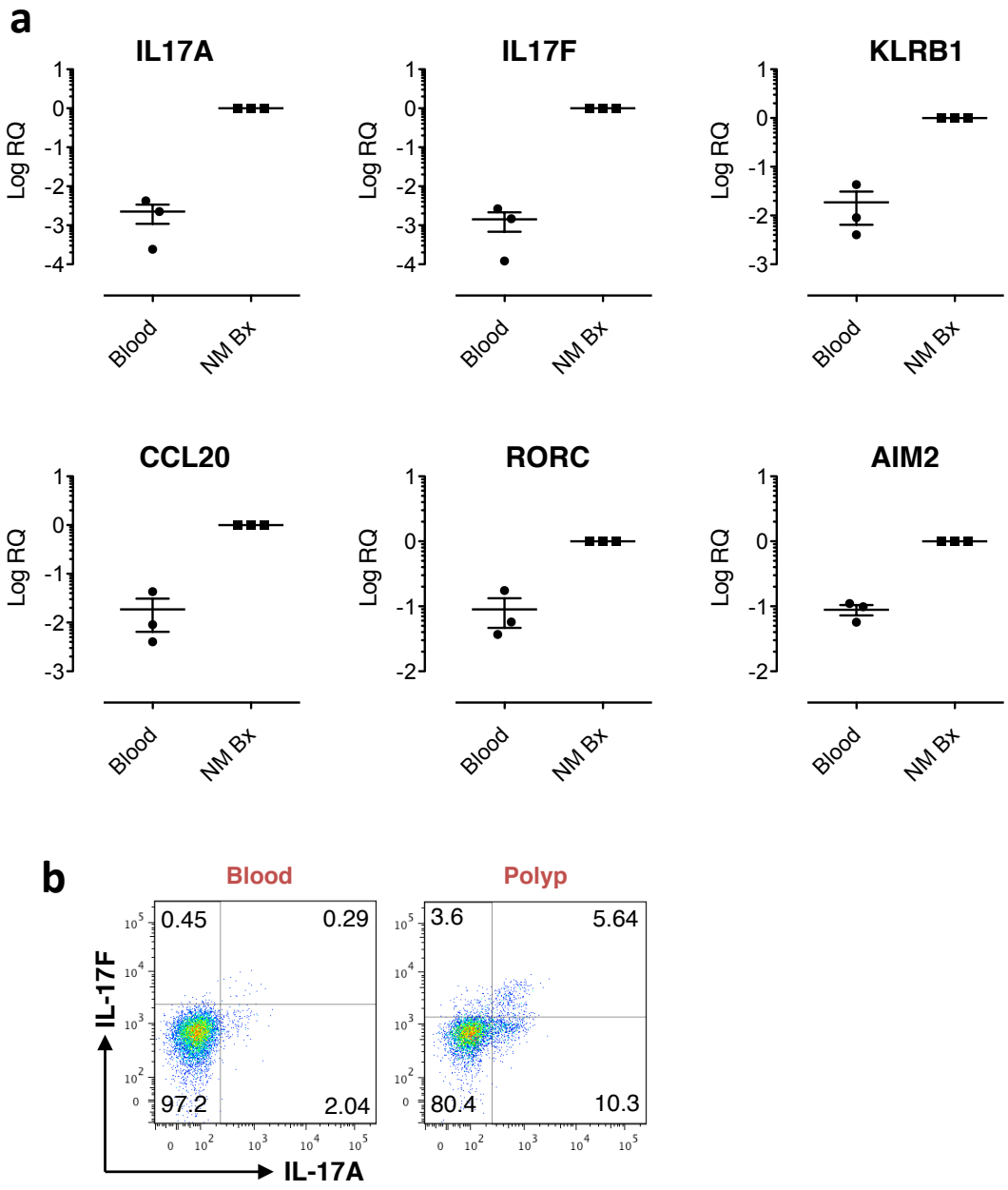


Figure 5.8 Validation of gene expression array data.

RNA from sorted $CD3^{+}CD4^{+}$ normal nasal mucosa (NM Bx) and blood cells was extracted following activation with PMA/ionomycin (n=3 per group). (A) Expression of the AIM2 gene and Th17-related genes were examined with TaqMan primer/probe sets by qRT-PCR. (B) Representative flow cytometry staining for IL-17A, IL-17F and IL-17A/F double producing cells from paired polyp- and blood-derived cells is shown.

5.2.3.4 Sub-analysis of microarray data for selected Th17 associated genes

Since analysis of the microarray data showed that normal nasal mucosal-derived $CD4^+$ T cells displayed a strong significant upregulation of Th17-related genes including *IL17F* and *KLRB1*, the expression of a larger number of selected Th17-associated genes was examined. These genes were analysed without the significance constraints (fold difference >2; $p<0.05$) in order to establish whether the Th17 signature was generally upregulated in nasal mucosal-derived $CD4^+$ T cells versus peripheral blood-derived $CD4^+$ T cells.

Twenty-six Th17-associated genes were examined and 21 out of the 26 genes showed upregulated expression in nasal mucosal $CD4^+$ T cells compared to peripheral blood $CD4^+$ T cells (Table 5.2). These ranged from *CCR6*, which showed only slight upregulation (1.05-fold increase) to genes such as *RORC* and *IL17A*, which were significantly differentially expressed but were omitted from the genes list obtained from the primary microarray analysis as differential expression was less than 2-fold (*RORC*: 1.98-fold increase; $p<0.001$, *IL17A*: 1.55-fold increase; $p=0.001$). Furthermore, the heatmap generated from hierarchical clustering analysis showed that a subset of the selected Th17-associated genes were consistently upregulated across the activated normal nasal mucosal $CD4^+$ T cell samples compared to parallel samples derived from peripheral blood (Figure 5.9). These included the cytokine genes *IL21*, *IL26*, *IL17A* and *IL23A* (IL-23 specific subunit p19), the Th17 transcription factor gene *RORC* and Th17-related cytokine receptors *IL23R* and *IL1R1*. Moreover, expression of *AHR* and *KLRB1* were also upregulated across both resting and activated normal nasal mucosal $CD4^+$ T cells. Overall, these results confirm that the Th17 signature is generally upregulated in the normal nasal mucosa compared to the periphery with the preferential expression observed in both resting and activated $CD4^+$ T cells.

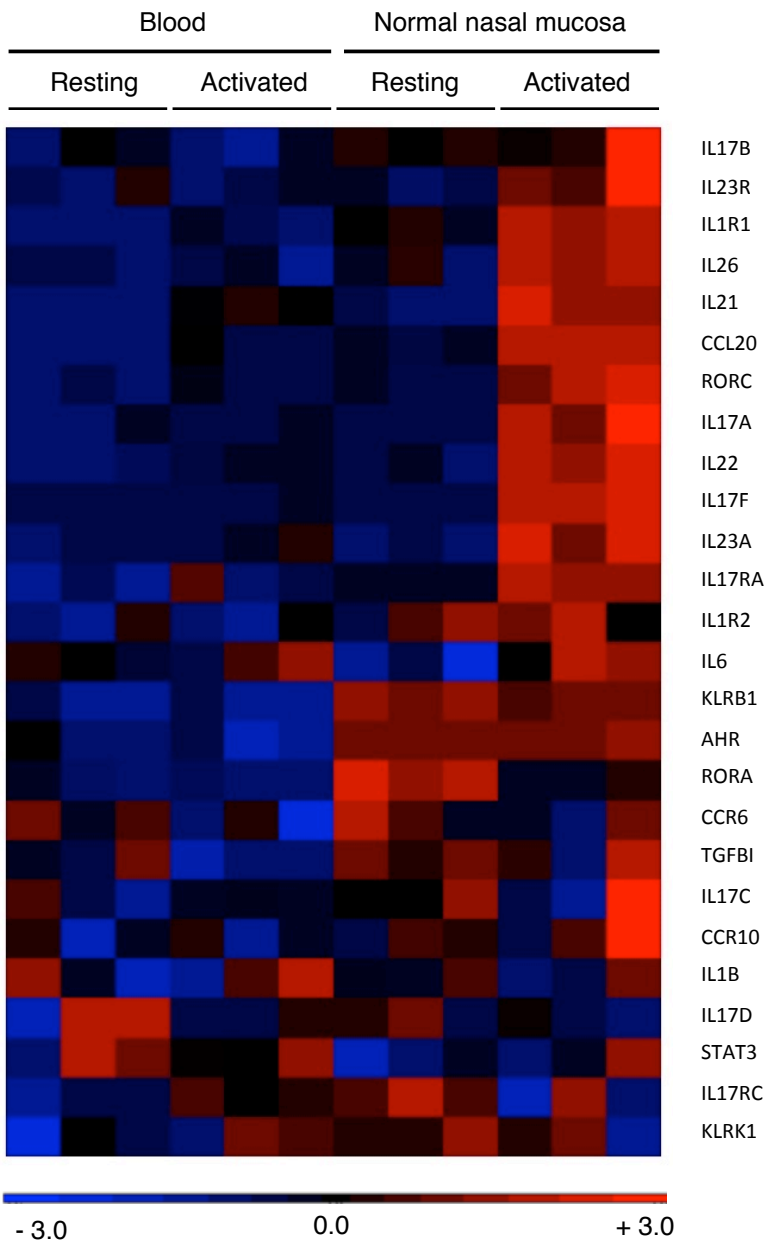


Figure 5.9 Th17 related genes are preferentially expressed in activated cells from the normal nasal mucosa.

Heatmap shows expression of selected Th17 related genes in resting and activated normal nasal mucosa and blood $CD4^+$ T cells.

Table 5.2 Th17 related genes in activated $CD4^+$ cells from blood and normal nasal mucosal explants.

Gene	p value	Ratio	Fold-difference
Blood (activated) down vs. normal nasal (activated)			
IL17F	<0.001	0.068	-14.672
IL22	<0.001	0.068	-14.622
CCL20	<0.001	0.084	-11.966
KLRB1	<0.001	0.110	-9.087
IL1R1	<0.001	0.164	-6.093
IL26	0.001	0.262	-3.811
AHR	<0.001	0.436	-2.292
IL23A	0.001	0.441	-2.268
IL21	0.001	0.474	-2.110
RORC	<0.001	0.506	-1.976
IL17A	0.001	0.648	-1.544
IL17RA	0.006	0.654	-1.529
RORA	0.009	0.736	-1.358
IL23R	0.011	0.786	-1.272
IL1R2	0.055	0.827	-1.210
TGFBI	0.085	0.860	-1.163
IL17B	0.015	0.874	-1.145
CCR10	0.233	0.922	-1.085
CCR6	0.346	0.956	-1.046
IL6	0.385	0.960	-1.042
IL17C	0.692	0.972	-1.029
Blood (activated) up vs. normal nasal (activated)			
STAT3	0.531	1.052	1.052
IL17RC	0.368	1.044	1.044
IL1B	0.597	1.026	1.026
KLRK1	0.837	1.015	1.015
IL17D	0.911	1.007	1.007

Genes are listed in order according to magnitude of difference in mRNA expression between $CD4^+$ normal nasal mucosal vs. blood cells.

5.2.4 Determination of Th17 cell function in the nasal mucosa

5.2.4.1 Polyp-derived Th17 cells show a less pathogenic phenotype compared to peripheral blood-derived Th17 cells

Th17 cells that co-express IL-17 and IFNγ have been described to display a pathogenic pro-inflammatory phenotype, whilst IL-17/IL-22 double-positive cells are thought to function in a more protective capacity through induction of AMP expression (Liang *et al.*, 2006, Annunziato *et al.*, 2007, Bending *et al.*, 2009, Boniface *et al.*, 2010). The percentages of IL-17$^+$ cells derived from polyp and blood cultures co-expressing IFNγ or IL-22 were therefore examined (Figure 5.10a).

A significantly smaller percentage of IL-17$^+$ cells from polyp explants co-expressed IFNγ compared to IL-17$^+$ cells derived from the periphery (polyp, 31.7% ± 8.9 vs. blood, 59.8% ± 7.2) (Figure 5.10b). No difference was observed in the percentage of T cells derived from polyp or blood cultures that co-expressed IL-17 and IL-22 (polyp, 47.6% ± 6.5 vs. blood, 51.8% ± 10.3). These results suggest that IL-17$^+$ cells present in nasal polyp explants are of a less pro-inflammatory phenotype compared to peripheral blood IL-17$^+$ cells.

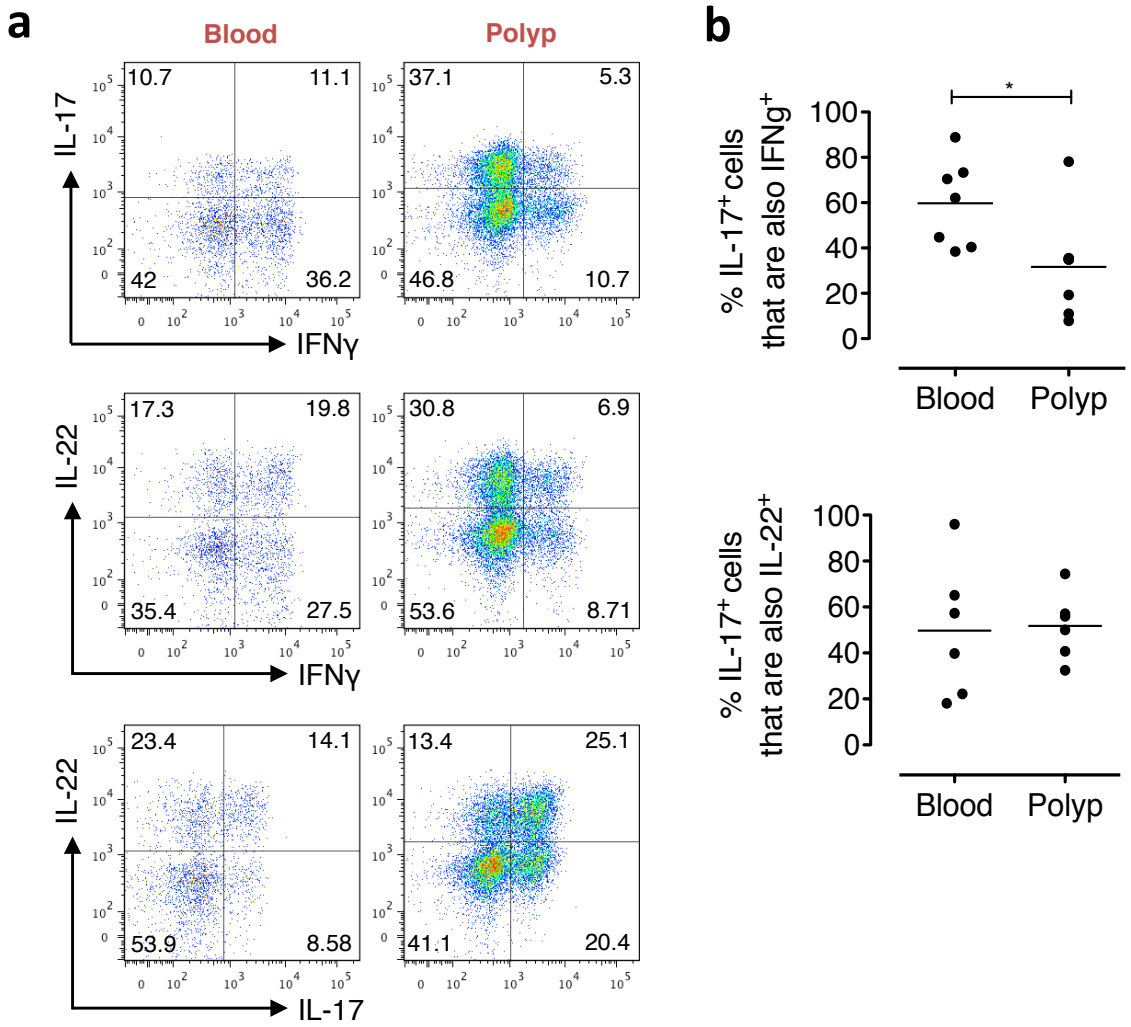


Figure 5.10 A smaller percentage of IL-17$^+$ cells derived from nasal polyp explant co-produce IFNγ compared to the periphery.

A) Representative flow cytometry staining for IL-17, IL-22 and IFNγ from paired blood and polyp-derived CD4$^+$ T cells is shown. (B) IL-17$^+$ cells from blood and polyp explants were examined for co-expression with IFNγ (n=7) and IL-22 (n=6). Wilcoxon matched-pairs signed rank test. *, $p<0.05$.

5.2.4.2 Effect of AIM2 inflammasome activation on nasal mucosal T cells

As the *AIM2* gene was significantly upregulated in nasal mucosal $CD4^+$ T cells expressing a Th17 signature, the next set of experiments aimed to determine if activation of the AIM2 inflammasome could potentiate Th17 cytokine production.

Nasal polyp-derived cells were transfected with the AIM2 inflammasome inducer, poly(dA:dT), and cultured for 36 hours before intracellular cytokine staining (Chapter 2, page 92). The percentages of IL-17, IL-22 and IFNγ producing cells were analysed relative to percentages observed in non-transfected cultures. As the experiments were preliminary in nature, only two cases were examined which precluded statistical analysis. However, initial results suggest that no or minimal differences were observed in the percentages of $IFN\gamma^+$, $IL\text{-}17^+$ and $IL\text{-}22^+$ cells upon AIM2 inflammasome induction (Figure 5.11a, b).

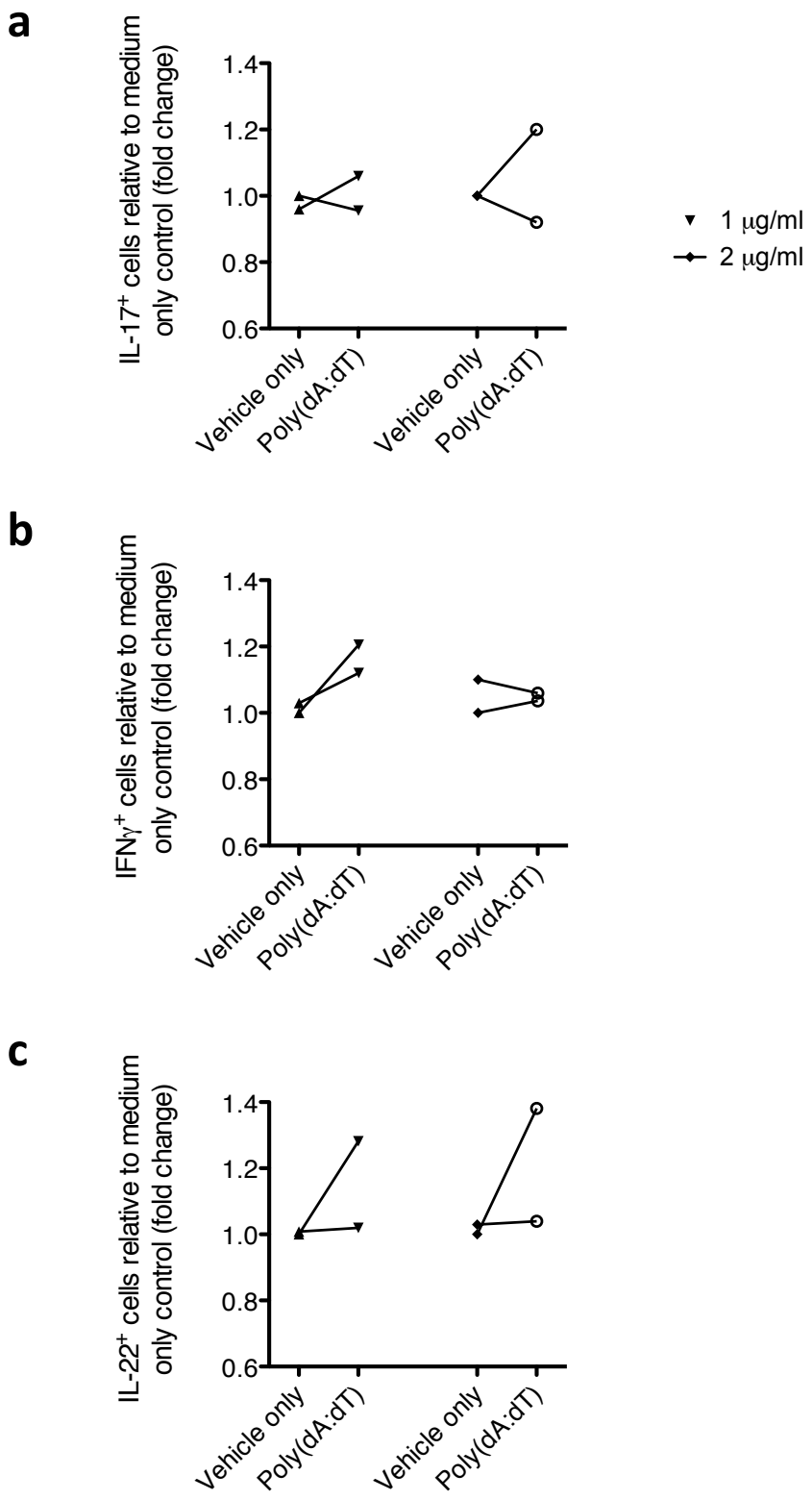


Figure 5.11 Effect of AIM2 ligand poly(dA:dT) on Th1/Th17 cytokine production.

Polyp explant-derived cells were transfected with poly(dA:dT)/LyoVec complex at concentrations of 1 μg/ml and 2 μg/ml (n=2). Intracellular cytokine staining was performed 36 hours later and expression was analysed relative to RPMI medium only control for (a) IL-17, (b) IFNγ and (c) IL-22.

5.2.4.3 Effect of nasal mucosal T cell supernatants on nasal epithelial cells

Nasal mucosal T cells have a predominant Th17 signature compared to the periphery. To determine whether this could potentially affect the function of nasal epithelial cells, the next set of experiments aimed to examine cytokine production in nasal explant culture supernatant/epithelial cell co-cultures. Non-diseased nasal turbinate tissue from patients undergoing surgery for non-CRSwNP related issues were selected for use in these experiments. Before co-culture experiments were performed however, the cytokine expression profile of turbinate-derived cells was first examined to ensure the cytokine profile was consistent with healthy nasal biopsies.

Nasal turbinates were cultured in an explant model in an identical fashion to nasal polyps. T cells derived from turbinate explants were analysed on day 7 following anti-CD3/CD28 stimulation for intracellular cytokine production. Figure 5.12 illustrates few turbinate-derived cells produced the Th2 cytokines IL-5 and IL-13. However, IL-17^+, IL-22^+ and IFNγ^+ cells were abundantly detected in turbinate explants in accordance with data observed from nasal polyps and normal nasal biopsies. Thus, epithelial cell co-culture experiments proceeded with the use of turbinate tissue (Chapter 2, page 64).

Turbinate culture supernatants (10%) were added to epithelial cells grown in BEBM medium or to BEBM medium alone. The presence of epithelial cells resulted in increased levels of the pro-inflammatory cytokines IL-6 (Figure 5.13b) and GM-CSF (Figure 5.13c) in co-culture supernatants compared to cultures with no epithelial cells (IL-6: with epithelial cells, 5304 pg/ml ± 2577 vs. without epithelial cells, 493.3 pg/ml ± 202.2; GM-CSF: with epithelial cells, 559.2 pg/ml ± 174.9 vs. without epithelial cells, 221.8 pg/ml ± 63). However, no difference was observed for levels of TNFα (Figure 5.13a), hBD1 (Figure 5.13d) or hBD2 (Figure 5.13e). In addition, epithelial cells cultured in the absence of turbinate supernatant did not produce any cytokines and only

production of hBD1 and hBD2 was observed. Overall, these results suggest that cytokines produced by nasal T cells are able to induce the production of pro-inflammatory cytokines from epithelial cells.

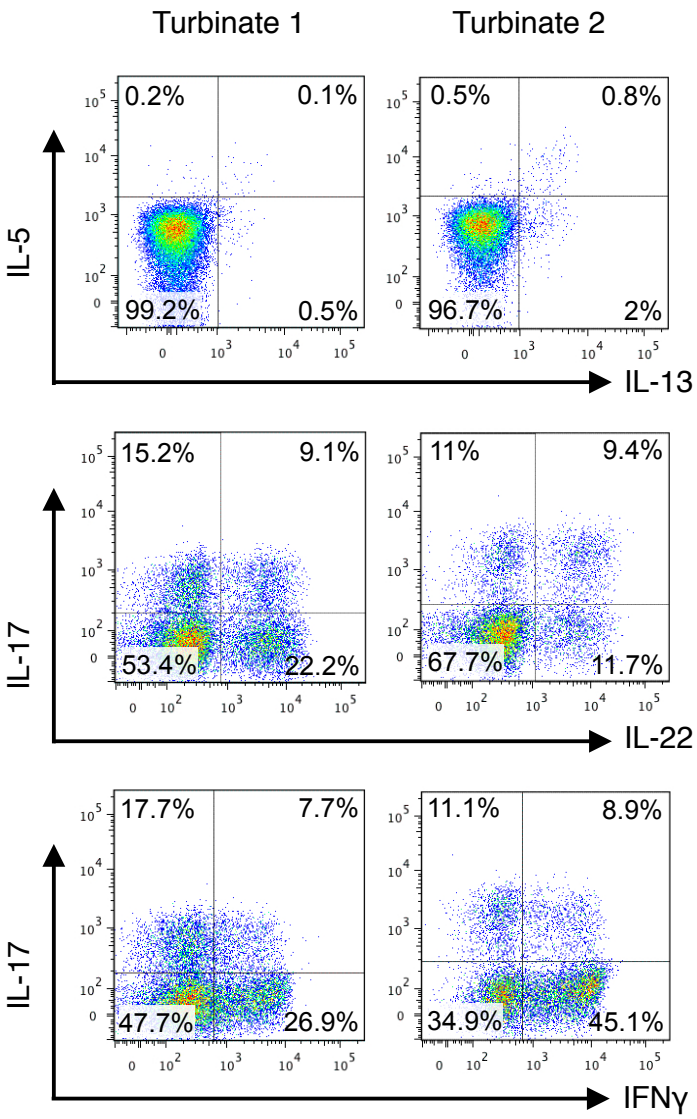


Figure 5.12 Turbinate-derived T cells have a similar cytokine expression profile to normal nasal mucosal-derived T cells.

T cells derived from nasal turbinate explants were analysed on day 7 following anti-CD3/CD28 stimulation and expansion. Cells were activated with PMA/ionomycin for 4 hours or cultured in medium only. Representative flow cytometry staining for 2 cases is shown.

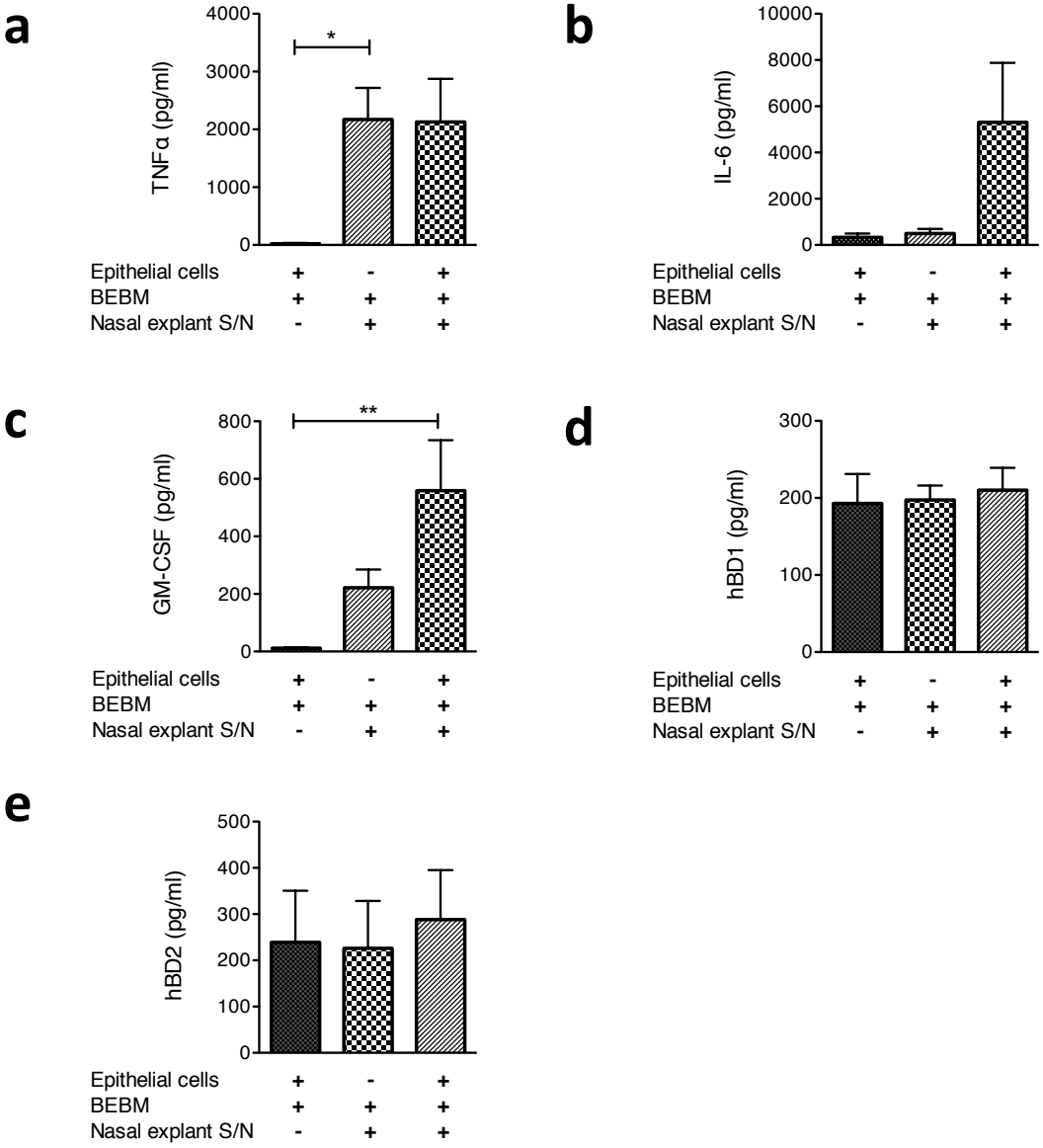


Figure 5.13 Effect of nasal turbinate explant supernatants on nasal turbinate epithelial cells in culture.

Supernatants from anti-CD3/CD28 stimulated nasal turbinate explants were cultured in the presence/absence of nasal turbinate epithelial cells for 24 hours (n=5). Levels of (a) TNFα, (b) IL-6, (c) GM-CSF, (d) hBD1 and (e) hBD2 were analysed in culture supernatants. Friedman test with Dunn's Multiple Comparison test.

5.3 Discussion

The function of Th17 cells in the nasal mucosal remains surprisingly ill defined and contrasting reports exist regarding the presence of Th17 cells in nasal polyps from CRSwNP patients. The aim of this chapter was to perform a detailed characterisation of the IL-17$^+$ T cell population detected in the nasal mucosa of both healthy volunteers and in polyps of CRSwNP patients. The surface phenotype, cytokine production profile and gene expression of nasal mucosal CD4$^+$ T cells was compared to CD4$^+$ T cells from the periphery. The possible protective role of nasal CD4$^+$ T cells was also examined.

5.3.1 *In vitro* differentiation of Th17 cells

Various groups have reported the ability to differentiate human Th17 cells from naïve CD45RA$^+$ cells *in vitro*. Burgler *et al.* (2009) examined the Th17 differentiating ability of different cytokine combinations and showed that the highest expression of IL-17 mRNA and IL-17 protein was from naïve cells cultured in the presence of TGFβ, IL-6, IL-23, IL-1β together with anti-IFNγ and anti-IL-12 antibodies. These cultures were performed in serum-free AIM-V medium. Manel *et al.* (2008) reported a comparable system using a cytokine combination of TGFβ, IL-1β plus IL-23/IL-21/IL-6 in the presence of anti-IL-4 and anti-IFNγ antibodies. In this study however, cells were cultured in serum free X-vivo 20 medium and by day 8 of culture, IL-17$^+$ cells represented 6% of the total population. In another study by Beriou *et al.* (2010), differentiation of naïve cells with the same cytokine combination, but in X-vivo 15 medium, resulted in an IL-17$^+$ population that constituted 3.3% of the total cell population following 7 days of culture.

In this thesis, examination of the effect of cell culture medium on Th17 differentiation showed that a high rate of cell death was observed for naïve T cells grown in RPMI and IMDM media in the absence of serum. As these culture media typically require serum

supplementation for viable cell cultures, this was not unexpected. Conversely, the absence of serum in cultures with AIM-V or X-vivo 20 media did not affect cell viability as these culture media do not require serum addition for optimal T cell growth.

Following 28 days of *in vitro* differentiation, IL-17$^+$ T cells constituted less than 2% (1.94%) of the total population with the AIM-V serum-free condition resulting in the highest percentage. Thus, these results suggest that even the published 'optimised' conditions for *in vitro* differentiation of Th17 cells from naïve peripheral blood cells result in low Th17 numbers compared to 'neutral' condition with nasal T cells. This suggests that the nasal microenvironment may favour a Th17 response either by inducing Th17 polarisation *in vivo* or by selective recruitment and retention of Th17 cells in the nasal mucosa. Furthermore, this is reflected with the minimal percentages of IL-17$^+$ cells observed in the periphery of both healthy controls and CRSwNP patients.

The ability of the nasal mucosal microenvironment to induce Th17 differentiation was not examined in this thesis although the presence of CCR6$^+$ and IL-17$^+$ cells in cultures from digested polyp tissue suggests that the tissue architecture is not essential for this process (Chapter 3). It has been reported, however, that β-defensins, produced by nasal epithelial cells, are chemotactic for memory T cells expressing CCR6 (Yang *et al.*, 1999) and thus may be involved in the recruitment of Th17 cells to the nasal mucosa. Moreover, microarray analysis in this study showed that CCL20, the ligand for CCR6 was significantly upregulated by nasal mucosal CD4$^+$ T cells compared to peripheral blood T cells (Table 5.1). CCL20 is produced by *in vitro* differentiated Th17 cells (Wilson *et al.*, 2007), as well as by psoriatic lesional skin-derived Th17 cells (Ghannam *et al.*, 2011) in addition to airway epithelial cells (Reibman *et al.*, 2003). These studies suggest that CCL20 may also play a role in the recruitment and/or retainment of Th17 cells in the nasal mucosal, resulting in the abundant IL-17$^+$ population detected.

5.3.2 Th17 cells are present in the nasal mucosa

The overwhelming majority of nasal $CD4^+$ T cells producing IL-17 and IL-22 co-localised with CCR6 expression. This was also observed for $IL\text{-}17^+IL\text{-}22^+$ T cells, consistent with $CD4^+$ T cells in the nasal mucosa being Th17 cells. Microarray analysis provided further validation that these cells were of a Th17 phenotype. A robust Th17 signature was detected with significant preferential expression of Th17-related genes by normal nasal mucosal $CD4^+$ T cells compared to peripheral blood $CD4^+$ T cells. *IL17F*, *IL22*, *CCL20* and *KLRB1* represented the genes with the highest fold expression and all have been extensively linked in the literature with Th17 cells (Hirota *et al.*, 2007, Seiderer *et al.*, 2008, Kleinschek *et al.*, 2009, Korn *et al.*, 2009). Together, these data strongly indicate that $IL\text{-}17^+$ cells in the nasal mucosa belong to the Th17 lineage.

$CCR6^+$ and $IL\text{-}17^+$ mononuclear cells were detected *in situ* in nasal polyp tissue by immunohistochemical staining and expression of the cytokines IL-17 and IL-22 remained stable in cultured polyp-derived T cells for up to 28 days. These results show that Th17 cells are present *in vivo* and are a stable committed lineage. In addition, the comparable percentages of $IL\text{-}17^+$ and $IL\text{-}22^+$ T cells observed from day 7 through to day 28 of culture shows that selective outgrowth of Th17 cells did not occur. Thus, these results provide further confirmation that the explant culture protocol and subsequent expansion step with IL-2 does not affect the profile of cells in culture.

The Th22 phenotype has been widely described in the literature, particularly in the skin (Eyerich *et al.*, 2009, Kagami *et al.*, 2010). The expression of CCR6 by Th22 cells has also been reported (Duhen *et al.*, 2009) and as such, a proportion of the $CCR6^+IL\text{-}22^+$ cells observed in polyp-derived cultures could theoretically belong to the Th22 lineage. The percentage of $IL\text{-}22^+IL\text{-}17^-IFN\gamma^-$ cells, which define the Th22 lineage population, was not examined in this study. Eyerich *et al.* (2009) have shown however that there is

reduced expression of the genes *CCL20* and *IL-17F* in Th22 clones compared to Th17 clones isolated from the skin of atopic eczema and psoriasis patients. Interestingly, these two genes showed the highest overexpression in nasal mucosal $CD4^+$ T cells compared to blood $CD4^+$ T cells in this study, suggesting that Th17 cells are the dominant phenotype over Th22 cells. Furthermore, IL-23R is selectively produced by Th17 cells and modest upregulation in the *IL23R* gene was also observed in this study (fold change 1.3). Conversely, *CCR10*, which is expressed exclusively by Th22 cells, showed no preferential expression in nasal mucosal $CD4^+$ T cells (fold change 1.08). Moreover, although *AHR* gene expression was upregulated in nasal mucosal T cells and is typically regarded as the pivotal Th22 transcription factor, it is also expressed by human Th17 cells (Veldhoen *et al.*, 2008a). Therefore, although the existence of a discrete Th22 population cannot be excluded, these data suggest that Th22 cells would constitute only a small population in the nasal mucosa compared to Th17 cells.

5.3.3 The protective role of nasal mucosal $CD4^+$ Th17 cells

Based on the inflammatory status of the nasal mucosa in CRSwNP, larger percentages of pathogenic IL-17^+IFNγ^+ cells were expected in polyp explant cultures compared to peripheral blood cultures. However, the lower percentages of IL-17^+IFNγ^+ cells in the nasal mucosa compared to in the periphery, as well as the upregulated gene expression of *IL17F* and *IL22*, indicate that Th17 cells in the nasal mucosa may be present in a protective capacity under both normal and non-Th17 mediated inflammatory conditions. Although the functional effects of IL-17F and IL-22 were not directly examined in this thesis, the ability of IL-17F and IL-22 to induce a protective immune response has been extensively described in the literature (see Section 5.1).

Moreover, the significantly upregulated expression of the *AIM2* gene by normal nasal mucosal T cells compared to peripheral T cells provides further indication of a potential protective role for these cells. The specific role of the AIM2 inflammasome in promoting a protective Th17 response has not been previously described. Preliminary functional experiments in this study with the addition of the AIM2 ligand poly(dA:dT) to polyp explant-derived cells elicited slight increases in the percentage of IL-22^+ cells observed but were not further pursued due to time limitations. Furthermore, as the *AIM2* gene was upregulated in $CD4^+$ T cells with a strong Th17 signature, Th17 cells may be able to respond to cytosolic DNA themselves, bypassing the need for antigen-presenting cells such as DCs to activate caspase-1. Further work will be required to validate this observation including functional characterisation of AIM2 protein expression in Th17 cells derived from the nasal mucosa. However, this is believed to be the first demonstration of T cells expressing the AIM2 inflammasome and suggests that Th17 cells may represent a previously unidentified link between the innate and adaptive immune response.

5.3.3.1 The effect of the nasal mucosal cytokine milieu on epithelial cell function

Culture of epithelial cells with nasal turbinate explant supernatants resulted in increased detectable levels of the pro-inflammatory cytokines IL-6 and GM-CSF compared to in the absence of epithelial cells. Surprisingly, no increases in the levels of hBD1 or hBD2 were detected. This data suggests that the cytokine milieu produced by turbinate T cells is able to stimulate the production of pro-inflammatory cytokines from epithelial cells. Indeed, production of pro-inflammatory mediators by epithelial cells in response to IL-17 has already been reported (Eyerich *et al.*, 2010, Way *et al.*, 2013).

Interestingly, it has been shown that T cells interact with the epithelium in a 'biphasic manner' in CRS - firstly by inducing the expression of beneficial pro-inflammatory cytokines via IFNγ, followed by the apoptosis and shedding of epithelial cells to limit inflammation (Basinski *et al.*, 2009). A large proportion of T cells derived from nasal turbinates expressed IFNγ, thus suggesting that results observed in this thesis could be due to the primary pro-inflammatory response of epithelial cells to IFNγ. However, the second phase of this response i.e. apoptosis of epithelial cells was not examined as analysis took place 24 hours following epithelial cell/turbinate supernatant co-culture. Another potential weakness was that epithelial co-cultures were not performed with supernatant from peripheral blood cultures from the same patients. Nevertheless, these data tentatively suggest that epithelial cells in the nasal mucosa may be stimulated by nasal T cells to display a 'protective' inflammatory phenotype to further corroborate with the host defence role of Th17 cells in the nasal mucosa.

5.4 Summary

In this chapter, IL-17$^+$ cells derived from the healthy and diseased nasal mucosa were examined in detail. The surface phenotype and cytokine profile of IL-17 producing T cells was examined. The genome wide expression profile of CD4$^+$ cells in the nasal mucosa was also compared to CD4$^+$ cells from the periphery. Lastly, the functional role of IL-17$^+$ cells was examined. The data presented in this chapter indicates that:

1. IL-17$^+$ cells detected in the nasal mucosa belong to the Th17 lineage.
2. Th17 is the dominant T helper cell phenotype in the nasal mucosa compared to the periphery.
3. Th17 cells are part of the normal nasal mucosal immune response.
4. Th17 cells may be present in the nasal mucosa in a protective capacity through upregulated expression of IL-17F and IL-22 although further studies will be required to verify the role of the AIM2 inflammasome in this process.

Chapter 6 General Discussion

6.1 Are IL-25R$^+$ T cells a subset of Th2 cells?

The upregulated expression of IL-25R mRNA transcripts has been shown in Th2-mediated diseases including Churg-Strauss syndrome, CRSwNP and allergic asthma (Terrier *et al.*, 2010, Corrigan *et al.*, 2011a, Iinuma *et al.*, 2015, Shin *et al.*, 2015). IL-25R expression by human Th2 cells has also been described (Fort *et al.*, 2001, Wang *et al.*, 2007, McDonald *et al.*, 2008). However, the majority of these studies examined gene transcript levels and the percentage of cells in the Th2 population expressing IL-25R was not examined or reported. The data in this thesis indicates that not all Th2 cells express the receptor for IL-25. Indeed, expression was variable (mean expression 15.4%). This is one possible reason for the modest increases in Th2 cytokines produced by nasal explant-derived cells upon IL-25 stimulation. Ideally, this experiment would have been strengthened by the addition of recombinant IL-25 and IL-33 to cultures consisting solely of IL-25R$^+$ T cells in order to determine the effect of these cytokines on an enriched population. Nevertheless, even in *in vitro* highly polarised Th2 cells differentiated from naïve peripheral CD4$^+$ T cells, IL-25R expression accounted for only 60% of T cells on day 28 of culture (Rana, unpublished data). It is therefore possible that IL-25R defines only a subset of Th2 cells.

Along this line, expression of CRTH2 has been reported to be absent on resting CD25$^-$ Th2 cells and production of Th2 cytokines is not restricted to CRTH2$^+$CD4$^+$ T cells (Nagata *et al.*, 1999). Heterogeneity in the Th2 lineage has also been described with the existence of IL-5$^+$ and IL-5$^-$ Th2 cells (Prussin *et al.*, 2010). IL-5$^+$ cells are postulated to represent a highly differentiated phenotype of Th2 cells with only 17% of Th2 cells in healthy individuals producing IL-5 (Upadhyaya *et al.*, 2011). Furthermore, Upadhyaya and colleagues showed that even in active diseases such as eosinophilic gastrointestinal disorders, only 23% of Th2 cells were found to be IL-5 expressing Th2 cells. These IL-

5 ‘super-effector’ Th2 cells are characterised by expression of haematopoietic prostaglandin D synthase (hPGDS) and respond to cytokines such as IL-25, IL-33 and TSLP with enhanced Th2 cytokine production compared to $hPGDS^{-}IL\text{-}5^{-}$ Th2 cells (Prussin, 2014). From this, it is possible that the subset of IL-$25R^{+}$ Th2 cells identified in this study may also represent a ‘super effector’ Th2 phenotype. Data from this thesis showed that IL-25R expression was significantly correlated with the percentage of IL-5 and IL-13 producing cells in nasal polyp explants. Moreover, microarray analysis showed that expression of the *HPGDS* gene was approximately 2-fold higher in IL-$25R^{+}$ T cells compared to IL-$25R^{-}$ T cells. It is therefore feasible that a ‘super effector’ population exists in nasal polyps and indicates that the targeting of IL-$25R^{+}$ T cells may be an attractive option for CRSwNP therapy.

6.2 How does the Th2 and Th17 response co-exist in the nasal mucosa?

The paradigm that Th2 cells, along with regulatory T cells, can inhibit the Th17 response is well established. However, many studies, including this thesis, have shown that the Th2 and Th17 response can co-exist in the same environment and the presence of neutrophils and Th17 cells in severe asthmatics shows that these responses are not mutually exclusive (Wakashin *et al.*, 2008, Cosmi *et al.*, 2011, Esnault *et al.*, 2012, Newcomb *et al.*, 2013). In addition, human T cell clones from $CD4^+CD161^+CCR6^+$ Th17 cells were found to include a small number that displayed a dual Th2/Th17 phenotype, including co-expression of the transcription factors GATA3 and RORγt (Cosmi *et al.*, 2010). Moreover, airway hyperreactivity and impaired lung function in asthmatics was positively associated with the number of dual Th2/Th17 cells present in BAL fluid with the most severe asthmatics displaying the highest number of Th2/Th17 cells (Irvin *et al.*, 2014).

Interestingly, a mouse study by Takahashi *et al.* (2011) has suggested that IL-22, produced by Th17 cells, is protective against OVA-induced allergic inflammation. IL-22 was shown to prevent allergic airway inflammation by inhibiting IL-25 production from lung epithelial cells. The decrease in $IL\text{-}25R^+$ eosinophil and Th2 cell recruitment and goblet cell hyperplasia was abrogated and enhanced upon administration of an anti-IL-22 antibody. This IL-22 protective effect was also detected in IL-22 transgenic mice compared to wild-type mice upon OVA-induced allergic airway inflammation (Fang *et al.*, 2014). Although these studies are in agreement with the protective Th17 signature that was observed in the nasal mucosa in this thesis, an increase in the number of IL-25 producing cells (eosinophils and epithelial cells) was also demonstrated in polyp tissue compared to nasal tissue from healthy volunteers. This suggests that IL-22 was unable

to prevent IL-25 production in nasal polyps. However, this was not confirmed experimentally. As the number of cytokine-producing T cells is not a reflection of the activity or potency of individual cytokines *in vivo*, whether the Th2 or Th17 response is dominant in CRSwNP remains undetermined. Furthermore, the majority of this thesis examined these two Th cell phenotypes in isolation and future studies would benefit from examining the interplay between the two Th cell phenotypes in the nasal mucosa. Nonetheless, the existence of nasal polyps themselves does suggest that IL-22 production by Th17 cells is not sufficient to inhibit IL-25 production and studies have shown that expression of the IL-22 receptor is downregulated in CRSwNP (Ramanathan *et al.*, 2007b). Thus, the IL-25/IL-25R-mediated Th2 response appears to be dominant, resulting in CRSwNP disease.

6.3 Would the study have benefited from a more stringent inclusion criteria for CRSwNP patients?

As discussed in chapter 3, the clinical status of CRSwNP patients whose surgical specimens were used in this study was diverse and included differences in aspirin sensitivity. Patients with AERD often present with the most severe cases of nasal polyposis and are associated with the highest rates of polyp reoccurrence following surgery (Kim *et al.*, 2007). The low number of aspirin tolerant samples collected precluded meaningful statistical comparison of the number of Th2 cytokine-producing T cells in AS vs. AT patients, although a trend increase in IL-25R expression was observed for AS compared to AT patients. This suggests that results observed throughout this study (Th2 cytokine production, IL-25R expression, IL-25^{+} cells etc.) could potentially have been diminished by the inclusion of data from AT patients. More profound differences may have been detected if data from AS patients only were included in this study.

This study was designed to examine the phenotype of the nasal mucosa in health and nasal polyp disease and as such, did not aim to distinguish between patients based on disease severity. Nevertheless, stratifying CRSwNP patients according to aspirin sensitivity may have resulted in a better idea of whether targeting the Th2 response was an effective therapeutic option for nasal polyposis. This has been shown to be beneficial in predicting subsets of asthma patients that will respond to certain therapies. For instance, clinical trials using the anti-IL-13 monoclonal antibody lebrikizumab resulted in significant improvement in lung function but this was only observed in patients with high serum periostin levels (Corren *et al.*, 2011, Hanania *et al.*, 2015). In addition, sputum eosinophilia has been demonstrated to be a biomarker for the identification of steroid refractory asthma patients that will respond to treatment with the anti-IL-5

monoclonal antibody mepolizumab (Haldar *et al.*, 2009, Nair *et al.*, 2009). These studies provide some support for the stratification of CRSwNP patients for effective therapeutic strategies, especially as CRSwNP is not considered to be one single disease entity but comprised of a heterogeneous group of diseases all associated with chronic nasal inflammation (Van Crombruggen *et al.*, 2011, Bachert *et al.*, 2014).

6.4 What is the pathomechanism of CRSwNP disease?

Although the aetiology of CRSwNP disease is unknown, the pathogenesis of CRSwNP is associated with a number of hypotheses including the *Staphylococcus aureus* superantigen theory (Van Zele *et al.*, 2004, Van Zele *et al.*, 2007) and the immune barrier hypothesis (Kern *et al.*, 2008) (as discussed in Chapter 1). These hypotheses were not directly examined in this study, as it was not the aim of this thesis. However, identification of CDR3 clones, with restriction to Vβ families potentially associated with *S. aureus* superantigens suggests that *S. aureus* may indeed play a role in CRSwNP disease. Examination of *S. aureus* colonisation in the nasal mucosa as performed by Van Zele *et al.* (2004) would substantiate this observation as well as serum and tissue measurements of *S. aureus* specific IgE and total IgE levels. Furthermore, functional studies such as proliferation assays following stimulation of polyp-derived cells with SEB or other *S. aureus* superantigens would help to determine whether T cell expansion is driven by *S. aureus*.

6.4.1 Summary of CRSwNP pathomechanisms

The pathogenesis of CRSwNP may be due to a combination of both the *S. aureus* hypothesis and the immune barrier dysfunction hypothesis. The results in this thesis extend these theories further with the potential recognition of an antigen or *S. aureus* superantigen by IL-25R$^+$ Th2 cells and the involvement of IL-25 and IL-33 in potentiating the Th2 adaptive immune response. How these results may theoretically interlink with the current postulated pathomechanisms for CRSwNP is summarised below.

Soyka *et al.* (2012) have previously shown that the epithelial barrier in CRSwNP is defective with decreased expression of tight junction proteins such as occludin and ZO-1. Together with epithelial cell apoptosis and shedding, this may facilitate the

infiltration of *S. aureus* into subepithelial reservoirs, leading to persistent colonisation of the nasal mucosa (Clement *et al.*, 2005). Moreover, internalisation of *S. aureus* into mast cells has also been demonstrated as another mechanism whereby *S. aureus* is able to evade the host immune system to survive in the nasal mucosa (Abel *et al.*, 2011). These intracellular reservoirs of *S. aureus* result in the persistent induction of IL-17A. Indeed, *S. aureus* superantigens such as SEB have been shown to induce the expression of IL-17A from nasal epithelial cells (Jin *et al.*, 2014). In conjunction with the reduced IL-22-mediated protective response due to downregulation of the IL-22R on nasal epithelial cells (Wang *et al.*, 2014b), this IL-17A signature may result in the chronic pathogenic inflammatory response observed in CRSwNP. This is separate to the protective Th17 response that is observed in the normal nasal mucosa in the absence of a chronic inflammatory stimulus.

The Th17 signature may also potentiate the pathogenic Th2 response observed in CRSwNP. OVA-induced Th2-mediated airway inflammation has been shown to be dependent on inflammasome activation with no inflammation observed in mice deficient in NLRP3 inflammasome components ($NLRP3^{-/-}$, $ASC^{-/-}$ and $IL\text{-}1R1^{-/-}$) (Ritter *et al.*, 2014). Furthermore, activation of the NLRP3 inflammasome by ATP and subsequent IL-1β production results in a pro-inflammatory cytokine milieu including IL-6, leading to enhanced Th17 differentiation (Besnard *et al.*, 2012). Moreover, Yu *et al.* (2015) have demonstrated that the major house dust mite allergen Derp2 was also able to activate the NLRP3 inflammasome. As expression of the AIM2 inflammasome was upregulated in Th17 cells, these studies suggest that *S. aureus* bacterial DNA may potentially be able to activate the AIM2 inflammasome in Th17 cells analogous to that for Derp2 and the NLRP3 inflammasome. AIM2 inflammasome activation may therefore further promote Th17 differentiation via IL-1β and contribute to pathogenic Th2 inflammation in CRSwNP.

A number of mediators including proteases and DAMPs are able to induce the release of IL-25 and IL-33 from epithelial cells (Reh *et al.*, 2010, Kouzaki *et al.*, 2013, Paris *et al.*, 2014, Snelgrove *et al.*, 2014). In addition, Soyka *et al.* (2015) have also shown that IL-33 is mainly induced by IFNγ – which was produced by a large proportion of T cells in the healthy and diseased nasal mucosa – as an 'alarmin' in response to IFNγ–mediated tissue damage. Thus, IL-25 and IL-33 may be able to stimulate ILC2 for the initiation of the Th2 innate immune response with recognition of a possible antigen leading to the clonal expansion and activation of the IL-25R$^+$ Th2 subset. *S. aureus* superantigens, in addition to polyclonal T cell activation, may also activate IL-25R$^+$ Th2 cells in an antigen-specific manner as TCR clones restricted to Vβ families associated with superantigens were detected. Together these mechanisms, coupled with the potentiation of the Th2 response by IL-25 and IL-33 via IL-25R and ST2 expressing Th2 cells, may potentially lead to the uncontrolled inflammatory response in CRSwNP that may well be driven by *S. aureus* in addition to local Th17 and Th2 cells in the nasal mucosa.

6.5 Are Th17 cells involved in innate immunity in the nasal mucosa?

Results presented in this thesis suggest that Th17 cells are present in the normal nasal mucosa and may be beneficial for host mucosal defence. However, gene expression studies suggest the possibility that Th17 cells may also be involved in innate immunity through the AIM2 inflammasome. Due to the limited number of cases, no firm conclusions can be drawn from the preliminary functional studies. Further work will be required to assess the importance of the AIM2 inflammasome in Th17 cells including protein studies such as western blotting to identify AIM2 protein expression. Furthermore, direct evidence for inflammasome activation with measurement of IL-1β and casapse-1 similar to the protocol by Gross (2012) will verify if the AIM2 inflammasome is functional in Th17 cells.

6.6 What is the therapeutic potential?

6.6.1 Targeting the IL-25/IL-25R and IL-33/ST2 pathways

The potentiation of Th2 cytokine production by IL-25 and IL-33 and expression of IL-25R and ST2 by a Th2 subset provides a rationale for the targeting of these cytokines and receptors in CRSwNP therapy. At present, only evidence from mouse models of allergic airway inflammation are available to suggest that this is a valid approach (Ballantyne *et al.*, 2007, Kurowska-Stolarska *et al.*, 2008, Kearley *et al.*, 2009, Gregory *et al.*, 2012, Endo *et al.*, 2015, Shin *et al.*, 2015). An anti-IL-33 monoclonal antibody, ANB020, is currently under development by AnaptysBio for the treatment of moderate to severe asthma, atopic dermatitis and food allergies (AnaptysBio, 2014). Approaches to inhibit downstream IL-25R/ST2 signalling and soluble antagonist mediators such as soluble ST2, similar to etanercept in the targeting of the TNF receptor, may also be beneficial. Furthermore, as ILC2 also express IL-25R and ST2, inhibition of IL-25/IL-33 could potentially affect both the innate and adaptive Th2 response.

Therapies targeting a single cytokine are associated with difficulties, as exemplified by the limited clinical success of anti-IL-4 monoclonal antibodies such as pascolizumab (Pelaia *et al.*, 2012). This may be due to redundancy in the biological activities of IL-4 and IL-13 and suggests dual targeted approaches may be beneficial. Recent positive clinical studies with dupilumab (Wenzel *et al.*, 2013, Beck *et al.*, 2014), a monoclonal antibody against IL-4Rα which is involved in both the IL-4 and IL-13 pathway, provides further verification that targeting both IL-25 and IL-33 concomitantly could be the optimum therapeutic strategy.

6.6.2 Targeting the antigen in CRSwNP

Identification of common CDR3 sequences in IL-25R$^+$ Th2 cells suggests clonal expansion of IL-25R$^+$ T cells following recognition of a common antigen. Although

these results are encouraging, further TCR Vβ sequencing in a larger number of cases will be required to validate these results. Furthermore, human leukocyte antigen (HLA) testing to determine the MHC haplotype of patients expressing common CDR3 clones would also help establish whether antigen recognition is involved, as this process is restricted to certain MHC class II molecules for each individual antigen.

Following confirmation that IL-25R$^+$ Th2 cells are responding to a common antigen, the next step would be to identify the antigen(s) that is being recognised by the CDR3 sequences. Ding *et al.* (2015) were able to identify novel *Mycobacterium tuberculosis* antigens by screening for peptides recognised by CDR3 sequences from peripheral blood of TB patients with a phage display library. A similar approach could be used to determine the antigen involved in CRSwNP disease following cloning of the TCR, further to the modelling approach suggested in Chapter 5. Furthermore, T cell epitope mapping with the use of synthetic peptide libraries could also be a possible method (Mimotopes, 2015). Identification of the antigen involved in CRSwNP aetiology could potentially pave the way for novel approaches in CRSwNP treatment such as targeted T cell therapy. Tregs bearing engineered TCRs recognising the antigen could be delivered locally to the nasal mucosa to dampen the inflammatory response and such approaches have already been studied for the treatment of autoimmune diseases (Fujio *et al.*, 2004, Boulter *et al.*, 2005, Fujio *et al.*, 2007). Peptide vaccinations such as that with synthetic peptide immune-regulatory epitopes (SPIRE) being developed against the major cat allergen, Fel d 1, may be another possible approach (Worm *et al.*, 2011, Couroux *et al.*, 2015).

6.6.3 Targeting Th17 cells

The protective function of Th17 cells in the nasal mucosa suggests that promoting the Th17 response may be advantageous, with the study by Takahashi *et al.* (2011)

suggesting that IL-22 is able to inhibit IL-25-mediated airway inflammation. However, this approach may be less feasible in practice as it is potentially associated with a number of disadvantages. Promotion of T cell activation requires a very careful approach as the clinical trial with the anti-CD28 monoclonal antibody TGN1412 showed (Suntharalingam *et al.*, 2006). The involvement of Th17 cells in autoimmunity has been extensively reviewed (Eisenstein *et al.*, 2009, Maddur *et al.*, 2012) and thus, promotion of the Th17 response specifically could potentiate autoimmunity through increased inflammation and mechanisms such as epitope spreading and bystander activation (Ercolini *et al.*, 2009). Together, these studies suggest that targeting of Th17 cells in the nasal mucosa may not be a suitable therapeutic option in CRSwNP disease.

6.7 Conclusion

In this thesis, IL-25R has been identified as a marker of Th2 cells in nasal polyps with Th17 cells present in both healthy and diseased nasal mucosa. IL-25R$^+$ T cells selectively produced Th2 cytokines and showed upregulated expression of Th2-related genes compared to IL-25R$^-$ cells. Co-expression of ST2 by IL-25R$^+$ Th2 cells was also observed. Although this has been established for ILC2, this is believed to be the first demonstration in human Th2 cells. Furthermore, IL-25R and ST2 expressed by Th2 cells were functional and responded to IL-25 and IL-33 *in vitro* to potentiate Th2 cytokine production. *In vivo* sources of IL-25 included eosinophils and epithelial cells and higher numbers of IL-25$^+$ eosinophils were detected in nasal polyp tissue compared to control nasal mucosal tissue. Thus, IL-25R$^+$ Th2 cells may respond to heightened levels of IL-25 in CRSwNP to drive Th2-mediated inflammation. IL-33 was also detected in epithelial and endothelial cells in nasal tissue as potential *in vivo* sources of this cytokine. TCR Vβ sequencing of IL-25R$^+$ and IL-25R$^-$ cells showed no skewing of the TCR Vβ repertoire. However, common CDR3 clones were found in IL-25R$^+$ cells from a number of CRSwNP patients, suggestive of clonal expansion following recognition of an as yet unidentified antigen, with possible involvement of *S. aureus* superantigens. Accordingly, these results suggest that targeting of the IL-25/IL-25R and IL-33/ST2 pathways may be beneficial in the treatment of CRSwNP.

IL-17$^+$ cells detected in the nasal mucosa belonged to the Th17 lineage. The Th17 phenotype was dominant in the nasal mucosa with Th17-related genes preferentially expressed by CD4$^+$ T cells in the normal nasal mucosa compared to in the periphery. This suggests that Th17 may be a default signature in nasal mucosal immune homeostasis. Importantly, this Th17 signature was associated with significantly upregulated expression of the *IL17F* and *IL22* genes and lower numbers of pathogenic

IL-17^{+}IFNγ^{+} cells were also observed in nasal explants compared to the periphery. Together, these results suggest that Th17 cells may function in a protective capacity. Furthermore, gene expression of the AIM2 inflammasome component was also upregulated in nasal mucosal CD4^{+} T cells with a strong concomitant Th17 gene signature. This may represent a previously unidentified link between the innate and adaptive immune response. Preliminary data showed that stimulation of AIM2 with its synthetic ligand resulted in minor increases in IL-22 production by nasal explant cells. Although the role of AIM2 in Th17 cells requires further validation, together these results indicate that Th17 cells may be present in the normal nasal mucosa for host nasal mucosal immunity.

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